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ORIGINAL ARTICLE

The repercussions of implementing flow cytometry as a single HLA antibody screening technique in prospective renal transplant recipients

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Summary

In an effort to replace the complement-dependent cytotoxicity test (CDC) with a more sensitive single technique we looked at flow cytometry as a possible replacement. The Flow PRA Bead technique (One Lambda) performed well in our laboratory. Although as expected this technique was more sensitive and specific than CDC, there remained 11 samples from eight patients which were flow negative, CDC positive. The results of various antibody identification tests on these samples prompted us to alter the positive selection criteria which we had been using on our routine screening with the Flow PRA Beads and persuaded us that the initial CDC result was correctly positive in nine of the 11 samples.

Introduction

Accurate determination of the level and specificity of human leucocyte antigen (HLA) alloantibodies in patients awaiting renal transplantation is an important factor in both the local selection of peak-positive patient samples for donor crossmatching and in the allocation of kidneys through the UK Transplant points allocation scheme. Samples selected at this Unit for crossmatching are representative of the full range of a patient's HLA antibody profile. As we consider a positive crossmatch result with any of the peak or current sera a contradiction for transplantation, it is essential that these alloantibodies have been fully investigated. UK Transplant awards points to potential renal transplant recipients based on recipient age, waiting time, donor/recipient age difference, balance of exchanged organs, frequency of patients' HLA type within the population and identification of clinically relevant HLA antibodies. Points are used to determine where a kidney is placed when several patients reach the same mismatching level based on HLA-A, -B and -DR antigens. The method used to identify clinically relevant antibodies is at the discretion of the local centre; however, 3 points are awarded to patients whose HLA antibody specificities have been fully determined and half of a point is given when they are not identified. Both patient and scheme benefit from maximum points as ischaemic times are not increased due to re-allocation of crossmatch-positive kidneys.

For many years laboratories involved in renal transplantation relied on complement-dependent microlymphocytotoxicity tests (CDC) to ascertain the level and specificity of HLA antibodies [1]. However, the advent of the less labour intensive and more sensitive screening techniques using enzyme linked immunosorbent assay [2,3] and flow cytometry [4] led this laboratory to examine one of these up-to-date methods as a possible replacement for CDC. While some laboratories have adopted a multi technique strategy [5], we aimed for a single technique which would be more sensitive and specific than our current CDC screening and complement our crossmatching protocol. Pretransplant CDC crossmatching is

carried out on all patients and is supplemented by flow crossmatching for our high-risk groups, namely live related, highly sensitized, paediatrics and regrafts. Flow crossmatching in these groups is in agreement with other studies [6,7]. As local crossmatching is performed using a mix of flow cytometry and CDC, we tested the Flow PRA Screening Beads (One Lambda Inc., Canoga Park, CA, USA) as our possible replacement screening technique. While we knew that there would be financial implications, we calculated that these would be offset by the reduced labour costs and the increased sensitivity.

Materials and methods

Complement-dependent cytotoxicity (CDC)

The 976 samples were screened by the complement-dependent microlymphocyotoxicity test [1] using a cell panel composed of normal mixed peripheral blood lymphocytes and lymphocytes from chronic lymphatic leukaemic patients selected to cover all HLA-A, -B, and -DR antigens normally detected in the local population.

Flow cytometric PRA screening

Screening of the 976 serum samples from potential transplant recipients by flow cytometry for the presence of clinically relevant HLA antibody was carried out using an EPICS XL Flow Cytometer (Beckman Coulter UK Ltd, High Wycombe, UK) and Flow PRA Screening Beads (One Lambda Inc.) along with an immunoglobulin-G (IgG) conjugate provided with the kit [4]. Further screening was performed on discrepant samples using an immunoglobulin-M (IgM) Conjugate Fluroscein Isothiocyanate (FITC)-IgM; Jackson Immunoresearch, West Grove, PA, USA). Flow PRA class I and II Screening Beads are composed of two pooled panels of microparticles coated with HLA antigens purified from 30 cell lines for HLA class I and 30 cell lines for HLA class II. The tests were carried out according to the manufacturer's instructions with the exception that half volumes of all test reagents and patient serum samples were used. This decision was based on previous results from in-house testing (data not shown). The samples that remained discrepant after all screening was completed were re-screened using the volumes recommended by the manufacturer.

