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Improved specificity and sensitivity when using pronase-digested lymphocytes to perform flow-cytometric crossmatch prior to renal transplantation

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Abstract Several laboratories have resorted to flow-cytometric crossmatch (FCXM) in an effort to prevent hyperacute and accelerated renal allograft rejections. The currently employed FCXM has problems with both false-positive and -negative reactions, largely as a result of irrelevant IgG binding to Fc IgG receptors. In 1980, we circumvented this problem by digesting Fc IgG receptors with pronase, and demonstrated that, with immunofluorescence microscopy (IF), detection of IgG anti-HLA antibodies was highly sensitive and specific. In 1995, we introduced the pronase technique to FCXM and showed that this enzyme did not decrease HLA expression. We present herein a prospective study at our institution to determine whether FCXM using pronase-digested (PD) lymphocytes is as sensitive and more specific than FCXM with undigested (UD) lymphocytes when compared with the highly sensitive and specific IF assay. In analyzing the 186 donor-specific pre-renal-transplant crossmatches, we found that PD FCXM was as sensitive and specific as IF and was able to detect weak IgG anti-HLA antibodies that bound to B cells. Fourteen of these patients would have been denied transplants if one were to have relied on UD FCXM. The data clearly indicate that PD FCXM can reliably be used to detect weak IgG anti-HLA antibodies before renal transplantation.

Keywords Pronase · Flow-cytometric crossmatch · Transplant crossmatch · Anti-HLA antibodies · Renal allografts

Introduction

Several laboratories are currently developing assays to detect weakly reactive, donor-specific IgG anti-HLA antibodies. Such antibodies, when undetected in a kidney transplant recipient, can cause hyperacute and accelerated renal allograft rejection. The widely used complement-dependent microlymphocytotoxicity (CdL) assays may fail to detect such low levels of IgG anti-HLA antibodies. As a result, between January 1988 and December 1998, 324 kidney grafts in the US were reported to UNOS as having been lost as a result of such rejections, of which 38 were from live donors.

Loss of such a scarce resource, especially due to a potentially preventable cause, has prompted several laboratories to use two-color flow cytometry for detection of IgG anti-HLA antibodies that bind to donor lymphocytes [1, 2, 3, 4, 6, 7, 8, 9, 11, 14, 15, 16, 17]. This immunofluorescence technique is highly sensitive for detecting weak IgG anti-HLA antibodies that bind to both B and T cells. However, the flow-cytometric crossmatch (FCXM) technique has run into problems with specificity, especially with the detection of IgG anti-HLA antibodies. One cannot differentiate by FCXM between irrelevant normal IgG binding to Fc IgG receptors present on B cells as well as activated T cells and specific IgG

bound to HLA antigens. The lack of specificity with FCXM has led many transplant institutions to ignore a positive T cell FCXM assay, especially in the setting of a primary renal transplant and when the CdL assay with B and T cells is negative [7, 8, 9, 14, 15, 16]. In these reports, between 16% and 40% of recipients would have been denied renal transplants if a decision of "not to transplant" were to have been based solely on a positive donor-specific FCXM. Better than 80% of transplants with a positive FCXM and negative CdL assay had functioning allografts at 1 year, clearly proving that FCXM has problems with specificity. In 1995, we modified the FCXM technique by digesting lymphocytes with an enzyme pronase to remove Fc IgG receptors (i.e., CD16 and CD32) [12]. We clearly demonstrated that such enzyme pretreatment of cells does not decrease the density of HLA antigens, but instead may increase the antigenicity of HLA antigens and, hence, improve the sensitivity of FCXM. Furthermore, we showed that this technique was specific and improved the detection of weak IgG B cell-specific anti-HLA class-I antibodies that could not be detected by FCXM using undigested cells [11]. All our observations with pronase pretreatment of cells prior to FCXM have now been validated by investigators from three separate histocompatability laboratories [18].

