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A highly sensitive, rapid screening method for the detection of antibodies directed against HLA class I and II antigens

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Abstract. Screening of potential transplant recipients for antibodies that can cause graft rejection is an essential part of the pre-transplant monitoring carried out by tissue typing laboratories. This is a time-consuming process and the rapid reporting of results is dependent on the maintenance of frozen cell panels. The usual procedure of screening against a panel of random cells takes up to 6 weeks. In this study we have used flow cytometric analysis of pooled chronic lymphatic leukaemia (CLL) cells to detect antibodies directed against HLA antigens. We show that FACS screening of pooled cells can accurately and rapidly detect these antibodies and that the method is suitable for routine use. An estimate of the degree of patient panel reactivity can be determined within a few hours. In addition, the technique is more sensitive than those conventionally used, an advantage that may be of importance in preventing graft damage.

Key words: HLA class I and II antibodies, screening – Antibodies, HLA, screening – Screening, antibodies, HLA

Introduction

Preformed antibodies directed against HLA antigens can cause graft failure in renal allograft recipients [4, 5]. They may also be associated with a poor prognosis in recipients of other solid organ transplants [12, 14]. These antibodies may be present as a result of blood transfusions, pregnancy or previous transplants. It is therefore important to regularly screen potential recipients for anti-HLA antibodies. Screening can determine the panel reactivity (PR) for each sample, an estimate of the degree of sensitisation against the panel of cells used for testing and can be related to the chance of a given donor kidney being suitable. Careful analysis of the results is necessary for a definition of the antibody specificity. The use of different cell targets and blocking experiments in conjunction with screening results can allow the determination of which antibodies are potentially damaging to a graft. In patients who have lost previous transplants, evidence of an antibody being formed against a mismatched antigen would prohibit a repeat mismatch for that antigen. If, however, it can be demonstrated that no antibody was formed to the mismatch at any time, a graft bearing the previously mismatched antigen would not pose a greater risk of rejection. The latter has, in fact, been found [13].

Analytical work of this type constitutes a major portion of any transplant tissue typing laboratory's work load. Each sample must be screened against a panel of normal cells of sufficient size to include all or most of the HLA antigens. Panel size may range from 20 to 60 cells in different centres, but the larger the panel, the more reliable the results. It can take several weeks for enough suitable cells to pass through the laboratory, and it also takes a considerable amount of time to process each sample for screening. Patients must be screened regularly and this complex process is often repeated monthly for several years. Immediate results can be obtained by using frozen cell panels [9]. However, the making up, freezing down and storage of these cell panels can be difficult and there may be problems with cell viability.

As less than half of the patients screened are likely to have antibodies, a rapid method to detect whether a sample is positive or negative by a single test could reduce the numbers of samples requiring lengthy screening by over 50 %. To cover all of the HLA specificities in a single test would require the use of pooled cells. Screening pools of cells would only be possible if each sample could be analysed against a large enough number of cells. Flow cytometry is a method that allows the analysis of a large number of individual cells in a very short time. The use of flow cytometric crossmatching of recipient serum against donor lymphocytes has shown it to be a more sensitive method of antibody detection than the conventional cytotoxic crossmatch [3].

The aim of this study was to determine if analysis of pooled chronic lymphatic leukaemia (CLL) cells by flow

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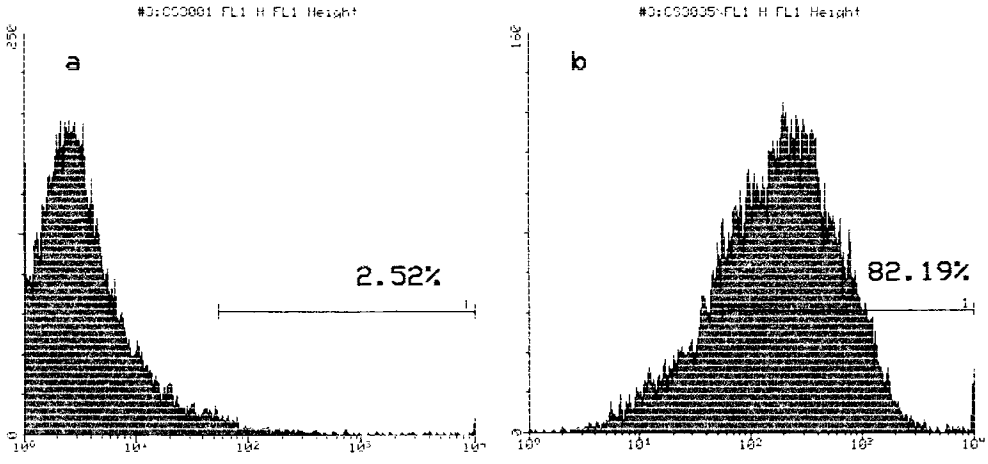

