Transpl Int (1997) 10: 69–73

BRIEF REPORT

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Hyperacute renal allograft rejection from anti-HLA class 1 antibody to B cells – antibody detection by two color FCXM was possible only after using pronase-digested donor lymphocytes

Received: 16 April 1996
Received after revision: 25 July 1996
Accepted: 16 September 1996

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Abstract We present a report of a transplant recipient who lost her renal allograft from hyperacute rejection. This was secondary to a weak IgG anti-HLA class I antibody that was only reactive to donor B lymphocytes. This antibody was not detected in her pretransplant serum by the conventional complement-dependent cytotoxicity assays using donor blood lymphocytes. Pretransplant sera were analyzed retrospectively by two-color flow cytometric crossmatching (FCXM). It was difficult to determine if the recipient's serum contained an IgG antibody specific for HLA on donor B cells since IgG from control AB sera and pretransplant sera bound equally well to CD19 B cells. However, when donor lymphocytes were pretreated with pronase to digest the membrane receptor for Fc domain of IgG (FcγR) on non-T-cells, control IgG in AB serum did not bind to B cells and, hence, it was easy to detect binding of IgG (in pretransplant sera) to HLA on B cells. This case underscores the importance of identifying weak anti-HLA class I antibodies reactive only to B cells. Moreover, it shows that the currently used two-color FCXM lacks the specificity to detect such antibodies.

Key words B-cell crossmatch, pronase · Pronase, B-cell crossmatch · Hyperacute rejection, flow cytometry · Flow cytometry, hyperacute rejection

Introduction

In 1994, 22 cases of hyperacute renal transplant rejections were reported to the United Network for Organ Sharing (UNOS), an organization that accumulates data on solid organ transplants in the United States. During the same period, UNOS registered an additional 36 renal transplant recipients who had lost their allografts less than 1 week post-transplant due to accelerated rejection. Other investigators have also reported that a small, but significant, number of renal allografts are lost due to hyperacute rejection or early humoral-mediated graft rejection [3]. Loss of such a scarce resource, especially when it can be prevented, is particularly distressing.

Two-color flow cytometric crossmatching (FCXM) was recently developed as a more sensitive assay for detecting anti-HLA antibodies that are responsible for hyperacute rejection [1, 2]. We present a report of a renal transplant recipient whose donor was her brother. She underwent hyperacute rejection and graft loss resulted. Her pretransplant serum was analyzed retrospectively with two-color FCXM. In this report we clearly demonstrate that two-color FCXM, as currently employed, failed to detect donor-specific antibodies. However, donor-specific HLA class I antibody reactive only to B cells was detected in the pretransplant sera when we used the recently described modified FCXM [6]. In this modified assay, which employs two-color FCXM, donor cells are initially pretreated with the enzyme pronase to remove the membrane receptor for Fc domain of IgG (FcγR) receptors to improve the sensitivity and specificity of FCXM.

Case report

A 37-year-old Caucasian woman had end-stage renal disease (ESRD) secondary to hypertension. In August 1991 she received a cadaveric renal transplant with the following HLA match: donor – A3, A11, B35, B51, DR4, DR13, DQ6, and DQ7; recipient – A2, A25, B18, B51, DR11, DR13, DQ6 and DQ7. The recipient developed irreversible rejection and began dialysis 5 weeks post-transplant. During the 5 weeks post-transplant, she developed two episodes of acute cellular and vascular rejection that were unresponsive to two courses of Solu-Medrol taper and OKT3. The patient did not undergo transplant nephrectomy. Five months post-transplant, she developed anti-HLA class I and II antibodies reactive to frozen donor cells and to a panel of B and T lymphocytes from random donors (T-cell PRA = 47 %, B-cell PRA = 58 %). The B-cell PRA was analyzed after sera were absorbed with platelets. The antibody was specific to HLA A1, A3, A11, B35, DR4 and DR7. The patients PRA reactivity to T cells declined over 10 months, and in September 1995 she received a second kidney transplant. This kidney was donated by her brother. It was a one-haplotype match, and the mismatched antigens were B55 and DR9. Pretransplant crossmatch assays with donor peripheral blood lymphocytes were negative with sera taken 1 day pretransplant and also with sera taken 3, 6, and 12 months pretransplant. Their respective T-cell PRA counts were 96 %, 77 %, and 69 %.