In the current prospective study beginning in July 1998 and ending in June 2001, we analyzed the sensitivity and specificity of FCXM, comparing the use of pronase-digested (PD) with undigested (UD) donor lymphocytes. FCXM was compared with a previously described immunofluorescence microscopy assay (IF) that we have been routinely using at our institution since 1980, i.e., before the introduction of FCXM by Garovoy et al. in 1983 [5, 10, 13]. In the IF assay, one uses PD lymphocytes, and binding of IgG to cells is detected by IF instead of flow cytometry. Prior to routine clinical application of the IF assay at our institution, we clearly showed that all six kidney transplants done in the setting of a positive IF assay were lost due to hyperacute or accelerated rejection, even though the CdL assay was negative, thus indicating that IF was not only sensitive but also specific [10]. Hence, in this study we compared two-color PD FCXM and UD FCXM with IF. In this study, kidney transplants were performed only if the IF assay was found to be negative. Secondly, in this report, we present details to standardize the PD FCXM technique. This study was approved by our Institutional Review Board on Human Investigation.

Materials and methods

Cell preparation

Mononuclear cells from blood, lymph node, or spleen were isolated by standard Ficoll-Hypaque techniques, the methods of which have been published previously [10, 13]. Contaminating polymorphonuclear cells, macrophages, platelets, and red blood cells were removed with T-B Kwik (One Lambda, Canoga Park, Calif., USA). These cells are then treated with pronase for use in IF or PD FCXM assays. Cells to be used in the CdL assay were further separated into B and T cells with Dynal magnetic beads conjugated with anti-CD19 (to positively select B cells) and anti-CD2 (to positively select T cells).

Sera

All sera used were centrifuged at 10,000*g* (in a microfuge) for 10 min immediately before the assay to remove large immunoglobulin aggregates or immune complexes. Several normal sera were obtained from either AB individuals or commercially prepared, pooled AB sera. Commercial sera included Sigma (Sigma Chemical, St. Louis, Mo., USA), Pel Freeze (Pel Freeze Clinical Systems, Brown Deer, Wis., USA), and C-Six (C-Six Diagnostics, Megvon, Wis., USA). Human sera containing anti-HLA-DR and anti-HLA-AB specificities were obtained from the National Institutes of Health (Bethesda, Md., USA). Human sera containing anti-HLA-AB with broad reactivity were obtained from hemodialysis patients who had rejected their renal allografts.

Cytotoxicity assays

The complement-dependent CDL assay was performed according to the modified Amos technique on T and B lymphocytes. An antiglobulin step (i.e., with goat anti-human κ) was used for T cells. Briefly, cells were incubated with sera at 25 °C for 30 min, washed three times, and then incubated with goat anti-human κ (C-Six Diagnostics) for 1 min before the addition of rabbit complement at 25 °C for 1 h. Cell death was determined by fluorQuench (One Lambda). Scoring of dead cells in excess of controls was as follows: 1 = 0%–10%, 2 = 11%–20%, 4 = 21%–49%, 6 = 50%–80%, 8 = 81%–100%.

Panel reactive antibodies

Patient sera were tested against a frozen panel of T or B cells representing all the class-I (T cells; Pel Freeze, Brown Deer, Wis., USA) or class-II (B cells; Bio Test, Denville, N.J., USA) specificities. The CdL assay was used to determine whether sera contained anti-HLA antibodies that were reactive to certain HLA specificities in the cell panel. We calculated the panel reactive antibodies (PRA) percentage by enumerating the number of wells that contained cells with a cell death greater than 21% and dividing the result by the total number of wells. Each well contained cells from a single individual and, hence, 60 individuals were represented in the T-cell panel tray and 30 in the B-cell panel tray.

Antisera and murine monoclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2, anti-human Ig, specific for either IgG (specific for Fc of γ chain) or IgM (specific for Fc of μ chain), was obtained from BioSource, Camarillo, Calif., USA. Murine monoclonal antibodies to HLA-DR (L243) and HLA-A, B (W6/32) were obtained from American Type Culture Collections (ATCC, Rockville, Md., USA). FITC-conjugated antisera were centrifuged at 10,000*g* for 10 min in a microfuge before being used. Phycoerythrin (PE)-conjugated mouse anti-CD3 monoclonal antibody (clone UCHT-1, Sigma) and FITC-conjugated mouse anti-CD16 and anti-CD32 were obtained from PharMigen International, San Diego, Calif., USA.

