Transpl Int (1998) 11 [Suppl 1]: S372–S376




J. E. Worthington
A. Langton
H. Liggett
A. J. Robson
S. Martin

# A novel strategy for the detection and definition of HLA-specific antibodies in patients awaiting renal transplantation

J. E. Worthington (✉) · A. Langton · H. Liggett · A. J. Robson · S. Martin
Tissue Typing Laboratory,
Renal Transplant Unit, St. Mary's Hospital,
Hathersage Road,
Manchester M 13 OJH, UK

**Abstract** Conventional testing for HLA-specific antibodies employs complement-dependent cytotoxicity (CDC) which is labour intensive and dependent on a supply of viable lymphocytes. Our strategy to minimise CDC screening is initially to screen sera by ELISA (Quikscreen) to detect HLA Class I-specific antibodies. Negative sera are then screened by flow cytometry (FCS) using lymphoblastoid cell line pools to detect HLA Class II-specific antibodies. Only Quikscreen- or FCS-positive sera are then tested by CDC and, when indicated, with an ELISA kit (PRA-STAT) for specificity definition. Of 3680 sera, 886 (24.1%) were Quikscreen positive. Of the 2794 Quikscreen-negative sera, 374 (13.4%) were FCS positive. Therefore, only 1265 of the 3680 (34.3%) sera contained HLA-specific antibodies requiring specificity definition. This novel screening strategy has significantly reduced the CDC workload of the laboratory whilst enabling the detection of additional HLA-specific antibodies.

**Key words** HLA-specific antibodies · ELISA · Flow cytometry · Complement · Dependent cytotoxicity

## Introduction

It has been established that preformed IgG antibodies specific for donor HLA antigens may result in accelerated graft failure [5] and the production of HLA-specific antibodies post-transplant has also been shown to be associated with graft failure [9]. An important role of the histocompatibility laboratory is to detect and define these antibodies to ensure prolonged transplant outcome. Definition of the HLA specificity of antibodies in patients awaiting renal transplantation avoids not only graft failure but also unnecessary crossmatching, which is a waste of both time and valuable resources.

The conventional method used to screen patient sera for HLA-specific antibodies is complement-dependent cytotoxicity (CDC) [8]. This test enables the detection and definition of complement-fixing IgG and also IgM antibodies which may be directed against HLA or non-HLA targets, including autoantibodies. CDC has a number of disadvantages, including the requirement for large panels of viable lymphocytes in order to cover all HLA specificities, subjective reading of the end test, the inability to detect non-complement fixing antibodies and the detection of autoreactive antibodies which are irrelevant to transplant outcome [12].

More recently, flow cytometry and ELISA-based techniques have been introduced which are not complement dependent. ELISA kits are now available commercially which detect HLA-specific antibodies alone. The Quikscreen kit is a solid-phase ELISA-based technique which detects the presence or absence of IgG, IgM and IgA HLA Class I (HLA-A, -B and -Cw) specific antibodies but does not allow definition of HLA specificities [6]. The antigen source is obtained from a pool of over 100 platelet donations, which are purified using column chromatography and immobilised directly onto microtitre trays. Quikscreen has been demonstrated to be a rapid, user friendly method for screening large numbers of sera for the presence of HLA Class I-specific antibodies [7, 13]. PRA-STAT is another ELISA-