Flow cytometric antibody identification

Identification of HLA antibody in the 11 discrepant samples from eight patients (Fig. 1) was carried out using Flow PRA Specific Beads and Flow PRA Single Antigen Beads (One Lambda Inc.) [8]. A Flow PRA Specific Bead test is made up of a panel of 32 microbeads coated with purified class I antigens, and divided into four groups. Within each group there are eight HLA types on eight different beads. These beads exhibit different channel shifts allowing separation of the HLA types. A Single Antigen Bead test performs in a similar way except that the beads are coated with a purified single HLA antigen. The tests were carried out according to the manufacturer's instructions.

Results and discussion

The screening of 976 samples from 298 patients for presence or absence of HLA antibody was accomplished using our conventional CDC method and Flow IgG PRA Screening Beads. Results of the Flow IgG PRA Screening Beads revealed 842/976 (86.27%) concordant with CDC (Fig. 1). Discordant samples were re-screened to eliminate any procedural errors. As expected, this up-to-date method demonstrated HLA antibody in 47 CDC negative samples. This left 87 samples positive by CDC but negative by flow IgG screening. After demonstrating by flow that 32 samples were CDC positive due to IgM alloantibody activity and establishing that 44 samples were from patients known to have IgM autoreactivity (not clinically relevant), there remained 11 samples positive by CDC and negative by Flow PRA Screening Beads. These 11 samples were re-screened using the volumes recommended by the manufacturer to ensure there had been no loss of sensitivity caused by the amendment to the original method. All samples remained negative. They were then tested with the more sensitive Flow Specific Beads and Flow Single Antigen Beads.

Eight of the 11 samples tested, from five patients, had demonstrated HLA specificities by CDC (Table 1). The specificities obtained by CDC in five of these samples (ER, FB, AB, SE1 and SE2) were confirmed by at least one of the flow identification procedures and additional specificities were assigned in four of the five samples. The three remaining samples in this group (GJ1, GJ2 and GJ3) were from one patient who appeared to exhibit fluctuating levels of non-specific reactivity, although in one sample (GJ1) HLA specificity was demonstrated.

Human leucocyte antigen specificity by CDC could not be determined in three of the 11 discrepant samples. When tested using Flow PRA Specific Beads one sample had HLA specificity, while the other two samples remained negative. Insufficient quantities of samples meant that the only sample which could be tested with the Flow PRA Single Antigen Beads was the one in which specificity had already been determined using Flow PRA Specific Beads.

We therefore identified HLA antibody in seven samples from six patients (three using Flow PRA Specific Beads, two using Flow PRA Single Antigen Beads and two using