Pronase digestion of cells

The details of this technique have been described previously [10, 13]. Briefly, 1×10^7 lymphocytes in 0.2 ml phosphate-buffered saline (PBS) or RPMI 1640 without fetal calf serum (FCS) were digested with 0.5 ml of the appropriate concentration of pronase (Type XIV,

Sigma) for 30 min at 37 °C to remove Fc IgG receptors on lymphocytes. The cells were then washed three times with PBS containing 10% FCS. DNAase (Sigma) was added to the first wash to dissociate cell clumps (500 k unity units/10^7 cells). Enzymatically treated cells were kept at 4 °C with PBS containing 2% FCS and 0.02% sodium azide. PD cells will regenerate Fc IgG receptors if cells are left in PBS without sodium azide. The cell viability of the lymphocytes as determined by trypan-blue exclusion is usually greater than 80%. Most contaminating phagocytic cells die and clump out with the enzymatic digestion.

Identification of IgG anti-HLA antibodies by IF

Aliquots of PD cells (0.3×10^6 in 0.2 ml PBS) were interacted with 25 µl of control AB sera or recipient sera at 4 °C for 30 min and washed twice. The cells were then incubated with 25 µl of appropriately diluted (usually 1:10) FITC-conjugated goat antisera specific for either IgM (specific for Fc of µ chain) or IgG (specific for Fc of γ chain) at 4 °C for 30 min. All assays were performed in Eppendorf tubes or 5-ml Falcon plastic tubes, and the PBS used for the washes contained 2% FCS and azide. We examined wet coverslip preparations immediately by phase-contrast fluorescence microscopy to determine the percentage of immunofluorescent-positive lymphoid cells. Contaminating phagocytic cells are easy to identify under phase microscopy. Dead cells have non-specific FITC intracellular staining and, hence, are easy to differentiate from live cells with membrane-bound antibody. We routinely interacted three normal AB sera with non-pronase-treated or pronase-treated cells to determine whether Fc IgG receptors were effectively digested. Peripheral blood lymphocytes, when incubated with control AB sera, had usually less than 3% of cells staining for membrane IgG. Test sera were considered to have anti-HLA antibody when membrane IgG-staining of cells was more than 5% than that observed with control AB sera. Ten to fifteen percent of lymph-node and splenic B lymphocytes expressed membrane-incorporated IgG that is pronase-resistant. Further studies revealed that these B lymphocytes secreted IgG. We therefore employed an additional control without human sera to quantitate IgG-positive B cells when using lymphocytes from lymph node or spleen.

Two-color FCXM analysis

The same aliquot of cells used in the IF assay was incubated with PE anti-CD3at 4 °C for 30 min, washed three times, and fixed with 2% paraformaldehyde prior to flow-cytometric analysis (FAC Scan, equipped with a Consort 30 computer program). We collected data from 10,000 cells, using logarithmic amplification of fluorescence parameters. A two-parameter dot plot display of FITC (X axis) versus PE (Y axis) was generated. The flow cytometer was calibrated and PE-anti-CD3-stained peripheral blood mononuclear cells (PBMCs) were used to set markers separating CD3-positive T cells from non-T cells. We routinely interacted both pronase-treated and non-treated cells with FITC anti-CD16 and anti-CD32 to determine whether Fc IgG receptors were effectively digested. We analyzed several control AB sera and sera from patients with 0% PRA to determine the specificity of the two-color flow cytometry. The two-color dot plot display (Fig. 1) provided the following data in the four quadrants: left upper quadrant (LUQ) cells that are PE-positive and FITC-negative (i.e., T cells); right upper quadrant (RUQ) cells that are PE- and FITC-positive (i.e., T cells binding to IgG); left lower quadrant (LLQ) cells that are PE- and FITC-negative (i.e., non-T cells negative for IgG binding); right lower quadrant (RLQ) cells that are PE-negative and FITC-positive (i.e., non-T cells binding to IgG or B cells secreting membrane IgG). Non-T cells include B cells and CD16-positive NK cells. FL-1 histograms of either T or non-T cells were created from the FL-1, FL-2 dot plot. We gated non-T cells (i.e., LLQ + RLQ) and T cells (i.e., LUQ + RUQ) separately to create these histograms.

