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The use of pronase-digested human leukocytes to improve specificity of the flow cytometric crossmatch

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Abstract Two-color fluorescence cytometry (FCXM) has recently been introduced to improve the detection of anti-HLA antibodies that react to donor cells, especially in recipients receiving kidney allografts. Although this assay system is highly sensitive, it lacks specificity. Between 70 % and 90 % of potential kidney recipients with a positive FCXM would have been denied transplant if such an assay had been used alone to detect antidonor antibodies. Lack of specificity is principally due to normal or irrelevant IgG in aggregates or immune complexes binding to Fcγ R receptors on lymphocytes including B cells and a significant subset of T cells. To circumvent this problem, we digested Fcγ R receptors on lymphocytes with pronase. We present data demonstrating that pronase digestion of lymphocytes does not alter HLA antigenicity. In addition, pronased lymphocytes allow one to use either single- or two-color FCXM. With single-color FCXM, one can quantitate antibody reactivity to lymphocytes via a cursor (on the fluorescence histogram) that separates lymphocytes that do not bind to antibodies. We present data demonstrating that this modification renders FCXM highly sensitive and specific. In addition, one can discriminate between IgG and IgM antibodies that react to lymphocytes.

Key words Crossmatch, pronase · Pronase, crossmatch · Flow cytometry, pronase

Introduction

Despite improvements in the sensitivity of the complement-dependent microlymphocytoxicity crossmatch (CDL), a small but significant number of renal allografts are lost due to hyperacute rejections or early humoral mediated graft rejections [3]. In 1977, our laboratory developed an indirect immunofluorescence technique (IF) that increased the sensitivity and specificity of the crossmatch assay [6]. Specificity was achieved in the IF assay by predigesting the donor lymphocytes with pronase to remove Fcγ R receptors that bind to the Fc region of normal or irrelevant IgG. Since the introduction of this IF assay in our laboratory, we have not had a case of hyperacute or early humoral mediated renal allograft rejection at our institution. Furthermore, IF was put to clinical use, as compelling data indicated that this assay was highly specific [7]. All of five kidney recipients with donor-specific crossmatches that were negative by CDL but positive by the IF assay underwent histologically confirmed humoral rejections and allograft loss in less than 1 week [7].

The IF technique utilizes phase-contrast ultraviolet microscopy (× 800 magnification) to differentiate lymphoid cells from macrophages or polymorphs. One then enumerates the percentage of lymphoid cells that have IgG fluorescence staining on the cell membrane. Pronase-digested lymphocytes are depleted of Fcγ R receptors and, hence, with normal control human sera, less than 5 % of the lymphocytes have membrane immunofluorescence that can be detected by the IF technique. This low background FITC staining of cells

makes it possible to easily differentiate normal sera from anti-HLA-containing sera. In addition, the IF assay can be quantitated, as irrelevant IgG binding is no longer a problem [6, 7].

In 1983 Garovoy and colleagues reintroduced indirect IF to detect anti-HLA antibody binding to lymphocytes, but they employed flow cytometry (FCXM) instead of microscopy to quantitate antibody binding to cells [2]. With the FCXM and normal control sera, one obtains a fluorescence histogram. A cursor is set at a point on the histogram where most or all of the cells to the left of the cursor are considered to be fluorescent-negative. A serum that increases fluorescence to the right of this cursor is considered positive. However, owing to the presence of FcγR receptors, predominantly on CD16-positive lymphocytes, it became difficult to differentiate the binding of IgG in normal patient sera from that of normal control AB sera [4, 5]. Various normal and control sera gave rise to large variations in IgG binding to lymphocytes, making it difficult to interpret sera that were truly negative for anti-HLA antibodies. Hence, the FCXM lacked specificity.

To circumvent this problem of "false-positives", other investigators modified the FCXM to a two-color system. That is, T or B lymphocytes were identified with phycoerythrin-labelled anti-T or -B murine monoclonal antibodies, and it was presumed that IgG binding to either T or B cells, detected by FITC $F(ab')_2$ anti-human IgG, was therefore not a result of irrelevant IgG binding to FcγR receptors predominantly on the non-B, non-T-lymphocyte subset (CD 16-positive) [1]. Unfortunately, this technique continues to be plagued with problems of "false-positives" [9, 10]. Furthermore, because of the variable binding of normal control AB sera to T or B cells, it is difficult to quantitate the percentage of lymphocytes that are positive using the cursor approach (unlike the IF technique). Currently, one uses an arbitrary approach that does not involve a positive-negative cut-off point. Firstly, a fluorescence histogram is obtained using a logarithmic scale to measure FITC binding. Secondly, the logarithmic values are mathematically converted to linear fluorescence channels. One then compares the median (or mean) linear fluorescence channel of a given serum with that obtained from several negative control sera. With a 256-channel scale, a channel shift to the right of more than ten with T or B cells is considered positive.

