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The flow cytometric detection of alloantibodies in screening for renal transplantation

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Abstract Flow cytometric screening of sera using pooled chronic lymphocytic leukaemia (CLL) cells has previously been reported as a quick method for detecting HLA antibodies of the IgG class. In this study we investigated the sensitivity of this method in the detection of IgG and IgM alloantibodies, and its performance in serum screening when compared to conventional microlymphocytotoxic screening. Results indicate that flow cytometric screening is more sensitive in the measurement of IgG alloantibodies by up to five doubling dilutions, whereas the converse is true for IgM. IgM autoantibodies were found not to be detectable by flow cytometry. By testing a large number of sera by both methods in parallel, we have found that a significant proportion of sera exhibiting no activity or IgM activity alone on cytotoxic screening contain IgG antibodies detectable with a pool of CLL cells on the flow cytometer.

Key words Flow cytometry, alloantibodies, kidney transplantation · Alloantibodies, kidney transplantation, flow cytometry · Kidney transplantation, alloantibodies, flow cytometry · Screening, alloantibodies, kidney transplantation

Introduction

The screening of renal sera constitutes a significant proportion of the workload in a transplant-associated tissue typing laboratory. In order to reduce the number of sera entering our cytotoxic screening programme, we have introduced a flow cytometric serum "prescreen" to eliminate IgG-negative sera from analysis, in which serum reactivity against a panel of pooled CLL cells is measured flow cytometrically using an anti-IgG FITC-conjugated antibody [6]. Any serum showing reactivity with the pool is further analysed by routine cytotoxic screening against individual cells to define antibody specificity. Currently, up to 50 % of all samples analysed flow cytometrically show no reactivity with the CLL pool, representing a substantial reduction in the routine cytotoxic workload. The test can be performed in a short space of time and lends itself to analysis of large numbers of sera simultaneously. It can also be helpful in distinguishing potential HLA-typing reagents in sera from multiparous women by eliminating lengthy cytotoxic analyses of IgG-negative samples.

Although flow cytometry is well established as a sensitive method in the detection of alloantibodies [2, 4, 10], there is little published data on the class of antibody identified in the test. Of the samples we have analysed by cytotoxicity and flow cytometry in parallel, a significant proportion of CLL pool IgG-negative sera contain antibodies of the IgM class demonstrable only by cytotoxicity. Since it is now widely accepted that many antibodies of the IgM class are not detrimental to graft survival [3, 9, 11], it is possible that some of the differences in sensitivity and clinical relevance attributed to the flow cytometric crossmatch between centres may be as a result of the class of antibody analysed by each method. We have therefore investigated the types of antibodies that can bind in a flow cytometric assay by analysing the reactivity of a series of well-defined HLA and non-

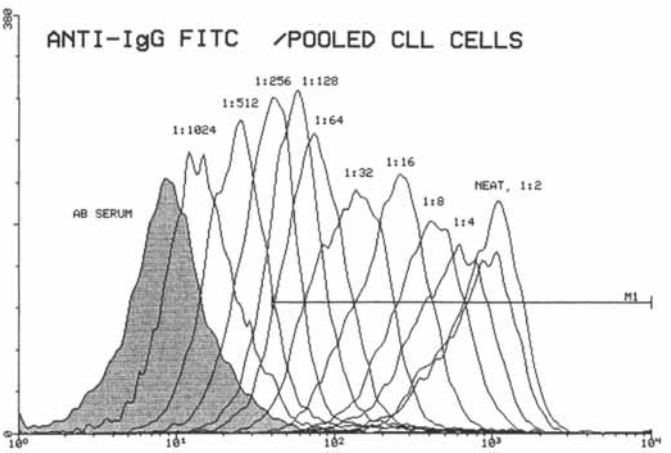

Fig. 1 Histogram overlays for titration of positive control (IgG) serum against pooled CLL cells. *M1* denotes "positive region" for histogram fluorescence

HLA antibodies by cytotoxicity and flow cytometry in parallel. The study is extended to include the flow cytometric/cytotoxic screening results of a large number of pre- and post-transplant sera to investigate the frequency of occurrence of alloantibodies defined by each method.

