Effect of Donor Cell Type on Complement-Dependent Cytotoxicity Crossmatch Outcome for Patients Immunized Against HLA Class-II Antigens

Marie-Luise Arnold,[1] Stephan M Ensminger,[2] Ilias I N Doxiadis,[3] Bernd M Spriewald[1]

Abstract

Objectives: **In the Eurotransplant zone, the crossmatch serum exchange program was established to reduce unnecessary organ shipment, using the complement-dependent cytotoxicity crossmatch as the reference to make the decision. Crossmatching at the donor center dictates whether the transplant should be shipped to the recipient center where a decisive crossmatch would then be done. However, in recent years, the target cell used for the crossmatching has changed from spleen cells to peripheral blood lymphocytes. In this study, we assess the impact of this change on the outcome of complement-dependent cytotoxicity crossmatches for patients immunized against HLA-class II.**

Materials and Methods: **The influence of the donor cell type was analyzed by crossmatching unseparated peripheral blood lymphocytes, separated T and B lymphocytes, as well as spleen cells from 12 organ donors with sera from 40 immunized kidney retransplant candidates. Negative sera and sera harboring only anti-HLA class-II antibodies were used as additional controls. We did more than 1200 complement-dependent cytotoxicity crossmatches.**

Results: **Crossmatches with sera containing anti-HLA class-I plus class-II alloantibodies (n=113 per cell type) were positive in 42% of peripheral blood lymphocytes, 72% of spleen cells, and 81% of B cells. Crossmatches with sera containing exclusively anti-HLA class-II antibodies (n=89 per cell type) were positive in 1% of peripheral blood lymphocytes, 30% of spleen cells, and in 31% of B cells. Overall, spleen or separated B cells identified approximately 30% more positive donor-recipient pairs.**

Conclusions: **The data show that the change from spleen cells to peripheral blood lymphocytes as donor target cells for complement-dependent cytotoxicity crossmatching increased risk of false negative results for patients harboring anti-HLA class-II antibodies.**

Key words: *Alloantibody, CDC crossmatch, Eurotransplant International, Virtual crossmatch*

The introduction of the complement-dependent cytotoxicity crossmatch assay in the 1960s was a milestone in solid organ transplant, strongly reducing the incidence of hyperacute and acute vascular rejections (1, 2). In Eurotransplant, the test format of complement-dependent cytotoxicity crossmatches is still the mainstay in the transplant decision-making process. More recently, the donor target cell for the complement-dependent cytotoxicity crossmatch in renal transplant changed from spleen cells to peripheral blood lymphocytes. This was due mainly to the introduction of reliable molecular-based HLA-typing done from peripheral blood lymphocytes before explantation. Using the same material for crossmatching to reduce cold ischemia time, the kidney allocation can be done before explantation. However, the possible effect of this change in cell source on complement-dependent cytotoxicity crossmatching has not yet been investigated.

Because the Eurotransplant zone covers a vast area with numerous transplant centers, the

From the [1]Department of Internal Medicine 3 and Institute for Clinical Immunology; and the [2]Center for Cardiac Surgery, Friedrich-Alexander University Erlangen-Nürnberg, Germany, and the [3]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Acknowledgements: *We are indebted to Ms Silvia Klöcker and Ms Martina Geithner for their expert technical support.*

Address reprint requests to: *Bernd M. Spriewald, Department of Internal Medicine 3, Institute for Clinical Immunology, University of Erlangen-Nürnberg, Glückstrasse 4a 91054 Erlangen, Germany*
Phone: *+49-9131-8536345* ***Fax:*** *+49-9131-8533399* ***E-mail:*** *bernd.spriewald@uk-erlangen.de*

Experimental and Clinical Transplantation (2008) 1: 1-6

crossmatch serum exchange program was established to avoid unnecessary organ shipment and reduce costs and cold ischemia times (3). For immunized patients awaiting kidney transplant, sera with alloantibodies of more than 5% panel reactive antibodies are sent from the recipient center to all participating Eurotransplant transplant centers. In the frequent event that the donor and the recipient center are not identical, a preliminary crossmatch for immunized recipients is done at the donor center. Should this preliminary crossmatch be negative, the transplant is shipped to the recipient center where a second definitive crossmatch is done. Interestingly, this second crossmatch is still preferentially done with spleen, accompanying the transplant.