The patient became anuric within 1 h post-transplant; 48 h later she underwent donor nephrectomy and was found to have classical changes of hyperacute rejection in the histology of her allograft. Antidonor IgG antibodies to T and B cells were detected by both cytotoxicity and indirect immunofluorescence (IF) assays from sera 48 h post-transplant.

Methods and results

Pretransplant crossmatch assays

Ficoll-Hypaque-separated mononuclear cells were obtained from donor blood. Contaminating polymorphonuclear cells and macrophages were removed by T-B Kwik (One Lambda, Canoga Park, Calif.). As is routine in our laboratory, two assays were used for the pretransplant testing to detect donor-specific antibodies. First, a complement-dependent microlymphocytotoxicity (CDL) assay that uses peripheral blood lymphocytes (PBLs) was performed according to the modified Amos technique with antiglobulin. The PBLs were found to contain both T and B cells. Secondly, the sera used were pretreated with Dithiothreitol (DTT) to remove IgM antibodies, and IF with PBLs that were pronase-digested was used to remove FcγR on the lymphocytes. The digested cells were combined with sera at 4 °C, washed, and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antisera specific for either IgM or IgG at 4 °C prior to determining the percentage of FITC-positive cells by phase-contrast IF microscopy. Incubation at 4 °C prevents modulation and disappearance of FITC-complexed Ig on the cell membrane. Both techniques are described in detail elsewhere [5, 6].

Using both assay systems, the patient was found not to have donor-specific T-cell IgG antibodies in her sera, either currently or previously. The reactivity of IgG antibody to B cells was determined by IF in our laboratory. IgG antibody to B cells is considered to be present when the percentage of lymphocytes staining for IgG is similar to the percentage of B cells. However, with this patients sera, it was difficult to determine by IF whether there was indeed a B-cell antibody, as PBLs from her donor contained between 3 % and 4 % B lymphocytes; 3 %–4 % of IgG staining was observed in the patient's sera. This value was not significantly different from background staining that uses control AB sera. As a rule, detection of B-cell IgG antibody by IF is not a problem since, in peripheral blood, most donors have 9 %–15 % B cells, and background IgG staining is not more than 3 % [5, 6].

Retrospective analysis of pretransplant sera

Loss of the allograft by hyperacute rejection prompted us to re-examine the patient's pre-transplant sera by two-color FCXM and also by performing a donor-specific CDL assay on enriched B cells. Two-color FCXM was performed with routinely described techniques that use PBLs subjected to T-B Kwik. In addition, we repeated the two-color FCXM with pronase-digested PBLs. Both techniques have been described in detail elsewhere [1,6]. The latter technique improves specificity without altering the sensitivity [6].

To acquire the data shown in Fig. 1, donor PBLs were incubated with human sera for 30 min at 4 °C, washed twice, and then reincubated with FITC goat $f(ab^1)_2$ antihuman IgG at 4 °C for 30 min. After three more washes, the cells were incubated with PE-anti CD19 at 4 °C for 30 min and washed four times prior to two-color FCXM. All sera used were centrifuged at 10000 *g*. Four control AB sera were used.

No antibody binding to T cells was detected in the patient's sera with either non-pronased or pronased PBLs by two-color FCXM. However, as can be observed from Fig. 1, there was no significant difference in IgG binding to CD19 B cells between the control AB sera and the patient's sera when nonpronased PBL were used in the two-color FCXM. In fact, IgG from all four control AB sera that we tested bound to B cells, making it difficult to determine if the patient indeed had a B-cell-specific antibody before transplantation. We have previously shown that normal or irrelevant IgG binds to B cells through their FcγR receptors. In Fig. 2, pronased PBLs were used in the two-color FCXM. The PBLs were stained with pycoerythrein (PE)-conjugated anti-CD3 antibody. Data from Fig. 2 clearly demonstrate that the patient's sera, but not the control AB sera, had an IgG antibody that bound to non-T-cells. Binding to pronased non-T-cells could not be demonstrated after using pretransplant sera absorbed onto platelets. Sera obtained 2 and 10 days post-transplant clearly bound antibody to T cells; this could be detected by a CDL assay, IF, and two-color FCXM (using nonpronased cells). Such findings support the concept that, before transplantation, the patient had a low titer donor-specific HLA class I antibody that bound only to B cells.

In separate studies, donor B cells were positively selected from the PBLs with Dynabeads HLA class II (Dynal, Norway) [11]. The CDL was assayed with recipient pretransplant sera and with donor B cells. The donor-specific B-cell crossmatch was positive for pretransplant sera at a dilution of 1:4 in the Amos-modified CDL assay. These findings confirmed our observations made when we used pronased PBLs with the two-color FCXM.