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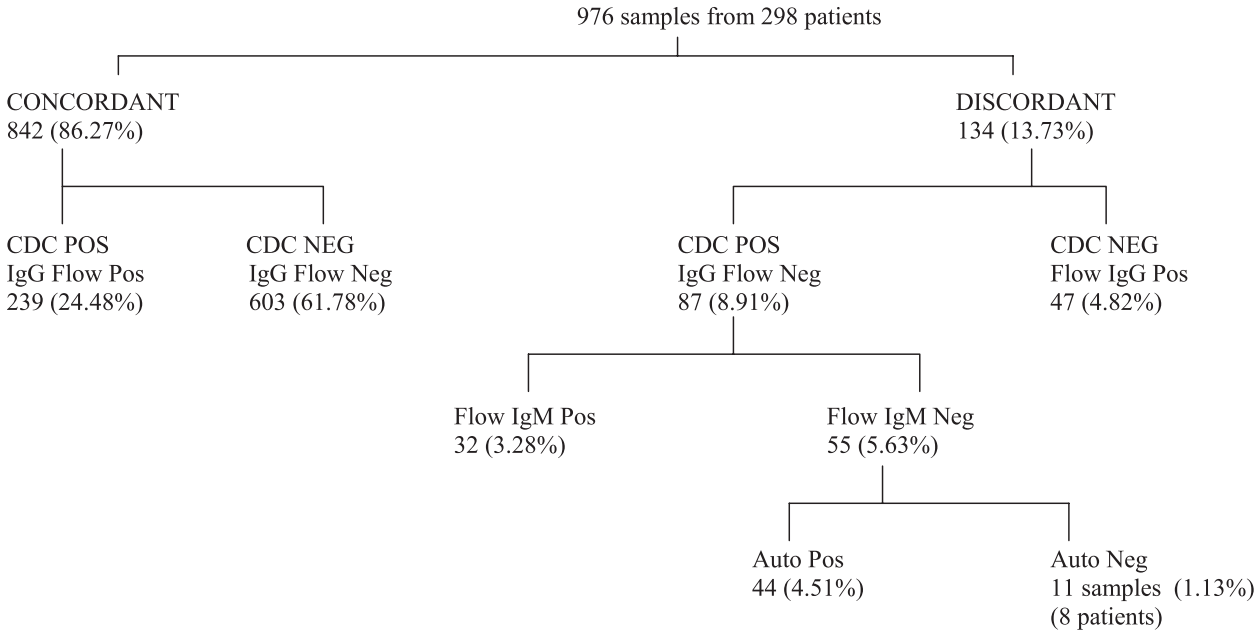

Figure 1 Comparison of CDC and Flow PRA antibody detection. CDC, complement-dependent cytotoxicity; POS, positive; NEG, negative; Auto, autoantibody; Flow, Flow PRA Screening Beads.

both types of beads) all of which were negative when screened with Flow PRA Screening Beads. It is also highly probable, had we been able to test the other two samples of patient GJ, that these samples would have been positive by Specific Beads using IgM conjugate as they were taken within 3 months of sample GJ1. The samples from MMcN and RMcK, although strongly positive by CDC, remained negative when tested with specific beads. Interestingly, samples taken within the preceding and subsequent 2 months were negative by CDC and flow cytometry, and all autoantibody screening was negative. An in-house nested polymerase chain reaction technique (not described) confirmed that these samples were drawn from the correct patients, both of whom had suffered from infection within the preceding 3 months.

The HLA class I and class II specificities of the antibodies identified were listed as unacceptable antigens for the appropriate six patients. Four of these patients have since been successfully transplanted and the remaining two patients were suspended from the waiting list due to other clinical issues. One of the two patients whose antibodies could not be identified has been successfully transplanted, while the other has been suspended from the waiting list for clinical reasons.

Discussions with the manufacturer and colleagues in other HLA laboratories have led us to a greater understanding of the screening histograms. These histograms show that in some instances, although there is insufficient fluorescence channel shift to designate a sample as positive for HLA antibody (we had used 10% as a cut-off), examination of the histogram shows a small but definite peak below the cut-off level. If we move the positivity marker by 0.5 in the first log decade, which would include these small peaks in the positivity range, positive results would be designated to SE1, SE2, VD, ER, FB and AB. This increased sensitivity range will vary with individual machine settings and laboratory protocols. The new marker gives us an increase in positive samples of 26/440 (5.91%). In order to determine if these were true HLA antibody-positive reactions, a random selection of five samples was tested with the Single Antigen Beads. These samples would have previously been assigned a negative result. All five samples were positive on the Single Antigen Beads, and we therefore assume that some of the others may also have been positive. Thus we believe to increase the sensitivity in order that we do not exclude any sample is justified, albeit that there is a small increase in samples used for crossmatching purposes, and some of these may be crossmatched unnecessarily.

The various investigations carried out on MMcN and RMcK would suggest that the original CDC results were false positives, i.e. non-HLA. However, there still remains the problem of the three results of GJ. Control beads which are automatically included in the Specific Bead and Single Antigen Bead kits may also be added to the PRA screening Beads, in order to determine if positive reactivity is non-specific. Although it seems that the samples GJ1, GJ2 and GJ3 were exhibiting this phenomenon in

Table 1. CDC positive/FLOW PRA negative.

