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Sequential analysis by immunoprecipitation-MALDI-TOF: A novel method for detection and identification of alloantibody specificities

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ABSTRACT

Alloantibodies are known to influence transplant outcomes. Apart from human leukocyte antigens (HLA), non-HLA targets have been suggested to play a significant role, but little is known about their nature. Here, we present a novel method for identification and characterization of cell surface antigens bound by alloreactive antibodies. Our method consists of 2 consecutive steps: first, immunoprecipitation of cell surface proteins is carried out with serum and, second, matrix-assisted laser desorption/ionization-time-of-flight is used to fingerprint the precipitated cell-surface proteins. As an example, we performed immunoprecipitation with peripheral blood lymphocytes, which had been incubated with an alloreactive serum; immune complexes were coupled to protein-G beads and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis; differential protein fractions were then analyzed by matrix-assisted laser desorption/ionization-time-of-flight. The method was validated with serum as well as with plasmapheresis material, which contained antibodies of known HLA specificities, demonstrating its applicability for clinical use.

1. Introduction

Antibody-mediated rejection is a major cause for graft failure in solid organ transplantation [1–3]. It is well established that in human leukocyte antigen (HLA)–mismatched transplants, anti-HLA antibodies may arise and exert damage to the graft through complement activation and/or antibody-dependent cell-mediated cytotoxicity [4]. In addition, there is increasing evidence that non-HLA antibodies also have a significant negative impact on allograft survival [5–8]. However, little is known about the nature of non-HLA antigens targeted by alloantibodies. Most studies reported detection of antibodies against monocytic or endothelial primary cells or cell lines, without further characterization of the protein structures bound by these antibodies [9–11].

In the literature, 2 methods have been used for characterization of previously unknown antigens recognized by antibodies in the sera of patients after organ transplantation [12,13]. One method is the serologic analysis of antigens by recombinant expression cloning (SEREX) [13,14]. The other method is based on protein arrays [12]. However, these methods use artificial expression systems, potentially representing denatured or improperly glycosylated proteins and, therefore, bearing the risk of missing clinically relevant antibodies.

Here, we present a novel method, which we designate as SIMT for Sequential analysis of Immunoprecipitation followed by Matrix-assisted laser desorption/ionization-Time-of-flight mass spectrometry (MALDI-TOF). This method allows the identification of antigens that are expressed on the cell surface and are able to induce a humoral immune response. To establish and validate the method, we used peripheral blood lymphocytes with known HLA types and antibodies with known HLA specificities. Because native human cells are used in our test, disadvantages associated with artificial expression systems such as those mentioned earlier can be avoided. At the same time, unlike some previous reports on detection of antibodies against intracellular structures [12,13], our method detects only proteins that are expressed on the cell surface and, therefore, are more likely to be relevant in the clinical transplantation setting.

2. Subjects and methods

2.1. Peripheral blood and plasmapheresis materials

Peripheral blood was obtained from voluntary healthy blood donors at our department in Heidelberg, Germany. Heparinized blood was diluted 1:2 with RPMI-1640 (GIBCO, Grand Island, NY) before adding onto Lymphodex (Fresenius, Bad Homburg, Germany). Lymphocytes were isolated by discontinuous density gradient centrifugation as recommended by the manufacturer, then adjusted to 2×10^7 cells/ml in RPMI + 7% dimethyl sulfoxide + 5%

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fetal calf serum and stored at −80°C until use. Just before immunoprecipitation, 8×10^7 cells were washed twice with 50-ml ice-cold Dulbecco's phosphate-buffered saline (D-PBS; GIBCO, Grand Island, NY). HLA typing was performed using Biotest Lymphotype HLA-AB 120 Special (Biotest, Dreieich, Germany) and Protrans S4 HLA-B Sequencing Kit (Protrans, Ketsch, Germany). HLA-B27–specific serum obtained from a voluntary blood donor was kindly provided by the CTS Laboratory, University of Heidelberg, Heidelberg, Germany. Plasmapheresis material (plasma that was removed through a plasmapheresis procedure) was collected from a patient who was found to have HLA-B7–specific antibodies and underwent desensitization in preparation for kidney transplantation at our hospital. Plasmapheresis material was aliquoted and frozen at −80°C. For immunoprecipitation, plasmapheresis material was thawed and centrifuged for 15 minutes at 4°C and 2,000*g*, and the supernatant was filtrated with the Stericup system (Millipore, Billerica, MA). HLA-B27–specific and HLA-B7–specific antibodies were characterized in a complement-dependent cytotoxicity assay using Lymphoscreen ABC 60-cell trays (Biotest, Dreieich, Germany). The study was approved by the ethics committee of the University of Heidelberg, Heidelberg, Germany.