Fig. 1. IgG binding to the pooled CLL for **a** negative control serum and **b** positive test serum, showing the percentage of cells with positive binding

Table 1. Comaprison of flow cytometric screening of 59 sequential sera with complement-dependent cytotoxic (CDC) screening for IgG antibodies

	FACS +	FACS −
CDC +	20	4
CDC −	9	26

Table 2. Comparison of flow cytometric screening of 30 sera with complement-dependent cytotoxic (CDC) screening for IgG antibodies

	FACS +	FACS −
CDC +	15	3
CDC −	4	8

cytometry was a reliable technique for detecting anti-HLA antibodies and if the method would be suitable for routine laboratory use.

Materials and methods

Serum samples were collected from 60 patients awaiting renal transplants. These comprised 30 sequential samples from each of the two centres involved in the study. Blood was collected and separated by laboratory staff not directly involved in the study. A further series of 30 samples was chosen to cover patients with a range of different panel reactivities.

Three different pools of CLL cells were used in the first series. Pool 1 consisted of 10 cells, pool 2 of 8 and pool 3 of 11 cells. Pools were made by combining equal numbers of each CLL cell, spinning down and resuspending at a concentration of 1×10^7/ml in FACS diluent (PBS/azide solution). A pre-screen of CLL cells was used to eliminate any that contained surface immunoglobulin. The 60 serum samples were split into three groups of 20 and each group was tested against one of the three CLL pools.

Each serum sample was incubated final dilutions of 1:4 and 1:8 with 30 µl of pooled CLL cells for 30 min at 22 °C. Normal AB serum was used as a negative control in every run. The cells were washed twice for 5 min at 1500 rpm. The incubations were carried out in duplicate with 4 µl of FITC conjugated anti-IgG added to one set of the samples and 4 µl of FITC-conjugated anti-IgM (Dako) to the remaining half. These were incubated for 30 min at 4 °C. The cells were washed twice for 5 min at 1500 rpm and were resuspended in a final volume of 500 µl.

Cells were analysed on a FACScan using LYSIS II software (Becton Dickinson UK). For each sample 10,000 events were collected for analysis. Immunoglobulin binding to the cells was determined by analysing FL1 histograms. Using the negative control a gate was set that would include cells with positive Ig binding but exclude those with normal background binding (Fig. 1). Test samples were analysed by determining the percentage of cells that fell within the gate. The small percentage of negative control cells that fell into the positive gate was subtracted from the percentage for each test sample. A test sample was considered to be positive if the corrected value was 5% or greater.

The second series of samples was tested using the same method but a single dilution of 1:5. Checkerboard titrations of serum had shown this to be the optimal dilution for use with CLL cells. Two separate panels of CLL cells consisting of a total of 18 cells were used. The cells were resuspended in, and all washing steps were carried out using, Terasaki Park medium (Life Technologies). A comparison with cells prepared in FACS diluent and Terasaki Park medium showed better cell viability with the medium.

Each sample was tested by conventional complement-dependent cytotoxicity (CDC) against panels of peripheral blood lymphocytes (PBL) and CLL cells with and without dithiothreitol. The overall panel reactivity and panel reactivity due to IgG were calculated. The specificity of the antibodies in positive samples was determined.