		Specific Beads		Single Antigen Beads	
CDC specificity		IgG	IgM	IgG	IgM
ER	*A28,B44,DR11	A2,A68 (neg class II)	NT	A2, B44 Plus	NT
FB	B27	NT	NT	Neg	B27
AB	B52	Neg	NT	A30	B52,B57,B55
SE1	A2	NT	A2,A68,A69	Neg	All bead population positive
SE2	A2	NT	A2,A68,A69	NT	NT
GJ2	A25	All bead population positive	NT	Control bead negative Patient antigen positive	NT
GJ1	A25	NT	A25	NT	All bead population positive
GJ3	A25	NT	NT	NT	All bead positive
VD	No specificity 70% PRA	B62,63	NT	B63	NT
MMcN	No specificity 70% PRA	Neg	NT	NT	NT
RMcK	No specificity 70% PRA	Neg	NT	NT	NT

SE1, SE2: samples from same patient within a 2-month period. GJ1, GJ2, GJ3: samples from same patient within a 3-month period. NT, not tested due to insufficient material.
*Unable to define specificity as all antigens present on same cell.

some of the tests, it would not affect the original screening results which were negative. The lack of IgM reactivity when screening this patient remains a problem, even though an IgM HLA-A25 antibody was detected when using the specific beads. We have however designated A25 as an unacceptable antigen for this patient.

The influence of the presence of HLA IgM alloantibody on graft survival is controversial [9,10] and we therefore continue to designate unacceptable antigens for transplantation based on the presence of HLA-specific IgG or IgM antibodies. Local policies also consider that a positive crossmatch result due to IgM reactivity in a sample which was taken in the preceding 6-month period is a contraindication for transplantation. Furthermore, any patient sample which produces an unexpected crossmatch-positive result is tested with the flow PRA Specific Beads and Single Antigen Beads. This includes any crossmatch-positive sample which was previously negative by Flow PRA Screening Beads. In addition, the screening tests are repeated to re-examine the architecture of the histogram. The presence of IgM autoreactivity is widely accepted as not being clinically significant in potential renal transplant recipients, but continues to be a complication of CDC crossmatching. We routinely auto-crossmatch all our recipients by CDC before they are registered for transplantation.

Some laboratories may advocate justifiably that more than one method be used. However, budget constraints, especially availability of staff, led us to replace CDC with Flow PRA Screening Beads. To use only the CDC method would have meant missing positivity in 47 samples from 30 of 298 (10.07%) patients, whereas using Flow PRA Screening Beads with both IgG and IgM conjugates meant that 11 CDC-positive samples from 8 of 298 (2.68%) patients were missed. It would appear that at least nine of these 11 CDC-positive, Flow PRA Bead-negative samples would be relevant and should be used in a crossmatch. However, we could have avoided missing six of these weak results by changing the criteria used when designating a sample as positive by Flow PRA Screening Beads.

We have concentrated on the false-negative results by Flow PRA, but we are aware that we may be detecting some false positives. This becomes obvious when these samples are tested with the Single Antigen Beads, as the control beads shift into the positive region, due to non-specific reactivity. To overcome this, we plan to include control beads in the screening procedure. If the samples

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exhibit positivity with control beads, they may be pretreated with 'Absorb out' beads. These beads should remove non-specific reactivity, but as yet they have not been tested in this laboratory.

Conclusion

Thus in this study, no single method confirmed the presence of all detectable HLA antibodies. However, the technique of Flow PRA Screening Beads proved to be more sensitive than CDC. The increased sensitivity and determination of isotype, along with the elimination of the false positives which were due to the presence of autoantibody and less labour required has led us to adopt this method for routine screening. The availability of control beads and 'Absorb out' beads should help to minimize false positives and continued monitoring of the histograms will assist in detecting irregular and unusual peaks of fluorescence. As with every available technique, experience in the use of the flow screening technique is invaluable when it comes to interpreting the results.

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