Despite these modifications, the FCXM lacks specificity, though it is superior to the microlymphocytoxicity crossmatch (CDL) in identifying anti-HLA antibodies [9, 10]. This lack of specificity stems to a large extent from FcγR receptors, which are also present on a significant T-cell subset and B cells [11, 12]. We, therefore, used pronase-digested lymphocytes in FCXM and compared the data to the highly specific IF assay. In this paper, we demonstrate that we can now use either the two-color or single-color FCXM with pronase-treated lymphocytes. In addition, we show that with this technique FCXM can be very specific and that recipient sera with anti-HLA antibodies to the donor can be accurately quantitated by setting a cursor on the fluorescence histogram, especially with single-color FCXM.

Materials and methods

Cell preparations

Mononuclear cells from blood, lymph node, or spleen were isolated by standard Ficoll-Hypaque techniques, the methods of which have been previously published [7]. Contaminating polymorphonuclear cells and macrophages were removed by 55 % percoll gradient prior to IF staining. Blood or spleen preparations from donors who received methylprednisolone were not used in cytotoxic assays, as this drug interferes with the removal of contaminating phagocytic cells.

For the CDL cytotoxicity assay, mononuclear cells were further purified with T-B Kwik (One Lambda, Canoga Park, Calif.) to remove polymorphs and macrophages.

Sera

All sera used were centrifuged at 10,000 *g* (in a microfuge) for 10 minutes 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.), Pel Freeze (Pel Freeze Clinical Systems, Brown Deer, Wis.), C-Six (C-Six Diagnostics, Megvon, Wis.), MSL (Bio-Bee, Boston, Mass.), Gibco (Grand Island, N.Y.), and Whittacker (Whittacker Bioproducts, Walkersville, Md.). Human sera containing anti-HLA-DR and anti-HLA-AB specificities were obtained from the National Institutes of Health (Bethesda, Md.). 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 microlymphocytoxicity assay (CDL) was performed according to the modified Amos technique with antiglobulin. Briefly, cells are incubated with sera at 25 °C for 30 min, washed twice, and then incubated with goat antihuman kappa (Atlantic Antibodies and Instar, Stillwater, Minn.) for 2 min before adding rabbit complement at 25 °C for 1 h. Cell death is determined by eosin staining followed by formalin fixation. Scoring of dead cells in excess of controls is as follows: 1 = 0 %–10 %, 2 = 11 %–20 %, 4 = 21 %–49 %, 6 = 50 %–80 %, 8 = 81 %–100 %. The CDL assay is not performed, especially on B-lymphocyte preparation, when cell viability is less than 85 % or when contaminating phagocytic cells are in excess of 15 %.

Panel reactive antibodies (PRA)

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.) or class II (B cells; Bio-Test, Dreieich, Germany) 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. Percent PRA was calculated by enumerating the number of wells with a cell death greater than 30% 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.

FITC-conjugated anti-sera and monoclonal antibodies

Goat $F(ab')_2$, antihuman Ig, specific for either IgG (Fc-specific) or IgM (mu chain-specific), were obtained from Jackson Immunoresearch (West Grove, Pa.). Murine monoclonal antibodies to HLA-DR (L243) and HLA-A, B (W6/32) were obtained from American Type Culture Collections (ATCC, Rockville, Md.). FITC-conjugated anti-sera were centrifuged at 10.000 g for 10 min before use.

Identification of antilymphocyte antibodies by indirect immunofluorescence (IF)

The details of this technique have previously been described elsewhere [6, 7]. Briefly, 1×10^7 lymphocytes/ml were digested with 2.0–2.75 mg pronase (Type XIV, Sigma Chemical, St. Louis, Mo.) to remove FcγR receptors on lymphocytes. Cells were washed three times with phosphate-buffered saline (PBS) containing 2% fetal calf serum. DNA ase (Sigma Chemical) was added to the first wash to prevent cell clumping (500 kunity units/10^7 cells). Enzymatically treated cells were kept at 4 °C in the presence of sodium azide to inhibit the regeneration of Fcγ receptors. (Pronase-digested cells will regenerate FcγR if they are incubated at 37 °C in culture media without sodium azide. The reappearance of FcγR takes 8–12 h. The cell viability of the lymphocytes, as determined by trypan blue exclusion, is greater than 90%. Most contaminating phagocytic cells die and clump out with the enzymatic digestion.) Digested cells were interacted with sera at 4 °C for 30 min and washed. Then, aliquots ($0.5–1 \times 10^6$ cell) were stained with FITC – conjugated goat antisera specific for either IgM (mu chain) or IgG (Fc chain). Wet cover slip preparations were examined immediately by phase-contrast fluorescence microscopy (× 800 magnification), care being taken to exclude any contaminating phagocytic cells in determining the percentage of immunofluorescent-positive cells. Unlike with cytotoxicity assays, contaminating phagocytic cells are not a problem, as they are easy to identify under phase microscopy. Dead cells take up FITC intracellularly and, hence, are easy to differentiate from FITC bound to antibody on the cell membrane. Three normal AB sera were routinely interacted with nonpronased and pronased cells to determine whether FcγR receptors were effectively digested. The concentration of pronase used varied with the different lots.

