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Analysis of platelet eluate for the elucidation of sensitization to HLA in kidney transplant candidate

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ABSTRACT. While a 42-year-old male patient was being prepared for deceased-donor renal transplantation, anti-HLA-A2 antibodies were detected in the serum by enzyme-linked immunosorbent assay (ELISA) method. The patient denied any transfusion history and previous transplant. Crossmatch by complement dependent cytotoxicity (CDC) and CDC with anti-human globulin (CDC-AHG) proved negative with a four-cell panel with positive typing for HLA-A2. Adsorption of antibodies with platelets and analysis of eluate were suggested to elucidate discrepancies in results by ELISA and by CDC-AHG. ELISA showed that adsorbed serum with platelets did not reveal antibodies for HLA-A2 specificity and suggested that they were removed by their specific binding with HLA-A2 antigens on the platelet surface. Eluate analysis by ELISA showed antibodies for HLA-A2 specificity. No antibodies for HLA-A2 specificity in the non-adsorbed serum were detected by CDC-AHG method. Revision of patient's data showed that a previous transfusion had occurred, which may have been the source of HLA sensitization. The suggested method may be a contribution towards the evaluation of sensitivity between CDC-AHG and ELISA methods for characterizing antibodies in the patient's serum.

Keywords: antibodies. immunologic cytotoxicity tests. enzyme-linked immunosorbent assay. HLA Antigens. kidney transplantation.

Análise do eluato de plaqueta para a elucidação da sensibilização ao HLA em um candidato ao transplante de rim

RESUMO. Enquanto um paciente do sexo masculino de 42 anos de idade estava sendo preparado para o transplante renal de doador falecido, anticorpos anti-HLA-A2 foram detectados no soro pelo método de ensaio imunoenzimático (ELISA). O paciente negava história de transfusão e transplante anterior. Prova-cruzada por citotoxicidade dependente de complemento (CDC) e CDC com antiglobulina humana (CDC-AGH) foram negativos com um painel de quatro células com tipagem positiva para HLA-A2. O método de adsorção de anticorpos com plaquetas e análise do eluato foi sugerido para explicar as discrepâncias dos resultados de ELISA e CDC-AGH. O método de ELISA mostrou que o soro adsorvido com plaquetas não revelou anticorpos para especificidade HLA-A2, sugerindo que eles foram removidos por meio de sua ligação específica com os antígenos HLA-A2 na superfície das plaquetas. A análise do eluato por ELISA mostrou anticorpos para especificidade HLA-A2. Nenhum anticorpo para especificidade HLA-A2 foi detectada no soro não adsorvido pelo método de CDC-AGH. Revisão dos dados do paciente mostrou que houve transfusão anterior, podendo ter sido a fonte de sensibilização HLA. O método sugerido é uma contribuição para avaliação da sensibilidade entre os métodos de CDC-AGH e ELISA em caracterizar anticorpos no soro do paciente.

Palavras-chave: anticorpos. testes imunológicos de citotoxicidade. ensaio de imunoadsorção enzimática. antígenos HLA. transplante de rim.

Introduction

Sensitization to human leukocyte antigens (HLA) in transplant immunology is the occurrence of alloantibodies in the serum of patients who desire to receive organs, directed towards HLA antigens. Sensitization in transplant candidates is normally associated to one or more risk factors such as previous transfusions, pregnancy or transplants (VONGWIWATANA et al., 2003; SOOSAY et al., 2003; MAO et al., 2007).

Alloantibodies cause graft destruction through several mechanisms such as complement activation and leukocyte recruitment (VONGWIWATANA et al., 2003). The need to identify sensitization against the donor's HLA antigens caused the standardization of routine crossmatch prior to any transplant. A positive

crossmatch against T lymphocytes is an absolute contraindication against transplant owing to high hyper-acute rejection risks and to the association of chronic rejection (GEBEL et al., 2003). Systematic research of anti-HLA antibodies in receptor serum also provides important information on the receptor sensitization with regard to immunologically compatible organs (TERASAKI; CAI, 2008).

Sensitization to HLA antigens is normally determined by Panel Reactive Antibodies (PRA) which evaluate serum reactivity against the lymphocyte panel of known HLA specificity. The best PRA interpretation is provided by the patient's epidemiological information with regard to risk factors for anti-HLA alloantibodies formation (multiple pregnancy, poly-transfusion and re-transplants) (HYUN et al., 2012; MISHRA; BALIGA, et al., 2013).