Results

Pronase digestion and FCXM data analysis

The optimal quantity of pronase needed to digest Fc IgG receptors on lymphocytes had to be determined with each new lot of the enzyme. Enzyme activity remained stable at –20 °C for at least 1 year. Enzyme concentration was considered optimal when cell viability at the end of digestion was more than 85% and no binding of normal IgG to non-T cells was observed. As depicted in Fig. 1, 2 mg/ml of pronase was found to be optimal. Pronase, when used at an optimal concentration, removed CD16 and CD32 Fc IgG receptors (Fig. 2) without decreasing antigenicity of the HLA and CD3 receptors. Instead, pronase digestion enhanced binding of anti-CD3 antibody to T lymphocytes (Fig. 1 and Fig. 2), presumably as a result of receptor "unmasking". Additionally, since optimal pronase digestion gives rise to a symmetrical histogram (especially for non-T cells), this improves reliability of a median channel value (Fig. 1). Excess pronase (at 3 mg/ml) significantly decreased cell viability and caused IgG to bind non-specifically to the cell membranes. Less pronase was needed to digest lymphocytes obtained from lymph nodes or spleen (usually 1 mg/ml).

We analyzed data by creating FL-1 histograms of either T cells or non-T cells from the FL-1, FL-2 dot plot. With PD FCXM, sera were considered to be positive for IgG anti-HLA antibody (reactive to B or T cells) when there was an increase of more than five median channels (MCs) when histograms of recipient sera were compared with control AB sera. This criterion was used as with PD FCXM; the MC for the control AB sera with B or T cells was low, i.e., three to seven channels, which is similar to the MC for cells exposed to the FITC goat anti-human IgG antibody in the absence of serum. We found FL-1 MC shifts of the T- or B-cell histogram to be more sensitive and specific in detecting IgG anti-HLA antibodies than was quantitating the percentage of IgG-positive T or B cells in a dot plot. Analyzing data in this manner was particularly relevant with lymph-node or spleen lymphocytes, which contained 10%–15% B cells secreting membrane IgG that was resistant to pronase digestion. Additionally, we did not amplify the MC fluorescence intensity for the control AB sera, as this maneuver altered the criteria for the increase in number of channels needed to determine the presence of IgG anti-HLA antibodies in the test sera. For example, as depicted in Fig. 3, there was a corresponding increase in the MC fluorescence with the positive anti-HLA-A1 sera when the flow cytometer was adjusted such that the MC fluorescence intensity for the negative control AB serum was increased from 9.14 to 73.65. The MC shift for anti-HLA-A1 sera diluted 1:16 (when compared with normal AB sera) changed from 12.08 to 105.78 channels. We therefore did not amplify the