2.2. Immunoprecipitation

Twenty milliliters of serum or 45 ml of plasmapheresis material (equal volumes of D-PBS for negative controls) were added to 8×10^7 cells, which had been resuspended in 500 μl of D-PBS. To each sample, 200 or 450 μl of 100 mM phenylmethylsulfonyl fluoride (isopropanol as solvent) were added, respectively. Samples were incubated for 90 minutes at 4°C on an agitator. After incubation, cells were pelleted, washed with 50 ml of ice-cold PBS, and lysed in 500 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid, 1% protease inhibitor cocktail [Sigma, Saint Louis, MO], and 0.01% Proclin300 [Sigma Aldrich Chemie, GmbH, Steinheim, Germany]) for 5 minutes at 4°C in ultrasonic bath and for 15 minutes on ice. Cell lysate was centrifuged for 10 minutes at 4°C and 10,000*g*. Supernatant was added to 40 μl of a 25% protein G bead slurry (Santa Cruz Biotechnology, Santa Cruz, CA), which was equilibrated and washed with immunoprecipitation (IP) wash buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, and 5 mM ethylenediaminetetraacetic acid). The mixture of cell lysate and protein G beads was incubated for 60 minutes at 4°C on an agitator and then washed 4 times with 800 μl of IP wash buffer. The pelleted beads were incubated with 23 μl of elution buffer (200 mM Tris-HCl pH 6.8, 6% SDS, 15% glycerin, 0.03 bromophenol blue, and 15% β-mercaptoethanol) for 5 minutes at 95°C on an agitator; 20 μl of the supernatant was saved.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 7.5% Ready Gel Tris-HCl Gel (Bio-Rad, Hercules, CA). Twenty microliters of each IP eluate were loaded and electrophoresis was performed at 100 mV. The gel was stained with the SilverSNAP Stain for Mass Spectrometry Kit (Pierce, Rockford, IL). Protein bands visible in specific lanes were excised and analyzed by mass spectrometry.

2.4. Matrix-assisted laser desorption/ionization-time-of-flight

The excised polyacrylamide gel pieces were destained with 15 mM potassium hexacyanoferrate (II) plus 50 mM sodium thiosulfate, followed by incubation with wash solution I (10-mM ammonium bicarbonate) and wash solution II (1:1 solution of acetonitrile with 10-mM ammonium bicarbonate) for 10 minutes each. Disulfide bond cleavage was performed with 10 mM dithiothreitol and carbamidomethylation with 55 mM iodine acetamide. The gel pieces were washed with wash solutions I and II for 10 minutes. The dried gel pieces were soaked in 5-μl trypsin solution (10 μl of 100 ng/μl trypsin + 20 μl of 10-mM ammonium bicarbonate), covered with 10-mM ammonium bicarbonate and incubated overnight. On the following day, 0.5 μl of the sonicated sample was mixed with 0.5 μl of matrix (0.05% trifluoroacetic acid, 50% acetonitrile, and 10 mg/ml 4-OH-α cinnamic acid) and spotted on a stainless steel MALDI carrier tray.

MALDI-time-of-flight (TOF)-MS was performed on a 4800 MALDI-ToF/ToF mass spectrometer (Applied Biosystems, Framingham, MA). Peak lists were generated using Data Explorer Software 4.0 (Applied Biosystems) and calibrated against a peptide standard mixture (des-Arg-Bradykinin m/z = 904.4681, Angiotensin I m/z = 1,296.6853, Glu-Fibrinopeptide B m/z = 1,570.6774, adrenocorticotropic hormone clip 1-17 m/z = 2,093.0867, adrenocorticotropic hormone clip 18–39 m/z = 2,465.1989). The tryptically digested proteins were identified using MASCOT database search engine (http://www.matrixscience.com) with National Center for Biotechnology Information and Swissprot database with a mass tolerance ≤ 0.1 Da of the monoisotopic peaks, a maximum of 2 missed cleavages per peptide, unrestricted taxonomy, and with the cysteine carbamidomethylation option enabled.

3. Results

3.1. Identification of HLA-B27 as antigenic target of a serum by SIMT

Figure 1 summarizes our method, called Sequential analysis of Immunoprecipitation followed by MALDI-TOF (SIMT), a procedure for identification of antigenic structures in two main steps: first immunoprecipitation, and second, analysis of the precipitated antigens by MALDI-TOF. We established the method by determining the antigenic target of a serum known to be specific for HLA-B27. Lymphocytes were isolated from two blood donors who differed in their serologic HLA-B types: one individual carried HLA-B27, B51 (B27-positive cells), whereas the other individual was typed as HLA-B57, B63 (B27-negative cells). After incubation with an HLA-B27–specific serum, cell lysate was added to protein G beads and the antigen–antibody complexes were eluted. After SDS-PAGE and silver staining (Fig. 2), a strong protein band at approximately 40 kDa was observed in the lane with B27-positive cells + B27-specific serum (lane 1) but not in the lane with B27-negative cells + B27-specific serum (lane 2). The 40-kDa protein band of lane 1 was cut out of the gel and analyzed by MALDI-TOF peptide mass fingerprinting. The results of MALDI-TOF are shown in Table 1. The amino acid sequence with the accession number AAL87223 reached the highest score (p = 0.00055) and proved to be identical with the amino acid sequence of HLA-B*2705, exon 2 to exon 4 (accession number AJ420238). To verify the HLA molecule, the person from whom the B27-positive cells were obtained was HLA-typed at high resolution level by sequencing. The sequencing result was HLA-B*2705, confirming that SIMT was able to correctly identify the protein that was expressed on the lymphocyte surface and recognized by the B27-specific serum. To assess the specificity of the method, a gel piece was excised at 40 kDa from lane 2 (*i.e.*, IP performed with the B27-negative cells + B27-specific serum) (Fig. 2). MALDI-TOF peptide mass fingerprint analysis of this gel piece revealed no significant results (data not shown); thus, no protein was identified in the B27-negative precipitate. Protein bands visible in lane 1 and lane 2 at similar intensity (Fig. 2) most likely