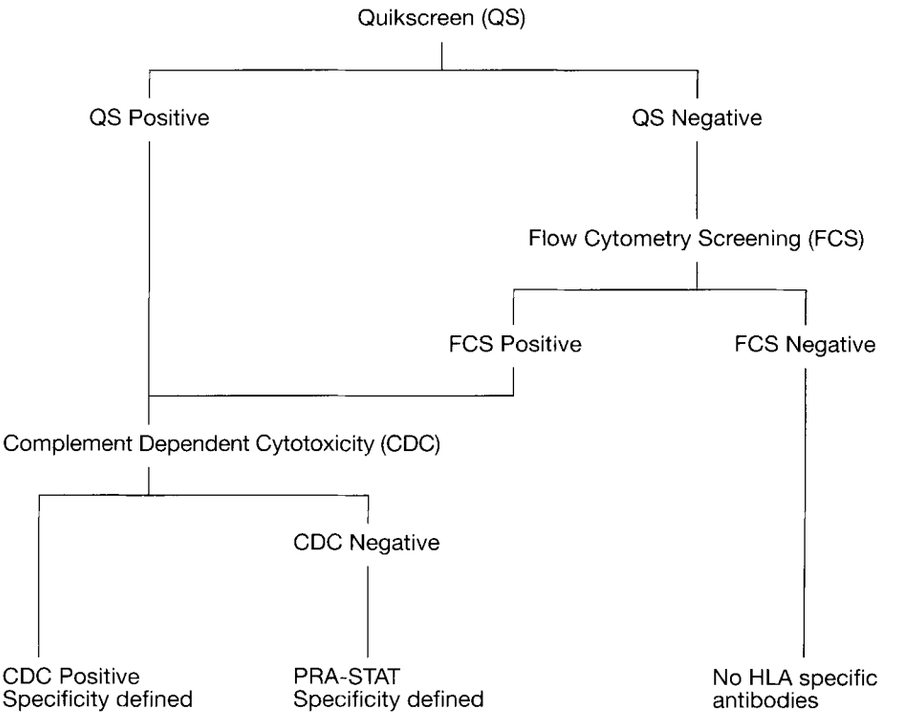


**Fig. 1** Flow chart to illustrate the screening strategy (*QS* Quikscreen, *FCS* flow cytometry screening, *CDC* complement-dependent cytotoxicity)

based assay which enables the detection and definition of IgG HLA Class I (HLA-A and -B) and Class II (HLA-DR, -DQ and -DP) specific antibodies [1]. It utilises 96-well microtitre trays which have been precoated with the pan-HLA monoclonal antibody TP25, which is directed toward the alpha-three domain of the HLA molecule, after which the trays are coated with a panel of soluble HLA antigens isolated from the culture supernatants of 46 different, EBV-transformed, HLA-phenotyped cells [4]. Advantages of an ELISA-based assay include the detection of HLA-specific antibodies alone, the ability to detect non-complement fixing antibodies and semiautomation of the technique, which removes the subjective reading of the end test.

Flow cytometry screening (FCS) techniques have been developed for the detection of IgG antibody binding to lymphocytes [3]. Two pools of EBV-transformed lymphoblastoid cell lines, each pool containing ten cell lines grown up individually, are counted and pooled in equal numbers. Sera are screened against both pools and antibody binding is detected using a FITC-conjugated anti-human IgG. This technique is not complement dependent and therefore detects IgG2 and IgG4. Disadvantages include the detection of non-HLA antibodies and the inability to detect IgM antibodies.

FCS and ELISA techniques have generally been considered as alternatives to one another, however, they both have their limitations. Consequently, we have devised a screening strategy that employs each method in turn to maximise the information obtained whilst minimising the amount of CDC screening required.

The strategy is to screen sera from patients awaiting renal transplantation with the ELISA Quikscreen kit to detect HLA Class I-specific antibodies. Positive sera are then tested by CDC using a 70-cell panel and, when indicated, with the ELISA PRA-STAT kit for specificity definition. Sera negative by Quikscreen are screened by FCS using lymphoblastoid cell line pools to detect HLA Class II-specific antibodies and confirm negativity for Class I. FCS-positive sera are also tested by CDC and PRA-STAT, as indicated for specificity definition. The aim is to screen out the negative sera which comprise the majority and focus on specificity definition for only those sera known to contain HLA-specific antibodies. The strategy is illustrated in Fig. 1.

## Materials and methods

### Specimens

Serum samples (3680) from patients awaiting renal transplant were screened for HLA-specific antibodies following the strategy outlined above.

### Quikscreen assay

The Quikscreen assay was performed according to the manufacturer's (GTI) instructions. Test sera and positive and negative con-

trols were diluted, dispensed into the precoated microtitre plates and incubated for 40 min at 37 °C. The plate was washed, alkaline phosphatase-conjugated goat anti-human IgG/IgM/IgA was added and the plate incubated for a further 40 min. The plate was washed again to remove any unbound conjugate and a chromogenic substrate (*p*-nitrophenyl phosphate) was added. After a 30-min incubation in the dark, the reaction was stopped by the addition of 1 M NaOH and the absorbance measured at 405 nm. Test results showing optical density (OD) values equal to or greater than twice the value obtained for the mean of the negative controls were regarded as positive results.