A well-known standard method for research in anti-HLA antibodies consists of the reaction of complement-dependent cytotoxicity **(**CDC**)** which detects complement fixers antibodies IgM and IgG. They may be directed against HLA and non-HLA molecules (TERASAKI, McCLELLAND, 1964; PEÑA et al., 2013). The CDC method may be restricted by its inability to detect low antibody rates associated to transplanted organ rejection (GEBEL et al., 2003).

New techniques for the detection of anti-HLA antibodies, such as flow cytometry, ELISA and LABScreen, have recently been introduced in routine protocols in histocompatibility laboratories (ZEEVI et al., 2006; COLOMBO et al., 2007). Comparative studies on different methodologies showed great improvement in sensitivity by new techniques with regard to the standard cytotoxic method (ALTERMANN et al., 2006; KOZMA; BOHATY, 2007; LEE; OZAWA, 2007; LEE et al., 2009; HO et al., 2008; CERVELLI et al., 2013). However, improvement in sensitivity may cause discrepant results among the available methodologies. It is thus necessary to confirm anti-HLA antibodies detected only by immuno-enzymatic methods (ELISA) which are employed in histocompatibility laboratories. Current study provides a case report of a kidney transplant candidate who denied a history of risk factors for HLA antigens sensitization. However, the patient's serum provided 32% PRA with specificity for HLA-A2 antibodies detected by ELISA. Antibody adsorption method by platelets and the study of eluate were suggested to confirm results (MUELLER-ECKHARDT et al., 1972).

Material and methods

Case report

The serum of a 42-year-old male patient on the waiting list for deceased-donor renal transplantation revealed anti-HLA-A2 antibodies which were detected by ELISA during routine research for anti-HLA antibodies. The patient denied any transfusion or transplant. Crossmatch proved negative by CDC and CDC with anti-human globulin (CDC-AHG) with a four-cell panel with positive typing for HLA-A2.

Discrepancies in results from ELISA and CDC-AHG methods and the patient's history of sensitization aired the hypothesis of a possible positive false result by ELISA. The patient's serum adsorption method with platelets and a study of the eluate as a confirmatory test were suggested to confirm the hypothesis.

Methodology

Suspension of platelets:

Platelets were obtained from 5 mL periphery blood with anticoagulant ethylenediaminetetraacetic acid (EDTA) (Vacutainer; Becton and Dickson, Oxford UK) from a serum-type HLA-A2 donor. After centrifugation at 1500 rpm for 10 min for platelet-rich plasma, the supernatant was transferred to a new tube and centrifuged at 3000 rpm for five minutes. The supernatant was removed and platelets were washed twice with PBS/EDTA buffer and final concentration adjusted for 1×10^6 platelets mL^{-1}.

Adsorption of Antibodies:

Suspension of platelets was incubated with 500 µL serum under analysis, during 60 min., at 22ºC. After two washings and centrifuges at 3000 rpm, the final volume was adjusted to 300 µL with PBS.

Elution of Antibodies:

Suspension of adsorbed platelets was acidified with HCl 10 N up to pH 3.0 during 10 minutes and then neutralized with NaOH 10 N. The supernatant with eluted antibodies was separated for analysis after being centrifuged at 3000 rpm.

Study on adsorbed serum with platelets and on the eluate of adsorbed platelets:

The two materials were submitted to research protocols for anti-HLA antibody by ELISA LAT1240 (One Lambda, Inc., Canoga Park, CA, USA), according to instructions by manufacturer. Samples were diluted in a diluting solution provided by the kit and incubated at room temperature with

pre-defined quantities of purified HLA antigens on wells in a Terasaki plate (One Lambda, Inc., Canoga Park, CA, USA). Specific binding between an antibody in the test sample and any antigens in the plate would be detected by a subsequent incubation, at room temperature, with a human Anti-IgG/alkaline phosphatase set, followed by an incubation at 36ºC with BCIP (5-Bromo-4-chloro-3-indolyl-phosphate). Whereas the substrate becomes bluish in the presence of the set bound to the specific anti-HLA antibody, in its absence the set is removed at the washing stage and the substrate remains colorless. Spectrophotometric interpretation by a 630 nm wave length determines the presence or absence of anti-HLA antibodies by comparison with a cutoff. Statistic analysis determines the specificity of the detected antibody.

Ethics

Current study was approved by the Committee for Ethics in Research of the Universidade Estadual de Maringá (Protocol n. 192/2011).