Discussion

Since one-third of transplant recipients are patients undergoing retransplantation with high levels of T-cell PRAs, it is not uncommon to encounter situations of low titer (or weak) HLA class I antibodies that bind only to B cells. During our retrospective analysis, we had insufficient pre-transplant recipient sera (i.e., sera

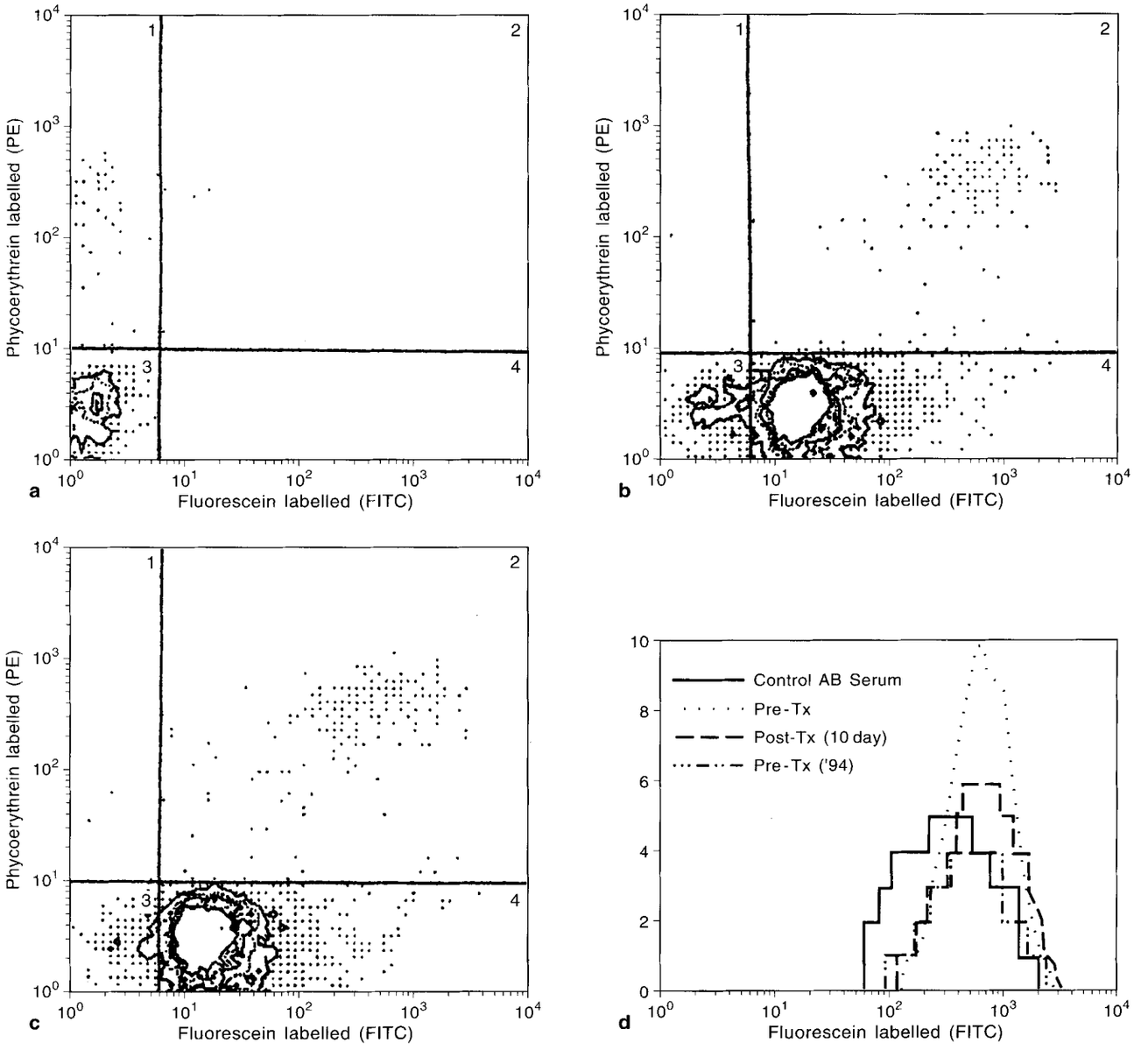

Fig. 1a–d Non-pronased cells examined for binding of FITC labelled IgG to PE-labelled CD19B cells: FITC-Labeled PE-CD 19 B cells: **a** control without AB serum; **b** a representative example of the control AB sera. Since the donor had 3 %–4 % $CD19^+$ B cells, there are very few double-stained cells in the second quadrant of (**b**) and (**c**); (**d**) FITC histograms after analyzing FITC labelled cells in quadrants 1 and 2 of (**b**), (**c**) and other data (not shown). Note that there is strong IgG binding to B cells with both AB sera and the patient's sera (pre- and post-transplant)