We have observed several instances in which the recipient center crossmatch gave a discrepant positive result compared with the negative donor center crossmatch. Most of these transplant candidates had been immunized against HLA-class II. This prompted us to investigate the effect of the donor cell source on complement-dependent cytotoxicity-crossmatch outcomes for sera containing anti HLA-class I and/or class-II alloantibodies, using unseparated peripheral blood lymphocytes, separated T and B lymphocytes, or spleen cells.

Materials and Methods

Study design

Unseparated peripheral blood lymphocytes, separated T and B lymphocytes from peripheral blood lymphocytes, and spleen cells from 12 deceased-donor organs were used for complement-dependent cytotoxicity crossmatching. To rule out interference with ABO-isoagglutinins, sera were selected according to blood group identity with the respective organ donor. Characterized sera were obtained from 40 immunized kidney retransplant candidates on the waiting list of the transplant center Erlangen-Nürnberg, Germany. These patients had no evidence for autoantibodies and had not received immunoglobulin preparations.

According to the anti-HLA alloantibody status, the patients' sera were divided into 4 groups: Group 1 consisted of 23 sera containing HLA class-I (mean panel reactive antibodies, 46%) and HLA class-II (mean panel reactive antibodies, 47%) alloantibodies, whereas group 2 consisted of 17 sera containing only HLA class-II alloantibodies (mean panel reactive antibodies, 38%). In both groups, sera were tested against all blood-group–identical organ donor cells, resulting in a total of 113 applicable donor-recipient pairs for group 1 and 89 for group 2. Four complement-dependent cytotoxicity-crossmatch tests using either unseparated peripheral blood lymphocytes, separated T and B lymphocytes, or spleen cells were done per donor-recipient pair, a total of 452 for group 1 and 356 for group 2.

As an additional control, crossmatching was done as described above using 16 anti-HLA–negative sera (panel reactive antibodies, 0%, resulting in 64 applicable donor-recipient pairs [group 3]) and 16 sera containing only anti-HLA class-I antibodies (mean panel reactive antibodies, 41%, resulting in 56 applicable donor-recipient pairs [group 4]).

Isolation of donor cell types

Peripheral blood lymphocytes as well as spleen cells were isolated using Lymphoflot (Biotest AG, Dreieich, Germany), and preserved in Lymphostabil medium (Biotest) until cell fractioning was completed. T and B lymphocytes were separated from peripheral blood lymphocytes using magnetic Dynabeads (Dynal Biotech, Oslo, Norway). Cell viability was assessed using eosin staining.

Complement-dependent cytotoxicity crossmatch test

Cells were stained with acridine orange (Sigma-Aldrich, ST Louis, MO, USA), and 2000 to 3000 cells were incubated with 2 μL of the respective serum for 45 minutes in quadruplicate at 20°C. Then 5 μL of complement and ethidium bromide solution (Sigma-Aldrich) was added for another 60 minutes. Afterwards, the percentage of dead cells was evaluated with ultraviolet light under a fluorescent microscope. A crossmatch was considered positive with a cutoff of 30% dead cells. Each test contained positive and negative controls in triplicate. The results were compared with respect to cell source for both serum groups. The frequency of positive crossmatches was compared between groups using the Yates corrected chi-square test using SPPS software (Statistical Product and Services Solutions, version 11.0, SPSS Inc, Chicago, IL, USA) and corrected for multiple comparisons by multiplying the P value with the number of comparisons.

Characterization of anti-HLA alloantibodies

Panel-reactive lymphocytotoxic antibodies (panel reactive antibodies) and anti-HLA class-I alloantibody specificities were determined by complement-dependent cytotoxicity in the standard micro-lymphocytotoxic assay using a frozen cell tray containing peripheral blood lymphocytes with a random panel size of 56 cell donors (Lymphoscreen, Biotest). Subsequently, a generic solid-phase assay, the LAT mixed (One Lambda, Inc., Canoga Park, CA, USA) was used to identify anti-HLA class-II antibodies. To determine the HLA specificity of the antibodies, a specific solid-phase assay based on Luminex technology was used for HLA class-I and class-II antibodies (Labscreen, One Lambda).