Single-color FCXM crossmatch

The techniques used were identical to those described under IF, except that cells to be pretreated with antibodies were used without enzymatic digestion (nonpronased) or after pronase digestion. FITC-labelled samples were analyzed by flow cytometry within 3 h. Paraformaldehyde-fixed cells were not used. FITC-labelled samples were analyzed on a FAC Scan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). Data from 10,000 cells were collected with single-color FITC and logarithmic amplification of fluorescence parameters. A consort 30 program converted logarithmic data into linear fluorescence channels (256-channel scale) and calculated the mean channel fluorescence. This program was equipped to overlay histograms for comparisons.

Table 1 Detection of anti-HLA antibodies – evaluating sensitivity and specificity of FCXM, IF, and CDL. Peripheral blood leukocytes from a single individual were interacted with various sera using different techniques to identify and quantitate anti-HLA antibody. FITC goat $F(ab')_2$ antihuman IgG (Fc-specific) was used in IF and single-color FCXM

Sera	CDL[a]	IF	Single-color FCXM	
		Pronased[b]	Non-pronased[c]	Pronased[b]
Autologous	Neg	1.5	77.1	4.1
Normal pooled AB sera				
– C-Six	Neg	1.8	82.8	3.9
– MSL	Neg	1.3	152.9	3.1
– Whittacker	Neg	2.0	163	3.7
– Blood Bank	Neg	1.6	84.03	4.8
– Pel-Freeze	Neg	1.5	123	3.4
– Sigma	Neg	2.0	87.2	4.7
– Gibco	–	–	–	3.1
HLA-Anti-DR				
– L243	2–4	36	109	40.7
– KC-SLBR 1 : 8[d]	2–4	27.5	131	29.8
– KC-BRA 1 : 8	2–4	29.3	137.3	26.3

[a] Values indicate extent of cell death
[b] Values indicate percentage of cells that are IgG immunofluorescent-positive
[c] Values indicate linear mean fluorescence channel on a 256-channel scale
[d] Dilution of sera used

Two-color FCXM analysis of normal and control sera

For this, we used a PE-conjugated anti-CD3 reagent (PE-Leu 4, Becton Dickinson) to identify T cells. FITC goat $F(ab')_2$ antihuman IgG (Fc-specific; Tago, Burlingame, Calif.) was used to identify IgG binding to T cells. Ficoll-hypaque-isolated peripheral blood mononuclear cells (PBMC) were further purified with T/B Lymphokwik (One Lambda). PBMC (0.5×10^6) were incubated with 50 lambda of normal human sera that had been previously centrifuged in a microfuge at 10,000 *g*. The cells were incubated with sera at 4 °C for 30 min, washed twice, and then reincubated with FITC goat $F(ab')_2$ antihuman IgG at 4 °C for 30 min. After three more washes, the cells were incubated with PE-Leu 4 at 4 °C for 30 min, and washed four times prior to flow cytometric analysis (FAC Scan, equipped with a Consort 30 computer program). Data from 10,000 cells were collected in a list mode using logarithmic amplification of fluorescence parameters. A two-parameter display of FITC (X axis) versus PE (Y axis) was generated. The flow cytometer was calibrated and PE-Leu 4-stained PBMC were used to set markers separating CD3-positive T cells from non-T cells.

Several control AB sera and sera from patients with 0% PRA were analyzed to determine the specificity of the two-color flow cytometry. The two-color display (Fig. 3) provided the following data in the four quadrants: (1) percentage of cells PE-positive (i.e., T cells); (2) percentage of cells PE- and FITC-positive (i.e., T cells binding to IgG); (3) percentage of cells PE- and FITC-negative (i.e., non-T-cells negative for IgG binding); (4) percentage of cells PE-negative and FITC-positive (i.e., non-T cells binding to IgG). Non-T cells include B cells and CD16-positive non-B, non-T cells.

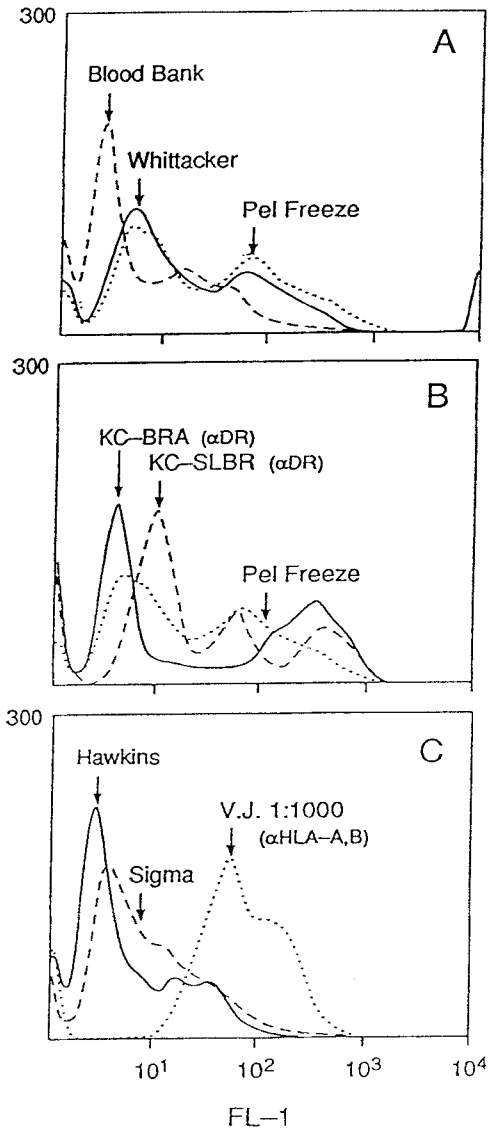

Fig. 1 A–C Single-color FCXM. Peripheral blood lymphocytes (nonpronased) were interacted with: **A** several normal AB sera (Blood Bank, Whittacker, and Pel Freeze); **B** sera with anti-HLA-DR alloantibody; and **C** patient sera (V.J.) with anti-HLA, AB activity. The latter was negative in the CDL assay at a dilution of 1 : 256