### FCS

Two pools each of ten HLA phenotyped EBV-transformed lymphoblastoid cell lines were selected to cover 51 HLA Class I (HLA-A, -B, -Cw) and 22 Class II (HLA-DR, -DQ) specificities and sera were tested against both pools. Cells and serum were incubated for 45 min at 22 °C. After washing in PBS, 0.1 % BSA, 0.1 % azide (PBS/azide) to remove unbound antibody, 3 µl of a 1 : 1 dilution of FITC-conjugated anti-human IgG was added and incubation continued at 4 °C for a further 20 min. After a final wash the cells were resuspended in 500 µl of PBS/azide and analysed using an EPICS-XL flow cytometer. The negative control threshold was set with a 2 % confidence limit and test samples were considered positive if the percentage binding was greater than 5 % from the negative control.

### CDC

Sera positive by either Quikscreen or FCS were batched and then dispensed in 1-µl aliquots onto Terasaki trays and screened in a microlymphocytotoxicity assay using fresh or frozen cell panels. The sera were screened initially with a random cell panel comprised of 40 HLA phenotyped individuals, followed by a panel of ten cells selected to cover any specificities omitted in the initial screen. A selected 20-cell panel of peripheral blood lymphocytes from patients with B cell chronic lymphocytic leukaemia was used to define HLA Class II specificities. The test was incubated initially for 1 h at 22 °C after which rabbit complement was added. The test was then incubated for a further hour at 22 °C after which a cocktail of fluorescent dyes were added. A black background was supplied by the addition of india ink. The reactions were measured using an inverted fluorescence microscope and the percentage cell death recorded for each well.

### PRA-STAT assay

The PRA-STAT assay was performed according to the manufacturer's (SangStat) instructions. Test sera and controls were diluted 1 : 101, dispensed onto precoated microtitre trays and incubated for 2 h. After washing, a peroxidase-conjugated goat anti-human IgG was added and the plates incubated for a further hour. The plates were washed again to remove any unbound conjugate and a chromogenic substrate (*o*-phenylenediamine dihydrochloride) was added. After a 15-min incubation in the dark, the colour development was stopped by the addition of 1 M HCl and absorbance measured at 490 nm. A run was considered valid if the positive and negative control values fell within the range specified by the manufacturer. A delta value for each test was determined as the OD of the well minus the OD of the corresponding no-antigen well. A test was considered positive when the delta value for the well was greater than 0.35 × the mean positive reference. The manufacturers recommend that the user does not totally rely on the cut-off but ranks the wells according to delta value in order to define specificities that fall partially below the cut-off value.

## Results

### Initial prescreen

Of the 3680 analysed, sera 886 (24.1 %) were Quikscreen positive and went forward for CDC testing. Of the remaining 2794 sera, 374 (13.4 %) were FCS positive and also progressed to CDC testing. Of the total 3680 sera, 2420 (65.7 %) were negative by both methods and therefore considered to contain no HLA-specific antibodies and were not tested further.

### Lymphocytotoxicity screening

A positive result for 1260 sera was generated Quikscreen by either or FCS and were screened by CDC. Of these, 691 have completed CDC testing and been analysed: 58 sera were negative, 25 having been FCS positive and 33 Quikscreen positive. These results are illustrated in Fig. 2.

### Specificity definition

Of the total tested, 633 sera were CDC positive, 458 specificities were defined in 276 of these samples. The remaining 58 sera were CDC negative, 15 specificities have been defined in 21 of these sera by testing with PRA-STAT. These results are shown in Fig. 3.

## Discussion

The main aim of implementing this screening strategy was to screen out the negative sera, which comprise the majority, and focus specificity definition on only those sera known to contain HLA-specific antibodies. CDC, the conventional method for antibody screening, is time consuming and absolutely dependent on a supply of viable lymphocytes from a spectrum of donors. A further disadvantage of CDC is the inability to detect non-complement fixing antibodies which have been shown to be graft damaging [14]. The more recently introduced ELISA and FCS techniques are capable of detecting non-complement fixing antibodies and, as the use of flow cytometry crossmatching is becoming more prevalent, it is important to use equally sensitive screening techniques.