Results

Table 1 shows the overall results of the first series of samples against the CLL cells for IgG antibody at titre 1:4 (there was 1 sample that was not screened by the conventional method). There were 20 samples that were found to be positive by the conventional screening method and by the FACS method. Twenty-six samples were shown to be negative by both methods. There were nine samples found to be positive by FACS that were CDC-negative. These samples had a percentage of positive cells in the range 5–22. Four samples gave positive reactions by CDC and were negative by FACS. Of these, three samples gave two weak reactions and the remaining sample gave three weak reactions against the CLL panel. There were no strong reactions that were missed by the FACS.

Three of the four missed reactions were samples that were tested against pool 2, the smallest pool with only 8 cells. The remaining missed reaction was against pool 1. The reactions that were positive by FACS and CDC-negative were evenly spread among all three pools.

The results of the 1:8 dilution were similar to those described above. There was one false-negative reaction at

this dilution, a sample with a CDC PR of 20% (this sample had a positive FACS reaction of 16% at the 1:4 dilution). The correlation between the CDC-determined panel reactivity and the percentage of positive cells by FACS was better at 1:8 for high PRs; however, for low PRs, the 1:4 dilution gave more accurate results.

The results of the IgM screening did not show such a high degree of correlation. There were, however, no samples in the series that were positive for IgM anti-HLA without also having IgG present that was detected by the FACS screening.

The second series of tests using sera with a variety of PRs were carried out at a dilution of 1:5 against two CLL pools. The results are shown in Table 2. Twenty-three samples gave the same results by both methods; four were CDC-negative but FACS-positive with positive percentages of 9, 16, 23 and 42. Three samples gave weak reactions by CDC but were negative by FACS with PRs of 6% in two cases and 10% in one case. The failure of the method to detect these weak reactions is most likely due to the appropriate antigen not being contained in the pool. In two other samples that had weak cytotoxic reactions giving panel reactivities of 10% and 13%, the FACS method gave PRs of 9% and 31%. Weak cytotoxic reactions should be easily detectable by the FACS method as it is known that flow cytometry is a very sensitive technique for the detection of IgG antibodies. A panel reactivity for the pooled cells was calculated by combining the positive percentages of the two pools, correcting for the background and dividing by two. The results of this set of samples gave a very good correlation between the conventional PR and the value obtained by FACS analysis. Figure 2 shows the corresponding panel reactivities of all 30 samples; regression analysis of these results gives an r^2 of 0.9052.

The HLA types of the CLL cells comprising the two pools are given in Table 3. This combination of cells covers all of the class II specificities and most class I specificities, with at least one antigen from each of the main cross reactive groups at the A locus being included. With further selection of CLL cells it may be possible to improve the representation of the small percentage of B locus antigens not included in the current pool.

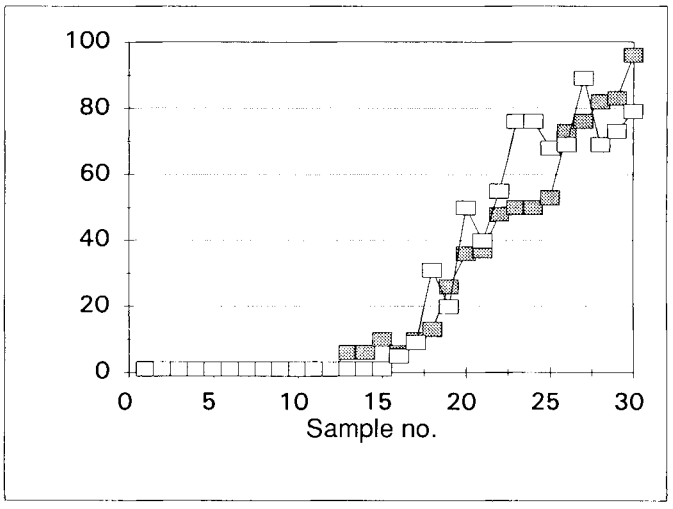

Fig. 2. Comparison of panel reactivities obtained by CDC (▨) and FACS (□) screening of 30 serum samples

Table 3. HLA types of CLL cells comprising the pools used in the second series screen