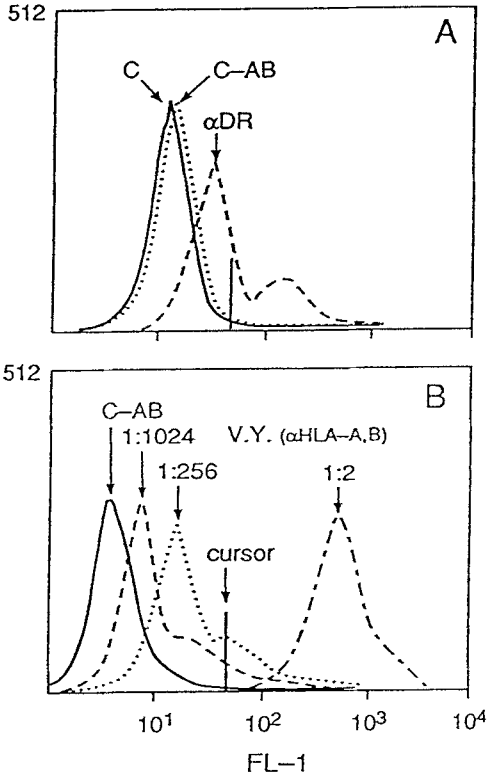

Fig. 2 A, B Single-color FCXM. Pronased peripheral blood lymphocytes from the same individual as in Fig. 1 were interacted with the same normal AB sera as in Fig. 1: **A** Interaction of cells with anti-HLA-DR antisera (KC-BRA); **B** Interaction with a patient's serum (V.Y.) at varying dilutions containing anti-HLA A, B antibody. The CDL assay was negative with V.Y. sera at a dilution of 1 : 128. Percentage of lymphocytes reactive with C-AB, V.Y. 1 : 1024, 1 : 256, and 1 : 2 were 3.9 %, 10.3 %, 23.3 %, and 99.7 %, respectively (*C-AB* the fluorescence histogram obtained with normal AB sera, Sigma; *C* the fluorescence histogram when lymphocytes are only interacted with the FITC antihuman IgG)

Results

Specificity of control sera lacking anti-HLA antibodies

An important facet of any donor-specific crossmatch assay is the use of human sera that lack anti-HLA antibodies (i.e., negative controls). The standard procedure is to use sera from nonpregnant, nontransfused individuals with an ABO blood type AB. Peripheral blood lymphocytes from six normal individuals were reacted with autologous sera, several AB-negative controls, and sera with anti-HLA activity. The sensitivity and specificity of cell-sera interactions were evaluated using nonpronased or pronased cells in the cytotoxicity (CDL) and indirect immunofluorescence (IF and FCXM) assays. A representative example using cells from a single individual is depicted in Table 1 and Fig. 1 and 2. With the single-color FCXM assay and using non-pronased peripheral blood mononuclear cells, it is evident that there is a wide variation in the linear mean fluorescence channel of IgG binding using different normal AB sera. Additionally, since there is a marked variability on the fluorescence histogram, one cannot apply a cursor with accuracy to denote cells that are fluorescent-negative (Fig. 1). This is evident even when staining lymphocytes in the absence of sera. This confirms previous observations and has been attributed to FcγR receptors on lymphocytes [1]. Finally, with these individuals cells, it is difficult to interpret data using

Table 2 Two-color FCXM-binding of IgG from control sera and patient sera using CD3-labelled nonpronased and pronased peripheral blood mononuclear cells. Reactivity of sera to cells from a single donor is depicted. With nonpronased cells, marked variability in binding of normal sera to T cells was observed. This was not observed with pronased cells