Following the strategy outlined in this paper, only one-third of the serum samples initially screened were

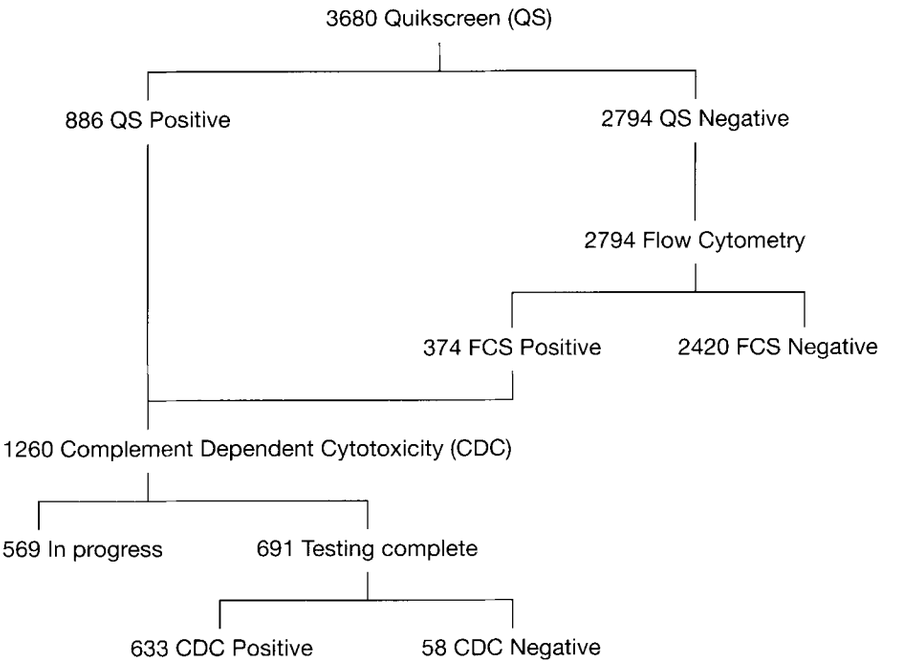


**Fig. 2** Results of initial prescreen and CDC testing (*QS* Quikscreen, *FCS* flow cytometry screening, *CDC* complement-dependent cytotoxicity)

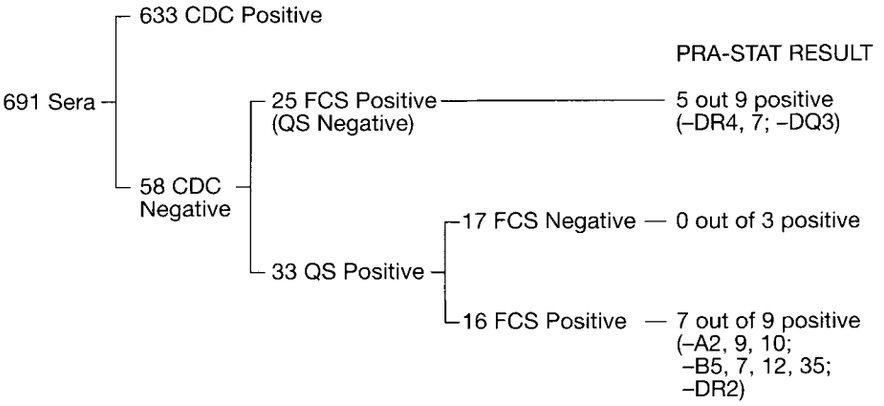


**Fig. 3** HLA specificity definition (*QS* Quikscreen, *FCS* flow cytometry screening, *CDC* complement-dependent cytotoxicity)

positive with either Quikscreen or FCS and required further testing by CDC. This substantial reduction in CDC workload has enabled the use of larger cell panels and, consequently, improved specificity definition. In addition, the introduction of ELISA and FCS techniques has enabled the detection of non-complement fixing antibodies and IgM HLA-specific antibodies, the relevance of which to graft outcome is still controversial [10, 11] but is under further investigation. A major advantage of both ELISA and FCS is that non-HLA IgM autoantibodies are not detected and do not give false indications of sensitisation. Whilst this is an advantage in terms of specificity definition, the identification of patients with non-graft-damaging IgM autoantibodies is essential in the crossmatch testing so that a patient is not unnecessarily denied a transplant. It is therefore essential that a laboratory has a system in place to detect autoantibodies during patient work-up for transplantation.

A total of 633 samples were CDC positive and 458 specificities were defined in 276 of these samples. In the majority of cases when specificities could not be defined, the patients had high levels of panel reactivity (greater than 65 %), which is recognised as making specificity definition difficult. It is sometimes possible to define specificities in highly reactive sera by testing in serial dilution. Alternatively, one advantage of PRA-STAT over CDC testing is that results can be ranked, using the delta value, in order of the strength of reaction. This facility allows definition of specificities in highly re-

active samples as the delta values for antibodies tend to cluster.