on the day prior to transplantation) to do platelet absorption studies to confirm that the B-lymphocyte antibodies were indeed HLA class I. However, the observation that, by day 2 post-transplant, the recipient developed antibodies to donor B and T cells (detected by cytoxicity and IF) provides indirect evidence that the recipient's positive B-cell donor-specific crossmatch was due to HLA class I antibody. In addition, the high level of PRAs to random T cells (69 %) on the day of her transplant made it difficult to determine antibody specificity.

As a rule, two approaches are used for detecting B-cell donor-specific antibodies: (1) a CDL assay using

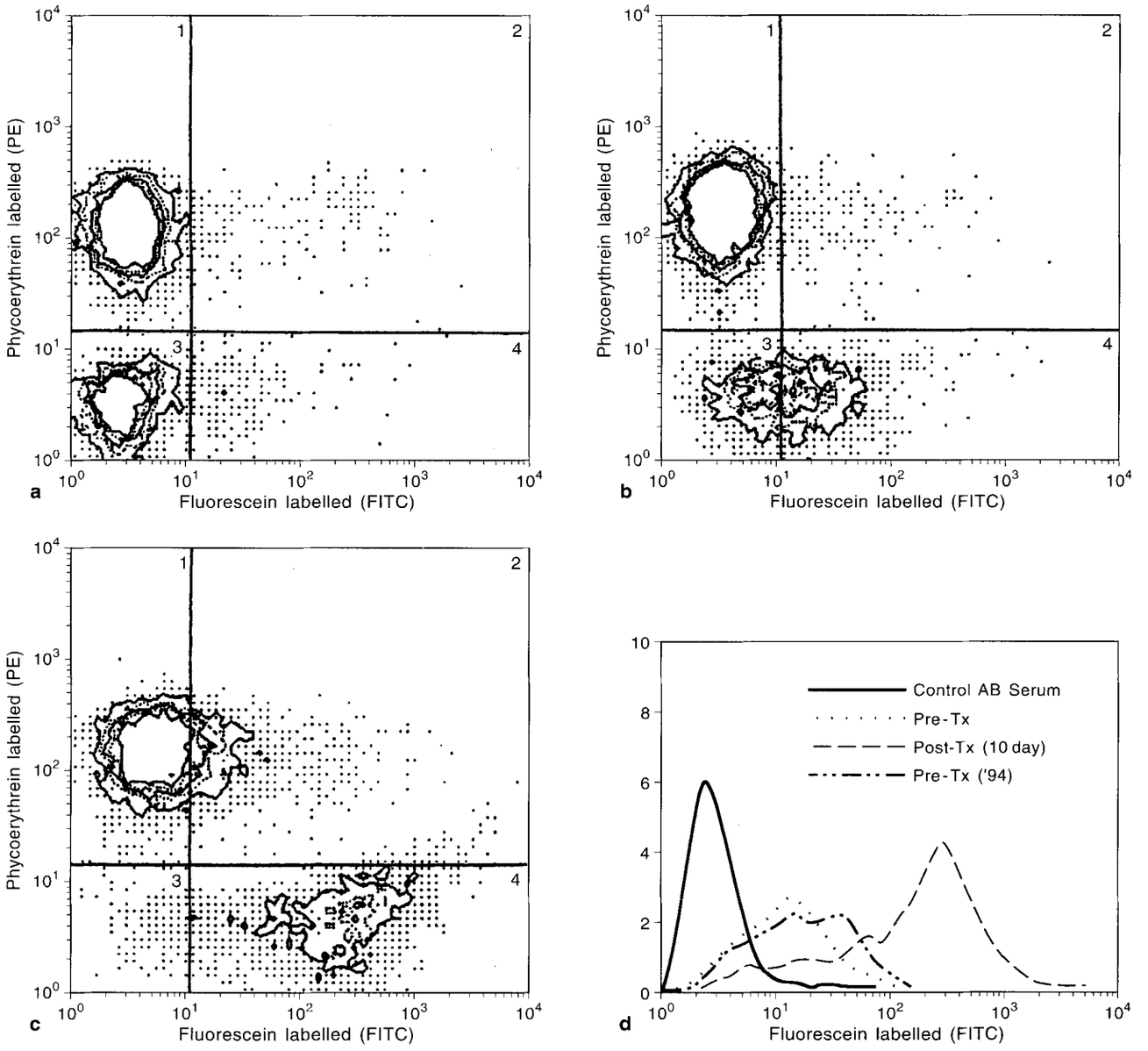

Fig. 2a–d Donor peripheral blood lymphocytes (PBLs) were initially pronased to remove (FcγR) [5] prior to being combined with sera. The sera and conjugates were combined with PBLs, as in Fig. 1. However, in Fig. 2, we examined the binding of FITC-labeled IgG to non-T cells since the PBLs were stained with PE-anti-CD3. Fig. **a** depicts control AB serum, **b**, pretransplant sera and **c** day 10 post transplant sera. The data in quadrants 3 and 4 were analyzed for FITC-IgG binding to non-T cells in the histogram shown in **d**. Note that the control AB serum had very low background IgG binding and, hence, it was easy to identify FITC-IgG binding to non-T cells in the pretransplant sera

highly enriched B cells separated from PBLs and (2) two-color FCXM with PE-conjugated CD19 or IF, as we do in our laboratory. It is necessary to use FCXM (or IF) in addition to the CDL assay, as weak HLA class I antibodies to B cells may not be detectable by the CDL assay, but only by IF or FCXM [5, 6]. Regarding the significance of donor-specific B-cell antibodies (i.e., T-cell-negative), there are data clearly demonstrating that weak HLA class I antibodies and HLA DR antibodies that do not react to T cells are indeed detrimental to graft survival. There is general agreement that transplantation should be avoided in recipients with B-cell-specific antibodies secondary to HLA class I anti-

bodies [4, 5, 8, 9]. Though IF is highly sensitive and specific, less than 5 % of histocompatibility laboratories use this technique, as one of the major concerns is that IF microscopy can be subjective and is dependent on the technologist's skill and experience. Additionally, histocompatibility laboratories prefer FCXM because, with IF microscopy, one examines 300 cells, while with FCXM one examines 5–10 000 cells.