Sera	% of T cells binding to IgG	
	Nonpronased	Pronased
Normal pooled sera		
C-Six	25.1	3.6
Whittacker	9.9	4.1
MSL	8.3	3.8
Blood Bank (AB)	4.2	4.3
Patients (PRA < 1 %)		
S.F.	10.7	1.0
M.G.	3.0	< 1.0
R.H.	2.1	< 1.0
K.K.	16.1	< 1.0
M.I.	3.6	2.6
M.N.	2.0	< 1.0
C.R.	16.6	2.5
N.C.	11.7	2.4

sera of patients with anti-HLA-DR antibodies in the non-pronased FCXM assay (Table 1, Fig. 1B). However, Fig. 1C demonstrates that FCXM with nonpronased cells, though nonspecific, is clearly sensitive in detecting anti-HLA, AB antibodies. To improve the specificity of single-color FCXM, we used pronased digested lymphocytes as previously described for the IF technique [6, 7]. Firstly, as depicted in Fig. 2, the fluorescence histogram of the negative control – AB sera is equivalent to the fluorescence histogram control where cells were only interacted with the FITC antisera (IgG-specific). Secondly, quantitation of fluorescent-positive cells is simplified, as one can now accurately place a cursor on the fluorescence histogram to denote cells that are fluorescent-negative (Fig. 2). Thirdly, Table 1 clearly demonstrates that with negative controls, the fluorescence histogram using pronased cells has less than 5 % positive cells. These data are equivalent to those obtained with the IF technique. Finally, one can easily differentiate sera containing anti-HLA DR and weakly reactive anti-HLA, AB antibodies. FCXM with pronased leukocytes was performed with cells from peripheral blood of seven different individuals and from lymph nodes and the spleen of three cadaveric donors. Thirty-two different sera with less than 5 % PRA from patients awaiting transplantation were tested with the various pronased cell preparations. In every instance, less than 5 % of an individual's cells were positive with such sera. These negative findings were confirmed by the CDL and IF techniques. Such data would indicate that the single-color FCXM using pronased lymphocytes is highly specific.

Specificity using two-color flow cytometry

Since currently used FCXM employs two-color immunofluorescence, especially with PE-stained T or B cells to circumvent false binding of IgG to CD16-positive non-B, non-T cells, it became important to verify whether false-positive FCXM, as reported elsewhere [8, 12], was indeed due to Fc IgG receptors, which are also present on B and T cells [11, 12]. We, therefore, employed two-color FCXM using nonpronased and pronased cells. Data in Table 2 and Fig. 3, using cells from a single donor, clearly demonstrate that there is marked variability in binding of pooled sera and renal dialysis sera to the donor T cells. Figure 3 exemplifies data on patient K.K.: with nonpronased cells, 16 % of T cells and all of the non-T cells (i.e., B cells and CD16 cells) bound to serum IgG. However, with pronased digested cells, IgG from K.K.'s sera did not bind to T cells, B cells, or the CD16 + cells.

Interestingly, there was a marked, unpredictable variability in the binding of a single serum (whether pooled or not) to different donor cells using nonpronased cells. This was not observed using cells predigested with pronase to remove FcIgG from B and T cells. These data support the concept that two-color FCXM can be made more specific by pronase digestion of cells.

Effect of pronase digestion on membrane HLA antigenicity

HLA class I and II antigens were quantitated using murine monoclonal antibodies and human alloantisera specific for HLA antigens. As can be seen from Fig. 4, pronase digestion did not decrease monomorphic HLA antigen expression on the cell membrane. Spleen cells were used to obtain the data in Fig. 4 and this would explain the high percentage of HLA-DR-positive cells. One could argue that pronase digestion alters HLA polymorphic determinants and, therefore, sensitivity. Figure 5 presents data using human sera containing antibodies to HLA-B7. The data indicate that alloantigenicity is not reduced after pronase digestion and that sensitivity is maintained. The same observations were noted with five different human alloantisera containing specificities for class I antigens (Bw4, A2, A1) and for class II antigens (DR7, DR2). Figure 5A clearly depicts that with nonpronased cells sensitivity (expressed as percentage of positive cells) is falsely reduced, based on where the cursor is placed.

Effect of pronase on IgM antilymphocyte antibodies

With IF and FCXM one can demonstrate IgM from normal sera binding to lymphocytes. This, in part, is related to the mu chain of IgM binding to FcμR on lympho-