Materials and methods

Materials

Lymphocytes from CLL patients were prepared by density centrifugation, washed and resuspended in 10 % dimethyl sulphoxide for storage in liquid nitrogen. Thawed aliquots were layered on Lymphoprep, washed in Terasaki Park Medium and resuspended in medium at a final concentration of 2×10^6/ml for use in microcytotoxicity, or 1×10^7/ml for flow cytometric analyses. Cell pools were prepared by combining 500 µl from 12 different CLL cell preparations. The selection of cells was based on maximising HLA allele content of the pools but eliminating any cells that produced a broad/positive peak when crossmatched using unsensitised AB serum.

Labelling antibodies used in the assay were fluorescein-conjugated $F(ab')_2$ fragments of rabbit immunoglobulins to human IgG or IgM (Dako).

Flow cytometric detection of IgG

Serum samples were tested against two CLL pools. Normal AB serum from untransfused males was used as a negative control and a mixture of HLA-Bw4 and -Bw6 typing sera for the positive control. In addition, a 1 : 256 dilution of the positive control in AB serum was included as a marker for the sensitivity of the assay. For each test, 10 µl serum was added to 40 µl cell suspension in a tube, mixed and incubated at 22 °C for 30 min. Cells were washed twice in 1 ml PBA (phosphate-buffered saline containing 0.1 % sodium azide and 0.1 % bovine serum albumin) and centrifuged at 200 *g* for 5 min. After the second wash, residual supernatant was carefully blotted from inverted tubes. Then, 50 µl of a 1 : 30 solution of anti-IgG or anti-IgM FITC-conjugated antibody in Terasaki Park Medium containing 0.1 % sodium azide was added to the resultant cell pellet, mixed and incubated at 4 °C for 30 min. Unbound label was removed by washing with 1 ml PBA and centrifugation at 200 *g* for 5 min before resuspension in 500 µl PBA. Samples were stored at 4 °C prior to analysis on a FACS can flow cytometer (Becton Dickinson) with a 15-mW argon laser at 488 nM. Fluorescein measurements (band pass filter of 530 nm) using logarithmic amplification and forward and side scatter profiles were collected on 10,000 cells per tube. Lymphocytes were gated on a dot plot of scatter characteristics and the fluorescence profiles analysed using Lysis II software. The degree of antibody binding was assessed by setting a marker on the "positive" region (generally covering the upper three decades of a log fluorescence scale) on a fluorescence histogram to exclude greater than 95 % of the negative control (AB serum) peak. For test sera, the percentage of cells lying within the positive region was calculated. The positive control serum consistently produced more than a 99 % shift of the cell population into this region. A 1 : 256 dilution of the positive serum gave shifts of 35 % ± 10 % of the total population.

Screening by microcytotoxicity

One microlitre cells at a concentration of 2×10^6/ml was added to 1 µl serum in microtest plates under paraffin oil. Plates were incubated at 22 °C for 30 min; 5 µl rabbit complement was added to each well and then incubated for an additional 120 min at 22 °C. One microlitre of acridine orange/ethidium bromide mix was added to each well and cell viability was assessed using a fluorescence microscope. For DTT treatment of IgM, 1 µl of a solution of 0.01M DTT in Terasaki Park Medium was added to 1 µl serum and incubated at 37 °C for 30 min prior to the addition of cells. Complement in DTT-treated wells was supplemented with 0.002 M L-cystine to block further digestion of IgG. Controls for the dilution effect of DTT addition were performed initially but are no longer routine. It is our experience from crossmatching using DTT that the incidence of sera reacting at neat, but not when diluted 1 : 1 with either DTT or Terasaki Park Medium, is extremely low (i.e. we have never observed one). We postulate that a reduction in serum protein concentration increases the sensitivity of the cytotoxic crossmatch, possibly by rendering cells more prone to complement lysis or as a result of improved access of antibodies in the reaction well, negating the dilution effect of the addition of DTT.