In retrospect, we should have done a B-cell donor-specific CDL crossmatch, especially since we had data that could not be interpreted with IF microscopy. Nonetheless, this case serves to illustrate the lack of sensitivity of the two-color FCXM currently used, especially when identifying IgG antibodies react only to B cells. Two-color FCXM was introduced on the assumption that CD19 B cells and T cells did not have FcγR receptors capable of binding to normal or irrelevant IgG present in control AB serum. Unfortunately, this is not the case [12, 13]. Hence, one cannot differentiate between the binding of normal IgG to FcγR on B cells and the binding of IgG to the HLA antigens on B cells (Fig. 1). Based on the same premise, it can also be difficult to differentiate between binding of weak anti-HLA class I antibodies and normal IgG to FcγR on T cells [5, 6]. Consequently, two-color FCXM lacks specificity. Between 70 % and 90 % of patients with a positive two-color FCXM would have been denied a kidney transplant if the decision to transplant had been solely based on FCXM with nonpronased PBL [7, 10].

To circumvent this problem, we used pronase-digested lymphocytes in the two-color FCXM. Pronase digests FcγR, but does not digest HLA and CD3 antigens [5, 6]. This case clearly illustrates that with two-color FCXM and nonpronased PBLs, one would have failed to detect the anti-HLA class I antibody that only reacted to B cells. The data in Fig. 2 clearly illustrate that pronase-digested PBLs improve the sensitivity and specificity of two-color FCXM, especially with an IgG antibody reactive to B cells. This is particularly relevant in situations when the B-cell-specific anti-HLA antibody is too weak to be detected by CDL assay and yet can cause graft loss within the 1st week [5]. Moreover, with pronased PBLs, one can expedite FCXM. By using PE-conjugated anti-CD3, one can simultaneously identify binding of IgG to pronased T or non-T-cells (Fig. 2). Pronase digestion removes FcγR present on non-T-cells (i.e., B and NK cells). Hence, one does not have to repeat the two-color FCXM with PE-labelled B cells.

Acknowledgements The research was supported by the Flippo Gift Fund 6–42415. The authors wish to thank Ms. Gail Maffett for her excellent secretarial support and Mr. Jing Xin Guan for his expert technical assistance.

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