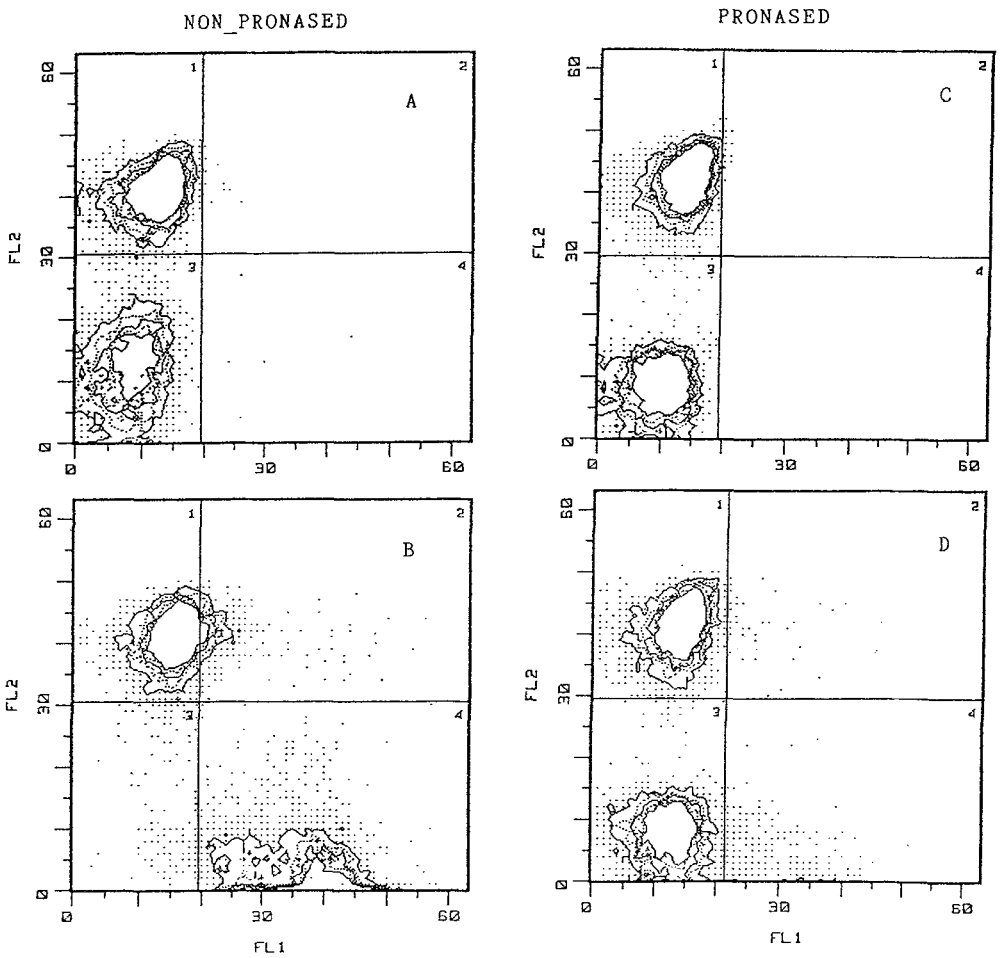

Fig. 3 Two-color FCXM on patient K.K. Serum was interacted with donor cells, washed, and then stained with FITC goat $F(ab')_2$ antihuman IgG (Fc-specific). After three washes, cells were restained with PE-anti T (CD3-Leu 4). FL-2 depicts PE staining while FL-1 depicts FITC staining. *Panels C and D* depict data with pronased cells. *Panels A and C* are controls where PBMC, in the absence of sera, are initially reacted with FITC goat $(Fab')_2$ and human IgG, washed, and then restained with PE-anti-T

cytes [8]. However, the majority of normal sera contain IgM that bind to non-HLA antigens on nonpronased lymphocytes. This can even be observed with IF or FCXM using lymphocytes that have been predigested with trypsin or pronase to remove FcR that bind to the mu chain of the IgM molecule [12]. In general, IgM binding from normal sera could not be observed after sera were diluted 1 : 8. Several normal sera were, therefore, screened to obtain sera that lacked IgM binding to lymphocytes. These sera were used as controls when examining patients' sera for IgM activity. Figure 6 depicts data on sera from a transplant recipient (C.R.). This sera gave rise to a positive crossmatch with the lymphocytotoxic assay. The crossmatch assay was negative using DTT-pretreated sera. As depicted in Fig. 6, IgM lymphocytotoxin could be detected at dilutions of 1 : 256. Pronase digestion did not decrease the sensitivity of FCXM in detecting IgM alloantibody. When using pronased cells, the negative control is distinctly negative, making it easier to use the cursor technique in evaluating the percentage of positive cells. Since peripheral blood lymphocytes contain 10 %–20 % B lymphocytes, with membrane IgM, the negative control has a subset of distinctly positive cells despite pronase digestion (Fig. 6 B).

Effect of pronase on non-HLA membrane determinants

It became important to determine first whether FcR was altered or removed after pronase digestion and, second, whether pronase interferes with CD3 receptors on T cells or membrane receptors specific for B cells, as some investigators have used two-color immunofluorescence to determine whether anti-HLA antibody binds to B or T cells [1]. Since CD16 and CD32 are the predominant FcγR receptors for IgG on lymphocytes (not macrophages), we investigated whether a lack of cytophilic IgG binding to lymphocytes after pronase digestion is due to removal of these receptors or secondary to alteration in receptor epitopes that bind to Fc of IgG. Data for CD16 are depicted in Fig. 6. After pronase digestion, CD16 was reduced from 18 % to 7.5 %, suggesting that the FcR receptor may have been re-

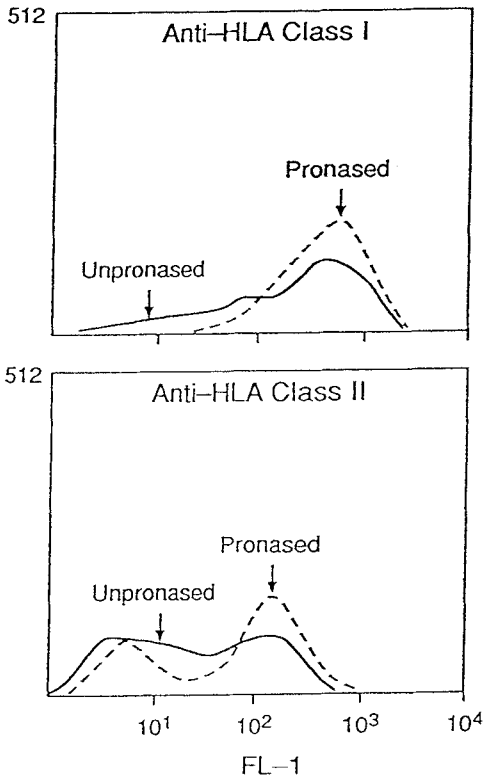

Fig. 4 Effect of pronase on HLA expression. Nonpronased and pronased spleen cells from a cadaveric donor were interacted with murine monoclonal antibodies reactive to HLA-DR (L243) or HLA-A, B (W6/32). Cells were stained with FITC goat $F(ab')_2$ antimurine IgG (Fc-specific) and then analyzed by single-color FCXM

