Cut-off value for absolute lymphocytes as an alternative for the immunophenotypic analysis of CD3+T cells in the monitoring of immunosuppressive therapy with thymoglobulin

Valor de corte para linfócitos absolutos como alternativa para análise imunofenotípica de células T CD3+ no monitoramento de terapia imunossupressora com timoglobulina

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Abstract

Introduction: Immunosuppression of T lymphocytes is required for preventing acute rejection after transplantation and for the treatment of chronic autoimmune and inflammatory diseases. The laboratory monitoring for this therapy is the measurement of T cells by immunophenotyping, aiming the target value of less than 20 cells per µL. **Objective:** To establish a cut-off point for the total number of lymphocytes in the automated blood cell count that reflects less than twenty T cells µL by immunophenotyping. **Methods:** We studied and evaluated 242 kidney transplant patients that had results of automated blood cell count and quantification of T cells by immunophenotyping technique. The patients were divided into two groups, depending on the T lymphocyte immunophenotyping rates established by lower and higher than 20 cells per µL. After, we evaluated the cut-off point for lymphocytes in the blood cell count with a specificity of 100% to exclude patients with high levels of T lymphocytes. **Results:** We found that the cut-off point of 70 lymphocytes per µL obtained by automated blood cell count showed 100% of specificity to exclude patients with T-cell counts higher than 20 cells per µL by immunophenotyping. **Conclusion:** The results found in this study may be helpful to monitor the immunosuppressive therapy in kidney transplant patients in places where a flow cytometer is not available, or when this equipment is not present in the full routine.

Keywords: flow cytometry; kidney transplantation; immunophenotyping; immunosuppressive agents.

Resumo

Introdução: A imunossupressão de linfócitos T é necessária para a prevenção da rejeição aguda em transplantes e no tratamento de doenças autoimunes e inflamatórias crônicas. O seu monitoramento laboratorial consiste na quantificação dos linfócitos T realizada pela técnica de imunofenotipagem, na qual o valor preconizado é manter inferior a 20 células/µL. **Objetivo:** Estabelecer um ponto de corte para o número de linfócitos totais no hemograma automatizado que reflita uma contagem de linfócitos T inferior a 20 células/µL por imunofenotipagem. **Métodos:** Foram avaliados 242 pacientes transplantados renais que continham resultados do hemograma automatizado e quantificação de linfócitos T por imunofenotipagem. Os pacientes foram divididos em dois grupos, conforme os valores de linfócitos T estabelecidos pela imunofenotipagem: inferiores e superiores a 20 células/µL. A partir disto, foi avaliado o ponto de corte de linfócitos no hemograma com especificidade de 100% para excluir os pacientes com valores elevados de linfócitos T. **Resultados:** Este estudo evidenciou que o ponto de corte de 70 linfócitos/µL obtidos pelo hemograma automatizado apresentou especificidade de 100% para excluir os pacientes com contagens de linfócitos T superiores a 20 células/µL na imunofenotipagem. **Conclusão:** Esta pesquisa poderá auxiliar no monitoramento da terapia imunossupressora em pacientes transplantados renais em locais que não possuem um citômetro de fluxo disponível, ou ainda quando este equipamento não se faz presente na rotina integral.

Palavras-chave: citometria de fluxo; imunofenotipagem; imunossupressores; transplante de rim.

Introduction

Kidney transplantation is recognized as a breakthrough in modern medicine, increasing the expectancy and quality of life of patients with irreversible renal failure. What was an experimental, risky and very limited treatment option 50 years ago, is today a routine clinical practice in more than 80 countries.[1]

Individuals in the terminal stage of chronic kidney disease (CKD) need to choose a substitution therapy: dialysis or renal transplantation.[2,3] In Brazil, transplantation is the most cost-effective substitution therapy,[4,5] being the best alternative for quality of life, morbidity, and mortality.[6,7] One factor that contributes substantially to this therapy being considered the best option for patients with CKD is the technological advance related to immunosuppressive therapy.[8,9]

The use of immunosuppressive therapy has contributed to the expansion of transplants, by inhibiting cell division and having anti-inflammatory properties. It is prescribed in the prevention of transplant organ rejection and in the treatment of autoimmune and chronic inflammatory diseases.[10] Because they modulate the effector T-cell response in the presentation of antigens, these drugs increase graft and patient survival.[11,12] As a therapy of Induction, associated with the best responses, polyclonal antithymocyte immunoglobulin (ATG) from horses or rabbits is used.[13,14]

Post-kidney transplant ATG is monitored by the quantification of absolute T-lymphocytes, carried out by immunophenotyping using Flow Cytometry,[15,16] which must be less than 20 cells/μL of total leukocytes.[17-19] Therefore, the dose is adjusted according to CD3 quantity.