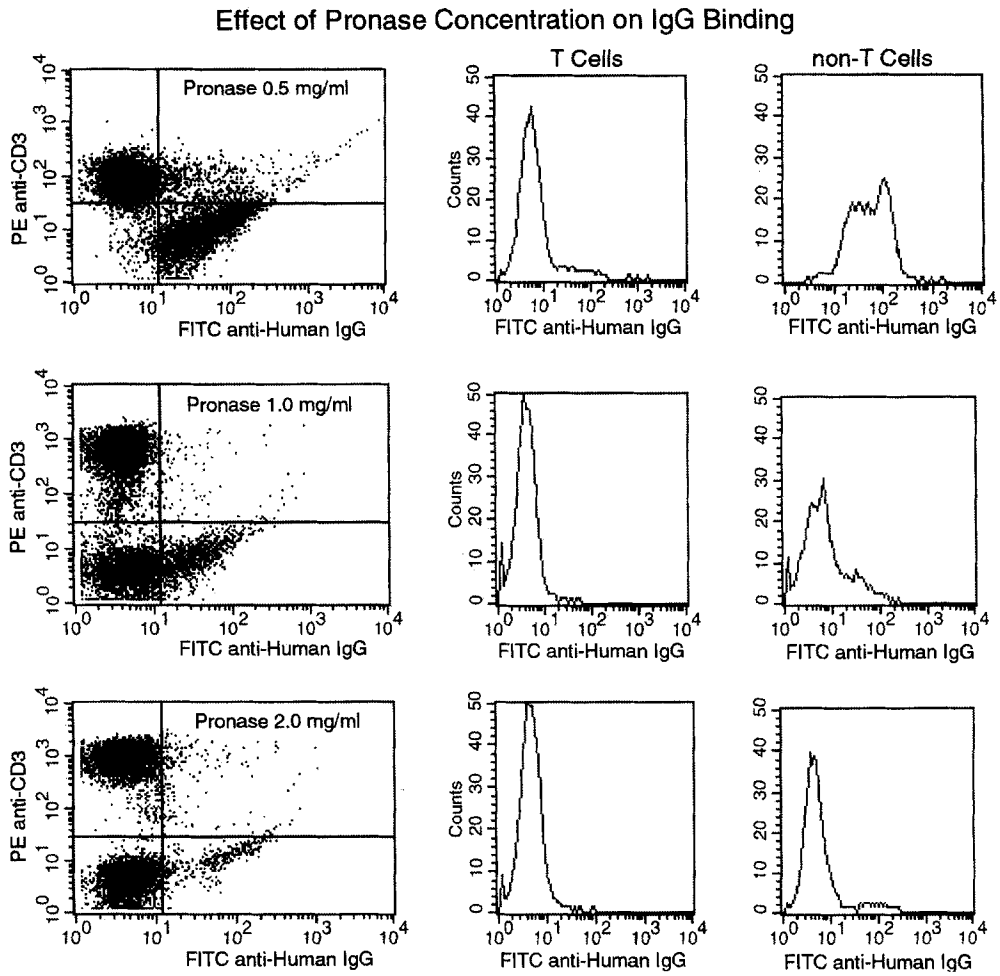

Fig. 1 Different concentrations of pronase were used to digest peripheral blood lymphocytes prior to interacting cells with a negative control AB serum and staining with FITC goat anti-human IgG and PE mouse anti-CD3. Note that pronase at 2.0 mg/ml was optimal in decreasing binding of irrelevant IgG to non-T cells, as is evident in the dot plot and the non-T-cell histogram

MC fluorescence intensity for the negative control AB sera as with PD FCXM; the criterion for a positive assay was a shift of five MCs. However, with UD FCXM, the MC when control AB serum was used varied from 8 to 100 channels for B cells and fewer than ten channels for T cells, even though the level of fluorescence intensity was similar to that of PD FCXM. The UD FCXM assay was considered positive if there was more than a five-channel shift for T cells and more than a ten-channel shift for B cells. The sensitivity of our criteria was tested after serial dilutions of sera with known anti-HLA antibody, comparing PD FCXM and IF, were used, as previously described [12]. With these criteria, detection of anti-HLA antibody by PD FCXM was found to be equivalent to that detected by IF.

Human sera known to have broadly reactive IgG anti-HLA antibodies were pooled and used as positive controls in our assays. With FCXM, it became apparent that there was a wide variability in the fluorescence intensity of IgG binding to lymphocytes of different individuals, when the same positive control sera were used. MC shift with positive human sera, when compared with normal AB sera, varied from 80 to 1,100 channels when cells from different individuals were used. In part, this variability could be explained by the density of HLA expressed on cells. We quantitated total HLA expression by using murine monoclonal W6/32 and L243 that bind to monomorphic determinants on HLA class I and class II, respectively. Hence, we routinely used these murine monoclonals to control for quantity of HLA expression, which can be important in the interpretation of a weakly positive donor-specific crossmatch.

Data in Table 1 summarize all the 186 donor-specific pre-renal-transplant crossmatches comparing IF with UD FCXM and PD FCXM. In this prospective study, care was taken to use the same source of lymphocytes in all the assays including the CdL cytotoxicity assay. Data clearly indicate that UD FCXM and PD FCXM are more sensitive than CdL in detecting IgG anti-HLA donor-specific antibodies. However, with UD FCXM there were 14 more positive IgG B cell-specific crossmatches (see group 4) that could not be detected by IF or by PD FCXM. One such case is exemplified in Fig. 4. In this example, with the UD FCXM assay, the MC of IgG binding to B lymphocytes, when control AB sera were used, was 16.5, and the MC increased to 41.79 with