Cell no.	HLA-A	HLA-B	HLA-DR
1	2	13, 15	2, 4
2	3, 11	7, 35	7, 8
3	1, 9	8, 18	3, 11
4	1, 28	8, 44	4, 8
5	23, 29	44, 13	7
6	1, 3	7, 37	4, 10
7	2, 24	18, 51	7, 11
8	2, 32	62	1, 11
9	1, 2	8, 44	2, 12
10	2, 24	7, 62	9, 13
11	3, 26	5	4, 12
12	2	51, 62	3, 9
13	2, 24	7, 55	14, 15
14	3, 31	7, 52	2, 14
15	2, 28	8, 39	1
16	2	8, 39	3, 13
17	2, 28	14, 60	12, 13
18	2, 24	35, 38	1, 13

Discussion

Flow cytometric analysis of pooled CLL cells gives a reliable positive or negative IgG result in a single test for each sample. This test covers both class I and class II antibodies. Whilst it is accepted that pre-formed antibodies directed against HLA class I antigens can cause hyperacute rejection, opinions are divided as to the significance of antibodies that react with B cells but not with corresponding T cells [1]. However, where it can be shown that antibodies to B cells are directed against HLA class II antigens, there is evidence of hyperacute or accelerated graft rejection [1, 7]. The use of blocking techniques with the pooled CLL cells would allow confirmation of the specificity of antibodies to class I or class II antigens.

It has also been shown that a panel reactivity can be determined for the pooled CLL cells. This panel reactivity is similar to that which is obtained by conventional screening against 30 random cells. A numer of samples found to be negative by conventional screening were found to be positive by CLL pool cytometry.

The increased sensitivity of the FACS screening method may be of importance. It has been shown that IgG antibodies detected by FACS but not by conventional screening can be directed against HLA antigens [6]. It is well documented that a number of patients have negative conventional crossmatches but positive FACS crossmatches. Some studies have shown that a positive FACS crossmatch is predictive of graft failure in both sensitised and non-sensitised patients [11], whilst others find a positive crossmatch is only significantly associated with graft loss in sensitised patients [3]. In all of these studies there are a number of patients with positive FACS crossmatches who do not experience any significant graft dysfunction – so called false-positive crossmatches. These apparent discrepancies may be explained by expanding the definition

of sensitised patients to include those with antibody detectable by FACS screening and the "false-positive" crossmatches may be those found in patients with negative FACS screening. Further studies are needed to determine if all the FACS-detectable antibodies are directed against HLA antigens and if these results would prove useful in interpreting FACS crossmatches.

The method has been found to be less sensitive for the detection or IgM antibodies. This is a definite advantage for IgM antibodies against some non-HLA determinants since these are not relevant to transplant survival [2, 10]. The significance of IgM antibodies directed against MHC class I and class II determinants in transplantation is not completely understood. It is possible that there may be IgM antibodies directed against HLA antigens, but in most cases IgG anti-HLA antibodies will be present along with the IgM antibody [8]. In the rare event of only an IgM anti-HLA antibody being present in a patient sample, this may not be picked up by the screening method. However, if that patient came to transplant, such an antibody would be picked up in the crossmatch test.

Any positive crossmatch result with a serum shown to be negative by the rapid screening method would then be further investigated and the antibody responsible for the positive crossmatch determined. Autoreactive antibody status is determined by crossmatching with autologous lymphocytes and would therefore be taken into account at the time of crossmatch despite negative results by the rapid screening method.

The ability to rapidly and accurately detect the panel reactivity of a sample is of particular value for new patients or for patients who have recently had a sensitising event. If patients are to be considered for transplant before there has been sufficient time to screen by conventional methods, an estimate of sensitisation is desirable. The necessity of performing a FACS crossmatch with the donor cells may be decided by the PR of the patient. Recipients of transplants can produce antibodies post transplant and rapid determination of an increase in panel reactivity post transplant may be required. The degree of sensitisation and changes in PR post transplant are important factors in determining the immunosuppressive therapy to be given to the recipient.

In conclusion, we believe that flow cytometric screening of patient sera against pooled CLL cells is a reliable and highly sensitive method for the detection of IgG anti-HLA antibodies. The technique is suitable for routine laboratory use and can provide a panel reactivity within 3 h.

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