Flow cytometry

Flow cytometry was done with an EPICS cytofluorimeter (Coulter, Hialeah, FL, USA). HLA class-I expression was detected using a phycoerythrin conjugated monoclonal antibody against HLA class-I (anti-ABC antibody, G4b-2.6, Becton-Dickinson, Franklin Lakes, New Jersey, CA), and HLA class-II expression was determined using fluorescein-isothiocyanat conjugated anti-HLA class-II antibody (anti-DR antibody, Immun-357, Beckman Coulter, Inc., Fullerton, CA, USA). A 100-μL cell suspension (1×10^5 cells) was incubated at 4°C with 4 μg of both antibodies. After 30 minutes, the cells were washed with phosphate-buffered saline and subsequently fixed with phosphate-buffered saline containing 1% paraformaldehyde unstained cells, which were use as negative controls. Because Dynabeads cannot be used in flow cytometry, cells were separated for this purpose using CD3 and CD19 MACS MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Results

An overall good discrimination between positive and negative crossmatch tests using the 30% threshold was practicable, as shown in Figure 1. When the number of positive crossmatches was compared between the 4 cell

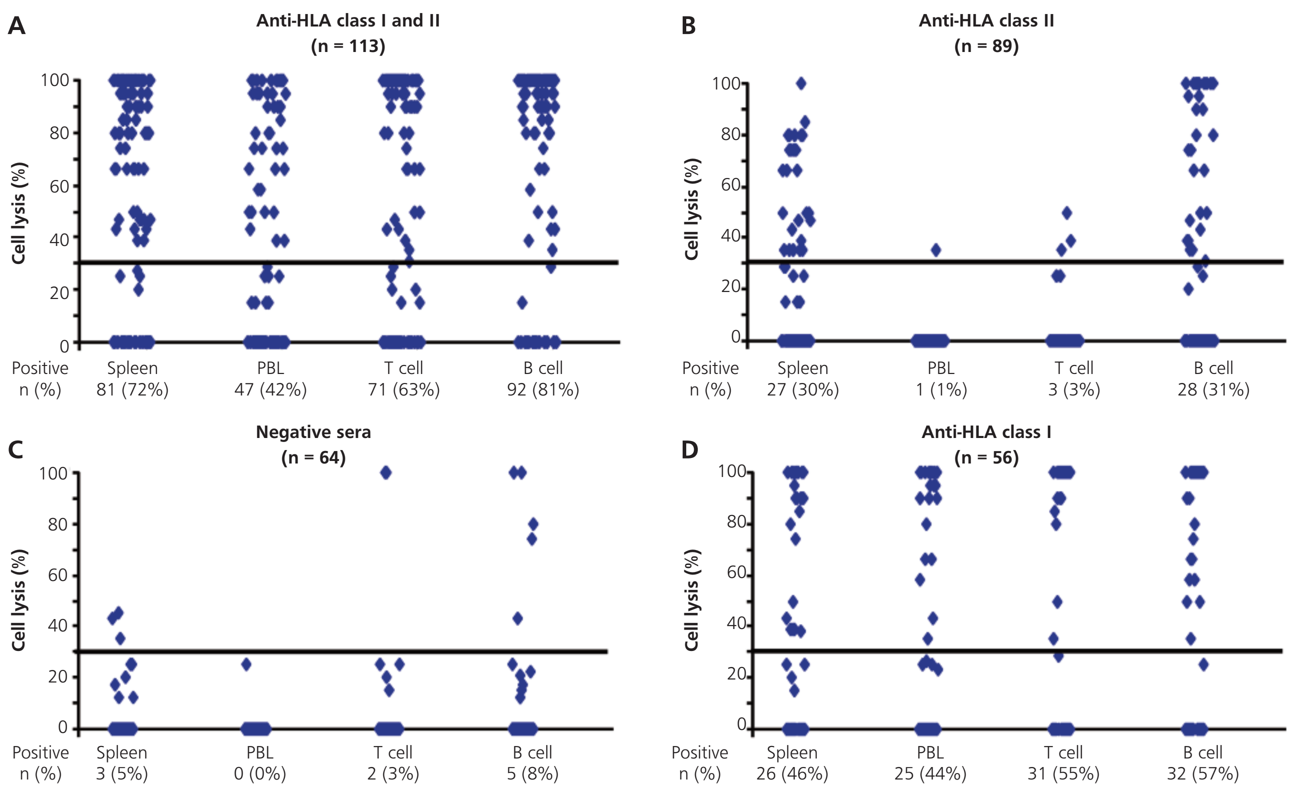

Figure 1. Complement-dependent cytotoxicity crossmatch results for kidney retransplant candidates immunized against HLA class I and II (A, n=113 per cell type) or exclusively against HLA class II (B, n=89 per cell type). As controls, complement-dependent cytotoxicity crossmatches were done using sera from nonimmunized patients (C, n=64 per cell type) or from patients immunized exclusively against HLA class I (D, n= 56 per cell type). The crossmatch was done with spleen cells, unseparated mononuclear peripheral blood cells (PBL), or T and B cells separated from peripheral blood, which were derived from the same respective donor. The dashed line depicts the 30% cell lysis cutoff line used to discriminate positive (≥ 30%) from negative (< 30%) test results. The number of positive test results is shown below the respective cell type.