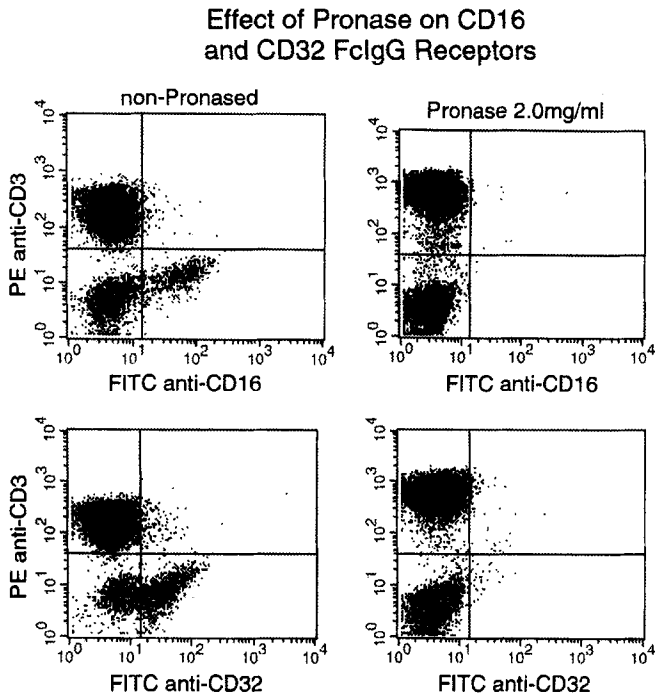

Fig. 2 The same cells as in Fig. 1 were either non-digested or digested with pronase at a concentration of 2.0 mg/ml and then stained with PE-labeled murine anti-CD3 and FITC-labeled murine anti-CD16 or CD32 antibodies. Note that a significant subpopulation of non-T cells has Fc IgG receptors, detected with murine anti-CD16 and anti-CD32 monoclonals

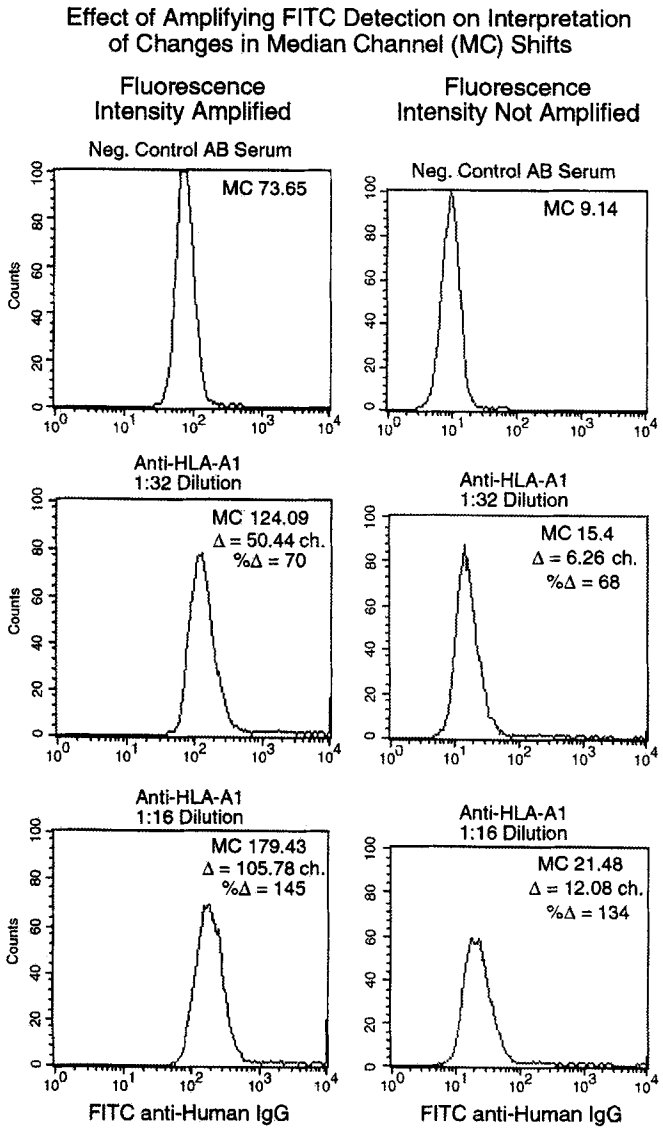

Fig. 3 Fluorescence histograms detecting FITC IgG binding were created of HLA-A1-positive T cells that were interacted with either negative control AB serum or anti-HLA-A1 antisera. *The panels to the left* display the effect of amplifying the fluorescence intensity of the same aliquot of FITC-labeled cells as in the right panels. *Delta* denotes the difference in MCs (i.e., MC shift) when compared with the MC for AB serum. *%Delta* denotes the percent change in channel shifts when compared with the MC for AB serum. Note that the amplification procedure significantly increased the value for MC shift, but did not appreciably affect the percent change in channel shifts