Results and discussion

Kidney transplantion is an option for the treatment of end-stage renal disease (GARCIA et al., 2012). Donor-specific antibodies in the serum of patients who should receive kidney transplants are an important risk factor (GEBEL et al., 2003). Patients may develop an immune response subsequent to blood transfusions (SCORNIK, MEIER-KRIESCHE, 2011; BALASUBRAMANIAM et al., 2012; TANHEHCO; BERNS, 2012, YABU et al., 2013) due to HLA alloantibodies produced as a response to HLA alloantigens (HENDRICKSON; HILLYER, 2009; SCORNIK, MEIER-KRIESCHE, 2011). Consequently, it is highly difficult to find a donor with compatible organs because of the above alloimmunization (RODEY, 2003).

The omission of information (such as blood transfusion) by the organ recipient may cause ambiguous interpretations in laboratory results. The person accountable for these analyses should pay attention to this fact. Register of the above case is of paramount importance to histocompatibility professionals and, from the immunological point of view, to those responsible for the patient's admission for transplant.

Limitations of the CDC method in the detection of low antibody rates and the introduction of more sensitive techniques for the detection of anti-HLA antibodies have led to different results which frequently need confirmatory tests (WU et al., 2013).

The development of the antibody elution method with HCl described above provides a decrease in HLA class I molecules from the platelets surface previously adsorbed by serum which, in current case, is immune from anti-HLA-A2 antibodies (KURATA et al,. 1989).

Table 1 shows results by ELISA (LAT1240-One Lambda INC) with immune serum, with and without adsorption of platelets, and eluate. Whereas non-adsorption serum was positive for anti-HLA-A2 antibodies up to dilution 1/64, serum with adsorption failed to react to anti-HLA-A2 antibodies. Data suggested that antibodies had been removed by specific bind with HLA-A2 antigens on the platelet surface. Current results corroborated those by Blumberg, 1984 (BLUMBERG et al., 1984) who eluted HLA-A2 and HLA-B7 antigens from platelets by elution with acid. Therefore, antigens originated from platelets through the adsorption of soluble HLA antigens in the blood. Other authors have described the partial loss of HLA antigens from the platelet surface by using chloroquine or acid treatment (KURATA et al., 1989; NEUMÜLLER et al, 1993).

Table 1. Result by ELISA method with patient's serum, with and without adsorption, with platelets A2+ and the respective eluate.

Dilution of patient's serum	ELISA reaction with non-adsorbed serum with platelets	ELISA reaction with adsorbed serum with platelets	ELISA reaction with eluate
Pure	Positive	Negative	Positive
1 / 2	Positive	Negative	Positive
1 / 4	Positive	Negative	Positive
1 / 8	Positive	Negative	Positive
1 / 16	Positive	Negative	Negative
1 / 32	Positive	Negative	Negative
1 / 64	Positive	Negative	Negative
1 / 128	Negative	Negative	Negative

Table 1 also shows that, since the eluate reveals the presence of anti-HLA-A2 antibodies recovered from absorbed platelets up to 1/8 dilution, the binding of antibodies on specific HLA platelets is proved.

According to results in Table 2, non-adsorbed serum reactive to molecule HLA-A2 in the ELISA method up to 1/64 dilution failed to have any reaction in the CDC-AHG method.

Table 2. Result of the CDC-AHG method between the patient's serum (non-adsorbed serum) with 4 different lymphocyte of donors with HLA-A2.

Lymphocyte source	HLA-A phenotype	CDC-AHG results
Donor 1	02, 68	Negative
Donor 2	02, 30	Negative
Donor 3	02, 11	Negative
Donor 4	02, 33	Negative

Results are consistent with the hypothesis that HLA-A2 antigens were adsorbed from the platelets' surface by acid elution. They also elucidate the difference in the sensitivity of CDC and ELISA methods to detect antibodies. When patient's data were re-analyzed, a previous transfusion to the test was confirmed. It had been omitted in the first report and may have been the probable source of HLA sensitization, specifically HLA-A2.

Conclusion

Adsorption of antibodies with platelets and eluate analysis contributed towards an evaluation of sensitivity between CDC-AHG and ELISA methods for the characterization of the antibody specificity under analysis. It also confirms a history of the patient's sensitization by a clarification of the report´s inconsistency. Additional evaluations of the protocol should be performed so that its potential as a confirmatory test in discrepancy cases between different antibody characterization tests may be evaluated.

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