The aim of this study was to analyze the quantifications of T-lymphocytes by immunophenotyping, of patients submitted to renal transplantation who used ATG, in a university hospital, and to establish a cutoff point for total lymphocytes in the automated blood count report, estimating a count Less than 20 cells/μL when compared to the standard technique.

Methods

Retrospective laboratory data was obtained from electronic medical records in the AGHU (Management Applications for University Hospitals) system of a University Hospital of Porto Alegre, RS, Brazil, from patients submitted to kidney transplantation from January 2014 to June 2015, totaling 242 samples.

We included samples from renal transplant recipients using ATG, submitted to T-lymphocyte quantification by immunophenotyping and total blood count or leukogram in the same whole blood sample. The following patients were taken off the study: those submitted to transplants other than renal; those using immunosuppressants to treat immune deficiencies; those without a blood count or leukogram; those without donor lymphocyte counts and external patients.

The samples were collected after the transplant, varying the number of samples from each patient according to the medical request. Some patients entered the study more than once.

The evaluated patients received rabbit ATG at the dose of 1.5 mg/kg, which consists of the same quantity reported by Buchler *et al*.[18] In the study by Castro *et al*.,[19] they suggested that the thymoglobulin dose should be 1 to 1.5 mg/kg, at a cumulative total dose ranging from 4 to 8 mg/kg, depending on the patient's immunological risk.

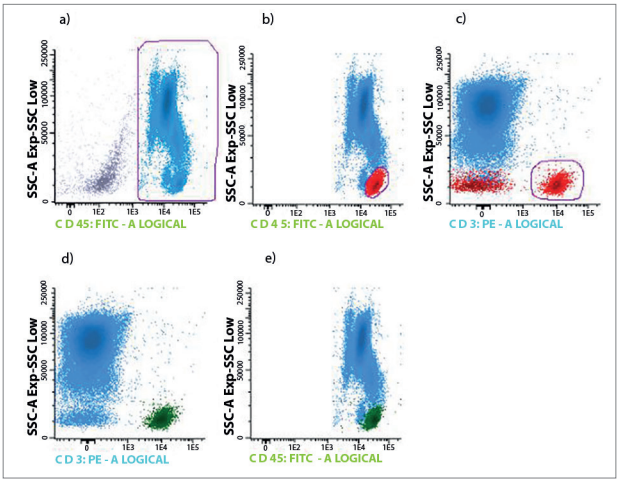
The study was developed according to the research ethics principles established by the Research Ethics Committee of the Hospital, CAAE: 50465815.4.0000.5327. Patients were not asked for any test or questioning, and we assured complete confidentiality regarding the identification of the individuals involved in the study. All the researchers signed the non-disclosure agreement to use data from the medical records.

Whole blood samples were used in K2EDTA anticoagulant tubes, which were processed within 2 hours after collection.

Blood counts and leukograms were performed on Sysmex® XE-5000 equipment (Sysmex Corporation, Kobe, Japan) to obtain the absolute leukocyte count and total lymphocyte count.

To characterize the T-cells, we used the immunophenotyping technique that identifies and quantifies these lymphocytes using specific monoclonal antibodies: CD45 (pan-leukocyte) conjugated with fluorochrome FITC (fluorescein isothiocyanate) and CD3 (T-lymphocyte marker) conjugated with PE (phycoerythrin). After labeling, the samples were obtained from the FACSCanto II Flow Cytometer (BD-Becton Dickinson, San Jose, California, USA) and analyzed in the Infinicyt® software, version 1.7 (Cytognos, Salamanca, Spain) (Figure 1).

Figure 1. Dot plot bivariate histograms illustrating the T-cell subpopulation of total leukocytes. (A) Gate strategy identifying CD45 + cells, corresponding to leukocyte cells in the sample (blue events); (B) selection of total lymphocytes within the leukocyte cells (red events) for recognition of T-lymphocytes in this population; And ending (c-e) gate of the T-lymphocytes within the total leukocytes.