Results

Relative sensitivity of flow cytometric assay for IgG HLA antibodies

The endpoint of the positive control serum in cytotoxic analyses was 1 : 32 for all HLA-Bw6-bearing CLL cells and 1 : 16 for homozygous HLA-Bw4 cells. Figure 1 shows histogram overlays for the flow cytometric assay using an anti-IgG FITC-conjugated label and pooled cells. This demonstrates a marked increase in fluorescence of the CLL pool for dilutions up to 1 : 1024 [median channel fluorescence separation of 1 : 1024 dilution and AB peak = 7.7 channels (standard deviation of AB peak = 1.91 channels)].

Table 1 Fluorescence data from flow cytometric titration of positive control serum against CLL cell pool

Serum	% Positive region (FL1)	Median channel fluorescence
AB (neg control)	3	8
Pos control N	100	898
1 : 2	100	806
1 : 4	100	542
1 : 8	100	379
1 : 16	100	255
1 : 32	99	124
1 : 64	94	72
1 : 128	85	56
1 : 256	57	38
1 : 512	20	25
1 : 1024	11	16
1 : 2048	3	11
1 : 4096	3	9
1 : 8192	4	12

Table 2 Fluorescence data on flow cytometric testing against the CLL pool of sera from ten untransfused male dialysis patients

Serum	% Positive region (FL1)	Median channel fluorescence
AB (neg control)	4	10
1	1	9
2	1	6
3	2	9
4	2	14
5	2	10
6	1	7
7	0	4
8	0	5
9	1	9
10	1	6

Analysis of the data by calculation of the percentage of cells shifting into a positive region on histogram fluorescence is shown in Table 1. A selection of sera from ten untransfused male dialysis patients, when tested against the pool, produced shifts in fluorescence of between 0 % and 4 % of the CLL pool (Table 2). We therefore consider a background of 0 %–5 % shift of the pool as negative for the assay. Using this criterion, the 1 : 1024 dilution of the control serum is the endpoint of the flow cytometric assay, with 11 % of the cell pool shifting into the "positive" fluorescence region.

In addition, three sera with weak but definable cytotoxic HLA specificities (from multiparous women) were tested flow cytometrically against the CLL pool to assess whether the method was able to detect low levels of antibody against rarer HLA antigens. When assayed flow cytometrically against the CLL pools, sera produced shifts in fluorescence as shown in Table 3. In the case of the B41/42 and B22 sera, the CLL pool showed an increase in fluorescence in spite of the absence of the HLA antigens against which cytotoxic reactivity was directed.

Relative sensitivity of flow cytometric assay for IgM alloantibodies

Table 4 shows endpoints for cytotoxicity and flow cytometry for IgM antibodies assayed against CLL cells. A human IgM monoclonal antibody with specificity for HLA DR 1303 [1], when titred against individual CLL cells, showed an endpoint of 1 : 1024 by cytotoxicity. In comparison, against the same CLL cells tested individually in a flow cytometric assay using an anti-IgM FITC-conjugated antibody, the monoclonal gave an endpoint of 1 : 64. Similarly, a human IgM monoclonal against HLA DR1301 with a cytotoxic titre of 1 : 128 showed a flow cytometric endpoint of 1 : 32. The sensitivity of this assay could not be improved by increasing the concentration of FITC-conjugated label. It was also noted that anti-IgM FITC-labelled cells given an additional wash after incubation with the labelling antibody showed a marked reduction in fluorescence not observed with the same use of an anti-IgG FITC label. A human IgM anti-class I monoclonal antibody (MP9) with specificity for HLA-A3/31 [8] showed a cytotoxic endpoint of 1 : 64 against CLL cells, whereas binding could only be detected flow cytometrically at 1 : 8. A serum sample with a specificity for HLA B8, removable by treatment with DTT, gave a cytotoxic endpoint of 1 : 16. When tested flow cytometrically, this serum did not produce a marked shift in fluorescence at neat or any dilution against CLL cells.