the recipient sera. However, with PD lymphocytes the MC of IgG binding to B lymphocytes with the same control AB sera had decreased to 3.4 as a result of digesting Fc IgG receptors, and the MC with recipient sera was 4.8. All 14 of these recipients underwent kidney transplantation with no hyperacute or accelerated rejection, indicating, therefore, that the UD FCXM with B lymphocyte was falsely positive, probably because IgG was binding non-specifically to Fc IgG receptors on B lymphocytes. Even though UD FCXM is highly sensitive, this assay failed to detect IgG B cell-specific antibodies in two patients (see group 2). Binding of irrelevant IgG to Fc IgG receptors clearly prevented detection of such weak IgG anti-HLA antibodies as were detected by IF and PD FCXM. In these two recipients, pronase digestion decreased the MC fluorescence of B cells with normal AB sera to fewer than four channels, thus revealing binding of IgG anti-HLA to B cells with an MC channel shift of between seven and nine channels. Both of these recipients were known to have an increased PRA to B lymphocytes, with anti-HLA class-II antibody specificity present in these donors. These two recipients were not given transplants.

Finally, we encountered three donor-specific crossmatches that were positive by PD FCXM but had a negative IF crossmatch (see group 3 and group 5). Two of these patients had a positive T-cell PD FCXM, but a negative B-cell PD FCXM. Both these patients underwent renal transplantation, as it was felt that these patients had non-HLA IgG antibodies. No rejections were observed in these two patients. An exception "not to transplant" was made on the third patient (group 5) with a negative IF and UD FCXM assay, but a positive PD

Table 1 Donor-specific crossmatches comparing FCXM with IF and CdL assay. *Number in parentheses* denotes total number of patients within the group

[a]Data include positive results with B or T cells or both

Parameter	Number of positive assays				
Assays	Group 1 (31)	Group 2 (18)	Group 3 (2)	Group 4 (14)	Group 5 (121)
CdL-B cell	31	0	0	0	0
IF	31	18	0	0	0
UD FCXM[a]	31	16	2	14	0
PD FCXM[a]	31	18	2	0	1

FCXM assay on B lymphocytes, as this was a re-transplant with the recipient known to have anti-HLA-A1 antibodies, and the prospective living donor was also HLA-A1-positive. In this one instance, we felt that the PD FCXM was more sensitive than the IF assay.

Discussion

The data clearly show that UD FCXM is highly sensitive (when compared with the CdL assay on B and T cells) but is wrought with significant false positives, especially with the detection of IgG binding to HLA on B cells and also on activated T cells which can express Fc IgG receptors. One can therefore deny transplants to a substantial number of patients based on the UD FCXM, which is currently performed by the majority of institutions. Hence, several institutions perform primary renal transplants (but not re-transplants) in the setting of a positive UD FCXM and a negative B- and T-cell CdL assay [7, 8, 9, 14, 16, 17]. A false-positive B- and T-cell crossmatch with UD FCXM is primarily a result of irrelevant IgG binding to Fc IgG receptors present on these cells. The pre-digestion of cells with pronase prior to FCXM circumvents this potential problem and renders FCXM both sensitive and specific [11, 12, 18].

Much less common is a situation where UD FCXM cannot detect weakly reactive IgG anti-HLA, B cell-specific antibodies (see patient group 2 of Table 1), again because one cannot differentiate by FCXM between binding of IgG to either Fc IgG receptors or to HLA antigens. Such a situation can be obviated with PD FCXM, thus preventing unnecessary hyperacute or accelerated rejections. Both we and others have previously reported cases of hyperacute and accelerated rejections when kidney transplants were performed in the setting of a weakly reactive IgG anti-HLA, B cell-specific antibody that could not be detected with UD FCXM [11, 18].