The patients were divided into two groups following the absolute values of T-lymphocytes obtained by immunophenotyping, according to the therapeutic target: less than 20 cells/μL and greater than or equal to 20 cells/μL. Subsequently, the cut-off point for total lymphocytes was determined by the automated blood count, with specificity of 100% for a T-lymphocyte count of less than 20 cells/μL.

We evaluated the results using the Receiver Operating Characteristic (ROC) curve analysis from the SPSS 18.0 software (Statistical Package for the Social Science). Data normality was investigated by the Shapiro Wilk test, lymphocyte count variables obtained from the automated blood count and immunophenotyping by flow cytometry were evaluated by the Pearson correlation, the patients' ages were presented as mean ± standard deviation, and the rest of the data as median (interquartile range).

Results

A total of 242 patients aged 51 ± 13 years were evaluated, and 50.4% were male. Leukocytes and total lymphocytes were counted in the automated blood count, and the quantification of T-lymphocytes by immunophenotyping of the patients were 5,550.00/μL (4,017,50 - 7,865.00/μL), 212.9/μL (132.7 - 328.5/μL) and 10.4/μL (4.0 - 28.3/μL), respectively (Figure 2). The correlation between the number of total lymphocytes obtained by immunophenotyping *versus* automated blood count yielded an r = 0.88; $p < 0.001$ (Figure 3).

Figure 2. Distribution of total lymphocytes by immunophenotyping and automated blood count.

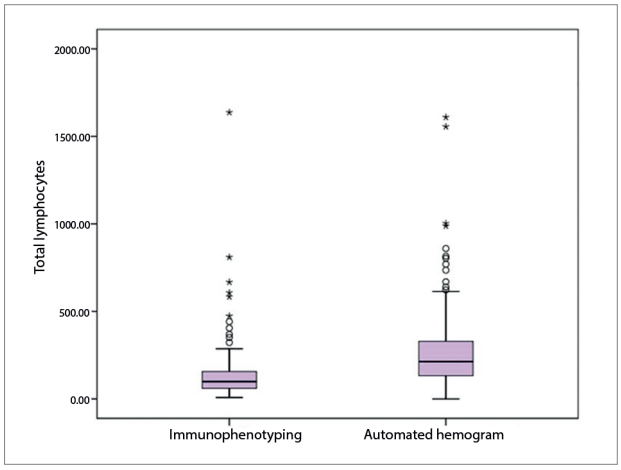
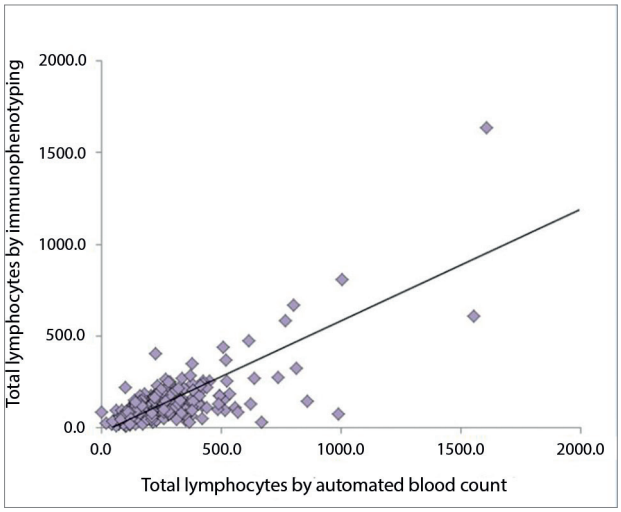


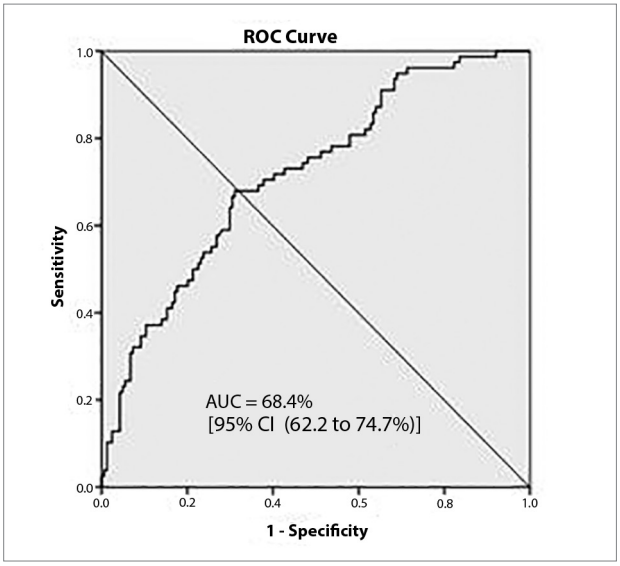
Figure 3. Correlation between the number of total lymphocytes obtained by immunophenotyping *versus* automated blood count (r = 0.88, $p < 0.001$).