The non-HLA IgM human monoclonal alloantibody DD5 [12], with cytotoxic profiles similar to autoreactive antibodies (i. e. high peripheral blood lymphocyte panel reactivity, reactivity with K562 cells), gave a cytotoxic endpoint of 1 : 128 but did not demonstrate binding by flow cytometry at neat or any dilutions using an anti-IgM FITC-conjugated label. Serum samples from unsensitised renal patients who displayed IgM panel reactivity by cytotoxicity were also tested to assess flow cytometric detection of non-HLA IgM antibodies. Cytotoxic activity in this group had previously been shown to be non-HLA by blocking experiments (in which incubation of target cells with non-cytotoxic mouse monoclonal antibodies directed against monomorphic determinants of HLA class I and II antigens did not reduce the cytotoxic titre of serum samples [3]). CLL cells with which sera reacted in cytotoxic tests were selected for use in the flow cytometric assay. Of five sera that fell into the non-HLA antibody category, none reacted with CLL cells when tested individually or as a pool with an anti-IgM FITC label.

Table 3 Flow cytometric analysis of sera with weak cytotoxic activity against less common HLA antigens

Cytotoxic reactivity	% Fluorescence shift		HLA antigens of CLL cells					
	Pool 1	Pool 2	Pool 1			Pool 2		
			A	B	DR	A	B	DR
HLA B41/42	65	77	3,31	7,52	2,14	2	44	4
			1,2	57,60	7,8	2,28	18,62	3,4
HLA B5 (52)	15	15	23,29	13,44	7	1,2	8,44	2,12
			3,11	7,35	7,8	2	62	1,11
HLA B22	24	57	2,24	7,62	9,13	2,24	35,38	1,13
			2	13,15	2,4	2,29	44,60	7,13
			2	18,60	4,13	11,30	7,13	2,7
			2,3	44,35	1,4	2,24	51,18	7,11
			1,25	17,18	1,4	1,28	8,44	4,8
			2,28	14,60	12,13	3,26	5	4,12
			3,29	7,44	2	1,9	8,18	3,11
			3,28	51	11,14	2,28	8,39	1

Table 4 Reaction endpoints for flow cytometric and cytotoxic analysis of IgM antibodies against CLL cells (*nd* not detected)

Antibody description/ specificity	Cytotoxic endpoint dilution	Flow cytometric (IgM-FITC) endpoint dilution
Human monoclonal anti-HLA DR1303	1024	64
Human monoclonal anti-HLA DR1301	128	32
Human monoclonal anti-HLA A31 + 3	64	8
Multiparous typing serum, anti-HLA B8	8	nd
Human monoclonal autoantibody	128	nd
Autoantibody sera from unsensitised dialysis patients	16	nd
	8	nd
	2	nd
	2	nd
	neat	nd

Table 5 Results of flow cytometric (IgG-FITC)/cytotoxic screening of patient sera

Cytotoxicity results	Flow cytometry	Number of sera
Negative (IgG/IgM)	Negative	47
	Positive	30
Positive IgM only	Negative	49
	Positive	25
Positive IgG	Negative	0
	Positive	80
	Total	231

Comparison of flow cytometric screening with conventional screening by microcytotoxicity

A series of 231 sera were screened by cytotoxicity (with and without DTT) and by flow cytometry using an anti-IgG FITC-conjugated label. These sera represented a batch of routine cytotoxic screening from patients awaiting transplantation (including untransplanted patients and those who had experienced previous transplant failure). As shown in Table 5, 80/231 sera demonstrated IgG antibodies in both cytotoxic and flow cytometric screening. There were no sera that contained IgG by cytotoxicity (with DTT digestion) not detectable by flow cytometry. An additional 74/231 sera contained only IgM antibodies by cytotoxic screening (i.e. all antibody reactivity being abrogated by treatment with DTT). Of this group, 25/74 were positive on the flow cytometer for IgG. Finally, of 77 sera that were negative on screening by cytotoxicity, 30 contained IgG antibodies demonstrable by flow cytometry. Overall, 49/74 of cytotoxic IgM-positive sera and 47/77 cytotoxic negative sera did not demonstrate any IgG antibodies in flow cytometric analyses. These sera would not routinely be analysed by cytotoxicity on account of a negative CLL pool/flow cytometer result, representing an overall reduction of 42 % in the number of samples entering our cytotoxic screening programme.