types, significant differences were noted for anti-HLA class-II positive sera. For sera containing both anti-HLA class-I and class-II antibodies (group 1), the number of positive results increased from 47 out of 113 for peripheral blood lymphocytes (42%) to 81 out of 113 for spleen cells (72%, $P < .005$, Figure 1A). The highest reactivity was seen for separated B cells, with 92 out of 113 (81%, $P < .005$, compared with peripheral blood lymphocytes). Since B cells also express high levels of HLA class I, we could not be sure which antibody class contributed to the respective test result.

On analysis of group 2, which was composed of sera containing exclusively anti-HLA class-II antibodies, the differences were even more pronounced (Figure 1B). Peripheral blood lymphocytes and separated T cells hardly reacted at all with the tested sera (1% and 3% positive results). However, crossmatches with spleen cells reached 27 out of 89 (30%, $P < .005$ compared with peripheral blood lymphocytes) and separated B cells 28 out of 89 (30%, $P < .005$ compared with peripheral blood lymphocytes). Apart from 2 crossmatch tests, the positive tests identified the same donor-recipient pairs. It is noteworthy that the difference in crossmatch outcome between spleen and separated B cells on the one hand and peripheral blood lymphocytes on the other was about 30% for both serum groups. This suggests that the use of spleen cells or separated B cells increases the rate of positive crossmatches by about one-third for sera containing anti-HLA class-II antibodies, either alone or in conjunction with anti-HLA class I.

On the other hand, for crossmatch results with negative sera (Figure 1 C) or sera containing only anti-HLA class-I antibodies (Figure 1D), no significant differences were noted.

Next, we analyzed group 2, composed of sera containing only anti-HLA class-II antibodies, to determine whether the positive crossmatches could be explained by donor-specific alloantibodies or repeated mismatches (Table 1). This was the case in about 80% of positive crossmatches; only 20% did not show donor-specific HLA-DR or DQ antibodies or repeated mismatches. This emphasizes the importance of defining alloantibody specificity in immunized transplant candidates.

To substantiate our observation, expression of HLA class I and II was analyzed using flow cytometry in all 4 cell populations, isolated from the respective donors. Figure 2 shows that spleen cells are strongly enriched for HLA class II, and that separated B cells consist nearly exclusively of HLA class-II expressing cells but also strongly express HLA class I, which is in agreement with previous findings (4).

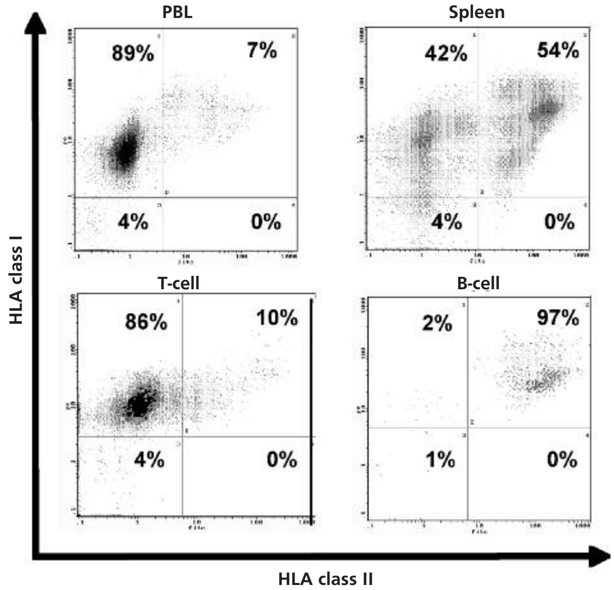

Figure 2. HLA class-I and class-II expression is shown for the respective cell populations used in the complement-dependent cytotoxicity crossmatch tests using flow cytometry. Cell populations were double stained for HLA class I and II. The result is representative of 12 analyzed donors.