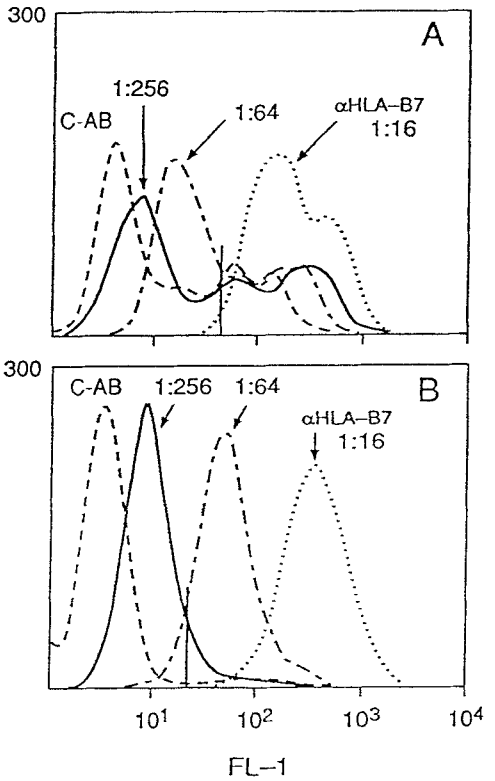

Fig. 5 A, B Effect of pronase on HLA allospecificity. Serial dilutions of human sera containing HLA-B7 alloantigen specificity were reacted with nonpronased and pronased PBMC. With the CDL assay, antibody could not be detected at 1 : 16 dilution: **A** With FCXM using nonpronased cells, the percentage of positive cells was: control AB sera 28.7 %; HLA-B7 sera at 1 : 16, 1 : 64, and 1 : 256 dilutions 99.4 %, 47.5 %, and 39.1 %, respectively; **B** With FCXM using pronased cells, the results were: control AB sera 5.2 %; HLA-B7 sera at 1 : 16, 1 : 64, and 1 : 256 dilutions 99.9 %, 94.6 %, and 14.1 %, respectively

moved. Similar observations were made with the CD32 receptor. In Fig. 7, it is clearly evident that pronase digestion does not alter or reduce detection of CD3 antigen on lymphocytes.

Discussion

Currently employed FCXM is highly sensitive but lack specificity despite the use of two-color immunofluorescence. This, in large part, is due to the presence of FcIgG receptors on B and T cells [11, 12]. Normal or irrelevant IgG can, therefore, bind to such cells, especially when sera contains IgG in an aggregated or complexed form [4, 11]. Bray et al. ultracentrifuged sera (100,000 *g*) to remove IgG complexes. This procedure is very cumbersome, impractical, and does not significantly decrease irrelevant binding of normal IgG to FcIgG receptors on B and T cells (Tables 1, 2).

The current data using pronase digestion and FCXM are identical to those obtained using IF (Table 1). With the IF assay, we have previously demonstrated that it is more sensitive than CDL and, in addition, is highly specific [7]. All of five renal transplant recipients with donor-specific IgG T-cell antibodies detected by IF, but not by CDL, lost their renal allografts either from hyperacute or accelerated vascular rejections [7]. Our institution decided to discontinue the prospective blind trial comparing CDL with IF because of the five successive rejections. No hyperacute or vascular rejections were observed in donor-specific crossmatches that were IF-negative. In the last 3 years, IgG T-cell antibody was detected by IF in 16 (3 %) of the 530 donor-specific crossmatches that were negative by CDL. Only one of these recipients received a transplant with a weakly positive donor-specific IgG antibody detected only by IF. This patient had an accelerated vascular rejection, further confirming that IF was specific. Using pronased lymphocytes and analyzing for donor-specific antibody, we have not detected any differences between IF and FCXM recently. However, with non-pronased cells and