These data reaffirm the flow cytometric/pooled CLL cell test as a reliable and sensitive screen for antibodies of the IgG class. In addition, 34 % of sera screened by cytotoxicity as positive for IgM alone, and 39 % of sera screened by cytotoxicity as negative demonstrated subliminal levels of IgG measurable only by flow cytometry.

Discussion

Flow cytometry is now well established as a more sensitive method than cytotoxicity for the detection of alloantibodies in crossmatching for renal transplantation. In the present study, using pooled CLL cells/flow cytometric analysis for screening sera, we have demonstrated increased sensitivity of five doubling dilutions in the detection of anti-HLA antibodies of the IgG class. For weak typing sera, the test demonstrated antibody binding to a much larger proportion of the CLL pool than would be predicted from cytotoxic profiles. Unpublished findings from the screening of sera from multiparous women for whom all family members have been HLA-typed show more antibody binding than would be expected from the sensitising paternal antigens. It is possible that the flow cytometric test detects antibodies bound to crossreactive groups or epitopes in densities too low to initiate complement activation in a cytotoxic assay. The finding of extra definable specificities for HLA typing sera in flow cytometric assays compared with cytotoxicity [10], in which flow cytometric reactivity to defined HLA specificities could be removed by specific platelet absorptions, suggests that the flow cytometric test is indeed able to detect antibody binding to HLA crossreactive epitopes not apparent by cytotoxicity. In addition, for patients whose HLA immunization events cannot be accurately defined, the broad spectrum of CLL reactivity seen by flow cytometry may be as a result of the binding of subliminal levels of antibody to extra HLA antigens not included in the crossreactive epitope group of cytotoxic specificity.

For the detection of IgM anti-HLA antibodies, however, the flow cytometric test is significantly less sensitive than cytotoxicity. Depending on the antibody used, cytotoxicity was more sensitive by two to greater than four doubling dilutions. In the case of non-HLA IgM antibodies, we were not able to detect by flow cytometry antibody binding to CLL cells in five patients with well-characterised autoantibodies or the human IgM monoclonal DD5. This is in accordance with a previous study [2] in which only 2 out of 43 sera with autologous lymphocytotoxic reactivity showed binding to T and/or B lymphocytes by flow cytometry. The reduction in cell fluorescence observed in multiply washed samples containing FITC-labelled IgM antibodies would suggest that the comparative insensitivity of flow cytometry may be due to the removal of low-affinity bound antibody (particularly in the case of autoantibodies) during washing steps. Combined with the relative efficiency of IgM in complement activation, it is doubtful that a non-complement-dependent flow cytometric assay could give comparable sensitivity to a conventional cytotoxic assay for antibodies of the IgM class. Nonetheless, a flow cytometric assay is still able to detect high titres of IgM HLA antibodies. For laboratories using anti-immunoglobulin labelling reagents that do not distinguish the class of antibody present in a crossmatch, it is possible that a proportion of the positive reactions are accounted for by IgM anti-HLA antibodies alone.

Early studies demonstrated the ability of flow cytometric crossmatching to detect pre-transplant anti-donor antibodies not detectable by cytotoxicity in 16 %–38 % of recipients [4, 5, 7, 13]. In the current modification of our flow cytometric crossmatch, replacing donor cells with pooled CLL cells enables the detection of subliminal levels of IgG alloantibodies by flow cytometry in 29 % of sera screened by cytotoxicity as negative, and 36 % of sera screened as containing IgM alone. It is possible that this method is able to predict a subgroup of patients who are more likely to produce an IgG-positive flow cytometric crossmatch. The clinical relevance of such antibodies to transplantation is as yet not known; however, analysis of the sensitising events in these patients may help to elucidate this. It is possible they represent residual subcytotoxic levels of antibody from previous immunizing events or an early stage of sensitisation in the transition of IgM to IgG production. A more precise understanding of the antibody response to antigenic stimulation by events such as transfusions and pregnancy may aid our ability to define patients for whom a positive flow cytometer crossmatch is an absolute bar to transplantation.

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