Analyzing the sensitivity and specificity of different lymphocyte absolute values obtained by the automated blood count, a cut-off point of 70 lymphocytes/μL was found, which then excluded 100% of the patients with T-cell counts above 20 cells/μL on immunophenotyping.

In the ROC curve analysis, we found sensitivity and specificity of 67.95% [CI: 95% confidence interval (CI)] (56.96 to 77.25%) and 68.90% [CI: 95%, 45 to 75.49%)], respectively, to identify patients with T-lymphocytes > 20 cells/μL. The area under the curve (AUC) was 68.4% [CI: 95% (62.2 to 74.7%)], $p < 0.001$ (Figure 4).

Figure 4. ROC curve representing the sensitivity and specificity obtained for the cutoff point of 70 lymphocytes/µL, when the T-lymphocyte counts were > 20 cells/µL on immunophenotyping. The area under the ROC curve, the usual summary measure of test performance was 68.4% [CI: 95% (62.2 to 74.7%)]. The diagonal line shows the best sensitivity value for the best specificity value.



The rates of false positive, false negative, positive predictive value and negative predictive value were 31.1% [CI: 95% (24.51 to 38.55%)]; 32.05% [CI: 95% (22, 75 to 43.04%)]; 51.0% [CI: 95% (44.14 to 57.75%)] and 81.9% [CI: 95% (76.33 to 86.37%)], respectively.

Discussion

In this study, the established quantity of total lymphocytes in the peripheral blood to represent a T-cell value of less than 20 cells/µL was 70 lymphocytes/µL, agreeing with Castro *et al.*,[19] who reported that the lymphocyte count should be lower than 100 cells/µL, with a range between 50-150 cells/µL. However, the study by Buchler *et al.*[18] shows that the value should be less than 200 lymphocytes/µL.

It is important to note that the lymphocytes quantified by the blood count include all subtypes of lymphocytes, i.e., in addition to T-lymphocytes, also B and Natural killer (NK). Therefore, the value of 70 lymphocytes/µL represents a safe margin to ensure that the T-lymphocytes in the sample are less than 20 cells/µL.

The values found in the automated blood count protocols of the patients included in the study were 5,550.0/µL (4,017,50 - 7,865.00/µL) leukocytes; 212,0/µL (132,7 - 328,5/µL) total lymphocytes and 10.4/µL (4.0 - 28.3/µL) T-lymphocytes by immunophenotyping, similar to those reported by Buchler *et al.*[18]

The mean age found in renal transplant recipients was consistent with that reported by Buchler *et al.*,[18] who reported an average of 48 ± 13 years in their patients. However, in this same study there is no data on gender prevalence. In our study, we found the average of 51 ± 13 years; among these, 50.4% were male.

The ROC curve allows to highlight the values for which there is greater sensitivity optimization as a function of specificity. The area under the curve is a performance measure of a test (Test Accuracy Index), where values above 0.70 are satisfactory.[20] In this study, we found a value of 68.4% [IC: 95 % (62.2 to 74.7%)].

Sensitivity results (defined as the ability of the test under investigation to provide a positive result) were 67.95% (CI: 95% (56.96 to 77.25%)) and the specificity result (defined as the ability of the test under investigation to provide a negative result) was 68.90% [CI: 95% (61.45 to 75.49%)], both considered to be satisfactory.

Conclusion

This study proposes a cut-off point of 70 lymphocytes/µL by the automated blood count, reflecting a T-lymphocyte number lower than 20 cells/µL, if performed by immunophenotyping, a value recommended for dose adjustment during ATG treatment.

The results of this study may help in the monitoring of this immunosuppressive therapy in renal transplant patients in places that do not have flow cytometry, or when this equipment is not present in the service routine.

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