A number of discrepancies between the three techniques was identified, all of which are under further investigation. There are several possible explanations for these observed discrepancies. In the Quikscreen assay, the antigen is immobilised directly to the well and it is possible that this affects the conformation of the HLA molecule in the trays, making them different from cell surface-bound HLA molecules. The Quikscreen kit is available with two different anti-globulin reagents. The one mainly used in this study was specific for IgG, IgA and IgM and the other one is specific for only IgG. Several studies have demonstrated that the anti-IgG reagent is more sensitive than the combined conjugate [2, 7]. It is therefore possible that some HLA-specific IgG antibodies were not detected in our study. Problems associated with FCS testing include the detection of non-HLA antibodies, which could account for sera found to be positive only by FCS. Although the FCS panel was selected to cover 73 HLA specificities, some less common antigens were not represented in the pools. Absence of some specificities is also a problem encountered with PRA-STAT. Some 17 sera were positive by Quikscreen, using both the anti-IgG and the combined conjugate, but were FCS negative. These are under further investigation to determine whether this was due to differences in sensitivity and it is intended that these samples be tested using Quik-ID, a specificity definition kit soon to be marketed by GTI.

Definition of the HLA specificity of antibodies in patients awaiting renal transplantation avoids not only graft failure but also unnecessary crossmatching, which is a waste of both time and valuable resources. Antibody screening is therefore a very important component of a histocompatibility laboratory's work. Implementation of this novel screening strategy has significantly reduced the CDC workload of the laboratory whilst enabling the detection of additional HLA-specific antibodies.

**Acknowledgements** Alison Langton is supported by a grant from North West Regional Health Authority (NHS), UK

## References


1. Buelow R, Mercier I, Glanville L, et al (1995) Detection of panel-reactive anti-HLA Class I antibodies by enzyme-linked immunoabsorbent assay or lymphocytotoxicity. Results of blinded, controlled multicentre study. Hum Immunol 44: 1–11
2. Brown CJ, Green T, Navarrete CV (1997) IgG HLA antibody detection by ELISA. Eur J Immunogenet 24: 75
3. Harmer AW, Sutton M, Bayne A, Vaughan RW, Welsh KI (1993) A highly sensitive, rapid screening method for the detection of antibodies directed against HLA Class I and II antigens. Transpl Int 6: 277
4. Harmer AW, Heads AJ, Vaughan RW (1997) Detection of HLA Class I and Class II specific antibodies by flow cytometry and PRA-STAT screening in renal transplant recipients. Transplantation 63: 1828–1832
5. Kissmeyer-Nielson F, Olsen S, Peterson VP, Fjeldborg O (1966) Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet 2: 862
6. Kao KJ, Scornick JC, Small SJ (1995) Enzyme-linked immunoassay for anti-HLA antibodies – an alternative to panel studies by lymphocytotoxicity. Transplantation 55: 192
7. Lucas DP, Paparounis ML, Myers L, Hart JM, Zachary AA (1997) Detection of HLA Class I-specific antibodies by the Quikscreen enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 4: 252–257
8. Martin S, Class F (1993) In: Dyer PA, Middleton D (eds) Histocompatibility testing. IRL Press
9. Martin S, Dyer PA, Mallick NP, et al (1987) Post-transplant anti-donor lymphocytotoxic antibody production in relation to graft outcome. Transplantation 44: 50
10. Martin S, Dyer PA, Scott PD (1992) Autoreactive IgM non-HLA antibodies are not entirely clinically irrelevant. Transplant Proc 24: 2504
11. Reddy KS, Clark KR, Cavanagh G, Forsythe JLR, Proud G, Taylor RMR (1993) Successful renal transplantation with a positive T-cell crossmatch caused by IgM antibodies. Transplant Proc 27: 1042–1043
12. Taylor CJ, Chapman JR, Ting A, Morris PJ (1989) Characterisation of lymphocytotoxic antibodies causing a positive crossmatch in renal transplantation: relationship to primary and regraft outcome. Transplantation 48: 953
13. Worthington JE, Thomas AA, Dyer PA, Moghaddam M, Martin S (1995) GTI Quikscreen for the detection of HLA Class I specific antibodies. Eur J Immunogenet 22
14. Worthington JE, Thomas AA, Dyer PA, Martin S (1998) The detection of HLA specific antibodies by PRA-STAT and their association with transplant outcome. Transplantation (in press)