Discussion

The aim of this study was to evaluate the influence of the donor cell source on direct complement-dependent cytotoxicity crossmatches for patients immunized against HLA class-II antigens. This was prompted by the fact that organ allocation in Eurotransplant is still based on the complement-dependent cytotoxicity crossmatch, and that the cell source for the initial crossmatch in the respective donor center has recently been changed from spleen cells to peripheral blood lymphocytes to reduce the cold ischemia time. The data obtained in this study indicate that for sera with anti HLA class-II alloantibodies, either alone or in conjunction with anti-HLA class-I antibodies, the positive rate of the complement-dependent cytotoxicity crossmatch will be reduced by approximately 30 percent.

Unseparated peripheral blood lymphocytes hardly detected anti-HLA class-II alloantibodies

(Figure 1B). If the crossmatch in the donor center is negative, the organ will be shipped to the recipient center where the crossmatch will be repeated, usually using spleen cells. As indicated by our data, a discrepant positive spleen complement-dependent cytotoxicity crossmatch can be expected in about one-third of patients immunized against anti-HLA class-II antigens. This will result in reallocation with a consecutively increased cold ischemia time. On reviewing the crossmatches done in our region for patients with anti-HLA class-II antibodies, we observed a discrepancy rate of about 20%. Because the overall advantage of using donor peripheral blood lymphocytes for typing and crossmatching is obvious for reducing cold ischemia time, the disadvantage for patients immunized against HLA class II must be resolved without changing crossmatch policy.

As indicated in Table 1, about 80% of cases with a positive B-cell crossmatch could be explained by either donor-specific anti-HLA-DR and/or DQ antibodies or by repeat mismatches. The ratio was the same for the spleen cell crossmatches (data not shown). This shows the importance of thoroughly screening the sera to identify specificities. This is further substantiated by the fact that about one-fourth of patients immunized against HLA class-II antigens had a negative B-cell crossmatch despite donor-specific antibodies or repeated mismatches (Table 1). Because nearly all retransplant candidates develop donor-specific alloantibodies (5, 6), the respective transplant center can decide about defining previous mismatches, especially HLA class II, as an unacceptable antigen, albeit there is no general consensus on defining repeat mismatches as unacceptable antigens (7).

Table 1. Correlation of donor-specific anti-HLA antibodies or repeated HLA class-II mismatches with B-cell crossmatch outcome.

	B-cell crossmatch (n=89)	
	Negative (n=61)	**Positive (n=28)**
Donor-specific anti-HLA DR or DQ antibodies	7 (11%)	10 (36%)
Repeated HLA class-II mismatches	7 (11%)	6 (21%)
Both	3 (5%)	6 (21%)
Neither	44 (72%)	6 (21%)

However, with respect to antibody screening, it is important to note that not all HLA class-II antibody specificities such as DP will routinely be identified because donors are not routinely typed for these antigens (8). Therefore, crossmatching, including direct complement-dependent cytotoxicity crossmatches, despite their known potential shortcomings (9), will remain valuable tools for immunized patients in the foreseeable future. In this respect, it was interesting to note that the rate of positive crossmatches for these patients was similar when spleen cells or separated B cells were used. This indicates that B cells as the target did not introduce an excessive sensitivity compared with spleen cells in this rather robust direct complement-dependent cytotoxicity test.

Although the debate about the impact of anti-HLA class-II alloantibodies on renal transplant is ongoing (10), there is accumulating evidence that transplant across a positive B-cell crossmatch has a negative effect on transplant outcome (11, 12), especially in patients immunized against HLA class II. Therefore, many centers will not transplant over a positive B-cell or spleen-cell crossmatch in immunized recipients.

In summary, the use of unseparated peripheral blood lymphocytes resulted in about one-third more negative crossmatches for patients with anti-HLA class-II antibodies. If the organ is transplanted locally, a crossmatch based on peripheral blood lymphocytes alone might not provide all the necessary immunologic information for clinicians and immunized patients alike to allow for an informed decision. If the organ is allocated to a national or international center, shipment of organ grafts based on peripheral blood lymphocytes crossmatches for transplant candidates immunized against HLA class-II should be avoided. This could be done in part by virtual crossmatching, which requires defining the alloantibody specificity, communicating the results, and as a future step, extending organ donor typing to HLA-class-II loci other than HLA-DR. Additionally, a B-cell crossmatch can provide valuable information for recipients immunized against HLA class-II antigens.

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