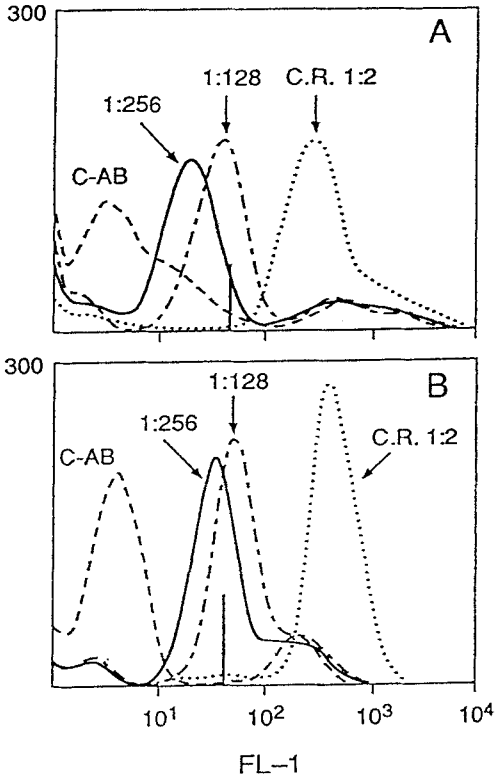

Fig. 6 A, B Effect of pronase on membrane determinant binding to IgM. Serial dilutions of human sera (C.R.) containing IgM lymphocytotoxins by CDL at dilutions of 1 : 32 were subjected to FCXM using nonpronased and pronased peripheral blood lymphocytes: **A** (nonpronased) Percentage positively with control AB and C.R. at 1 : 2, 1 : 128, and 1 : 256 dilutions was 18.9 %, 90 %, 40 %, and 22.6 %, respectively; **B** (pronased) Percentage positively with control AB and C.R. at 1 : 2, 1 : 128, and 1 : 256 dilutions was 15.7 %, 96.4 %, 68.5 %, and 42.8 %, respectively. Note that control AB sera has a positive fluorescent curve beyond the cursor (approximately 15 %–20 % of cells) due to pronase-resistant *IgM* on membrane of B cells

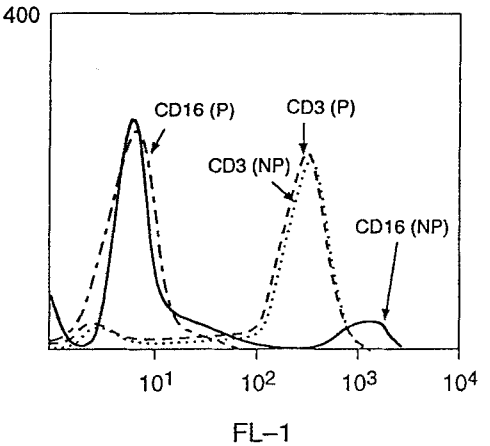

Fig. 7 Effect of pronase on Fcγ RIII and CD3 expression. Nonpronased *(NP)* and pronased *(P)* peripheral blood lymphocytes were interacted with murine monoclonals (B73.1, anti-CD16), washed, and then reacted with FITC goat antimurine IgG1 to detect B73.1 binding. FITC-conjugated anti-CD3 was intracted with *NP* and *P*, washed, and then analyzed. Percentage positive CD16 with *NP* cells and *P* cells was 18 % and 7.5 %, respectively. Percentage positive CD3 with *NP* and *P* cells was 66.8 % and 65.9 %, respectively

two-color FCXM, we have detected donor T-cell-specific IgG antibodies that could not be detected using pronased cells as depicted in Fig. 3. Such transplants have done well. Similar observations have been made by others who demonstrated that two-color FCXM increases the detection of IgG antibodies to donor T cells by 18 % (using nonpronased cells) [9]. One-year graft survival was 74 %, indicating that in the majority of instances, these IgG donor-specific antibodies do not react to HLA antigens [9].

Pronase digestion of lymphocytes is simple and very reproducible. FcIgG receptors are removed (Fig. 7) without altering HLA antigenicity (Figs. 4, 5) or non-HLA antigens that bind to IgM antibodies (Fig. 6). Data presented clearly indicate that pronase digestion preserves the sensitivity of single- and two-color FCXM (Tables 1, 2) but also renders these assays highly specific. In addition, one can now quantitate the donor-specific crossmatch assay by determining the percentage of lymphocytes binding to the antibody.

With the IF assay one routinely determines the percentage of cells with membrane IgM in the absence of sera. Previous studies have shown that pronase digestion does not remove membrane IgM on B lymphocytes [7]. In the presence of serum, if the percentage of IgG-positive cells is significantly more than the percentage of IgM-positive cells (i.e., B cells), one can assume that the serum contains an anti-HLA class I antibody. Difficulty arises when the percentage of IgG-positive cells is similar to or lower than the percentage of IgM-positive cells. Without platelet absorption one cannot exclude the possibility of a weakly reactive HLA class I antibody unless the patient had a previously high PRA level that gradually decreased. However, two-color FCXM can be employed in these difficult situations with pronased cells to verify whether the antibody binds predominantly to B cells or to T cells.

In conclusion, with the current two-color FCXM to evaluate donor-specific crossmatches, one can deny transplants to a significant number of patients [9, 10]. In these previous reports, between 70 % and 90 % percent of patients with a positive FCXM would have been denied kidney transplants if the decision to transplant had been solely based on FCXM. Thus far, in our hands, FCXM, both single and two-color, using pronased lymphocytes, is very similar to the IF technique that we have clinically used for donor-specific crossmatches since 1981. We plan to compare IF and FCXM

using pronased cells on a prospective basis to determine whether FCXM is, indeed, equivalent to IF in predicting specificity and sensitivity.

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