One cannot emphasize the need to use the optimal amount of pronase when digesting lymphocytes. With each new lot of pronase, one has to evaluate the optimal dose (as exemplified in Fig. 1 and Fig. 2) required to digest Fc IgG receptors present on lymphocytes obtained from peripheral blood, spleen, and lymph nodes. In general, less pronase was required with lymphocytes obtained from lymph nodes. Excess pronase will lead to excess cell death and non-specific binding of IgG to cell membranes. Secondly, we did not see the need to amplify the fluorescence intensity for detection of fluorescein-positive cells. Amplification simply increases the MC for the cells exposed to control AB sera, but does not necessarily increase sensitivity of anti-HLA antibody detection (see Fig. 3). Indeed, variable amplification of the fluorescence intensity for control AB sera will lead to a marked variation in criteria (i.e., actual difference in MC shift) for assigning a positive FCXM result. For example, the MC shift criteria for a positive B-cell UD FCXM has varied in different laboratories from ≥40 to ≥150 channels, probably as a result of variable amplification in the fluorescence intensity for control AB sera [3, 7, 8, 9, 14, 16]. In the present study, with PD FCXM the MC with control AB sera (for both B and T cells) could be maintained at approximately five channels, as there was no binding of irrelevant IgG to cells. Hence, with PD FCXM, an increase or shift of five channels or a percent change of 100 above the control AB sera was

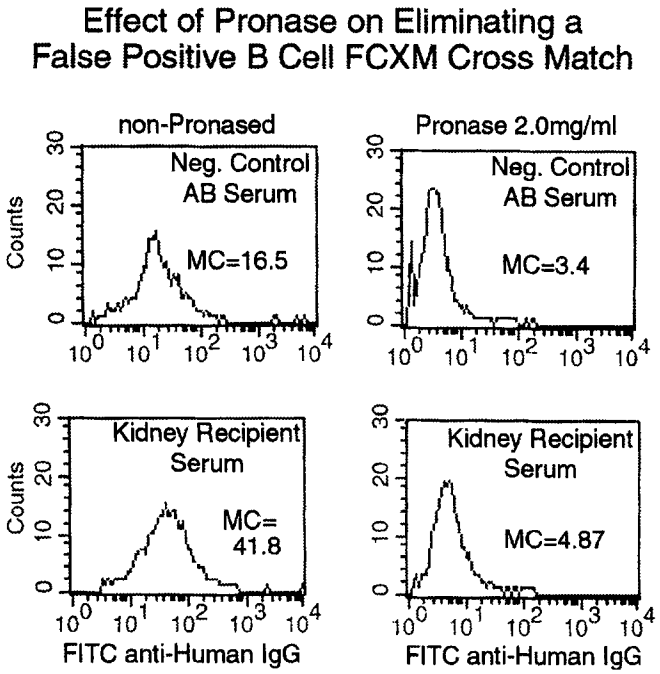

Fig. 4 Fluorescence histograms detecting FITC IgG binding. Histograms were created of non-pronase-treated and pronase treated non-T cells from donor peripheral blood lymphocytes that were interacted with the recipient serum. Note that the UD FCXM is positive while the PD FCXM is negative

significant and indicated the presence of IgG anti-HLA antibodies. In contrast, with UD FCXM, the lowest MC for non-T cells using control AB sera varied from 8–100 channels, and it was difficult to set a standard for the shift in MC to denote presence of anti-HLA antibody. However, in our hands, an increase of ten channels for non-T cells was felt to be significant in detection of IgG anti-HLA antibodies. Such a criterion maintained sensitivity of UD FCXM and paradoxically decreased the false-positive rate (see Table 1) when compared with other studies that have amplified several-fold the MC value for the negative control AB sera [7, 8, 9, 14, 16, 17, 18]. Based on data in Fig. 3, when one analyzes data, it would be more accurate to set standards for the percent change in MC shifts rather than the actual difference in MC shifts between the positive and negative control sera, especially when the fluorescence intensity is amplified. However, setting such standards for UD FCXM may not be practicable, as the MC for negative control AB sera varies, based on the source of non-T lymphocytes, i.e., different donors have different levels of Fc IgG receptors expressed on their peripheral blood lymphocytes, and these differences can vary even more with lymph nodes or spleen lymphocytes. Conversely, with PD FCXM, one can set standards for either the percent change or the actual difference in MCs when pronase-treated lymphocytes are used, as there is minimal variation in MCs with the negative control AB sera.

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