



Predictive value of the Luminex single antigen panel for detecting flow cytometry cross-match positivity

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ABSTRACT

Anti-human leukocyte antigen (HLA) antibodies are a major cause of allograft loss. Solid-phase immunoassays, notably Luminex technology, have lately begun to replace traditional techniques for detecting these antibodies. This platform, however, carries some restrictions in the type of antibodies it detects. For this reason, results using these new technologies must be correlated with results using traditional techniques that have proven clinical significance. We have correlated flow cytometry cross-match (FCXM) outcomes with results from Luminex assays. Serum samples from patients awaiting transplantation who had known anti-HLA antibodies as detected by Luminex were incubated with lymphocytes expressing (a) 1 of the HLA antigens detected by the sera or (b) several of them. Of the 169 T-cell FCXMs we performed, in 92 cases the target cell expressed only 1 of the HLA antigens detected by the serum. The results obtained correlated well with Luminex data ($r = 0.84$). A cutoff mean fluorescence intensity value of 6,500 for the Luminex single antigen assay yielded a sensitivity of 85% and specificity of 82% for detecting a positive FCXM. In the other 77 cases, the target cell expressed 2 or more of the HLA antigens detected by the serum. In this situation, the same cutoff proved a useful tool for differentiating negative from positive FCXMs.

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1. Introduction

Human leukocyte antigen (HLA) antibodies are a major cause of allograft loss, as research has clearly established [1–6]. These antibodies have been traditionally detected by cytotoxicity techniques and more recently by flow cytometry [7–9]. Currently, solid-phase immunoassay (SPI) using coated beads with HLA antigens offers a higher level of sensitivity in the detection of anti-HLA antibodies [10–13].

The identification of anti-HLA antibodies in the serum of patients awaiting renal transplantation allows strategies to facilitate the allocation of grafts, and the practice of virtual cross-matching is common in many transplantation centers [14–16]. Therefore, efforts are required to reconcile cross-match titer outcomes with the results of the new solid-phase assays to assess antibody strength as accurately as possible.

Some technical issues surrounding the solid-phase Luminex technology (reviewed by Tait et al. [17]) may affect these results, requiring further investigation. These issues include the detection of complement- and non-complement-binding antibodies, the failure to detect immunoglobulin (Ig)-M antibodies, structural

changes in HLA molecules bound to beads, and the role of naturally occurring HLA antibodies [18], among others. Focusing on the use of Luminex technology to detect IgG anti-HLA antibodies and correlating findings with less sensitive cross-match techniques may aid in avoiding some of these issues.

Although a number of approaches have been designed to study this correlation and define the cutoff values of solid-phase techniques, we believe that the most appropriate is to correlate flow cytometry cross-match (FCXM) results with data from the Luminex single antigen assay, in which only 1 antigen is bound to each bead. These types of assays involve techniques that detect a low level of antibodies, far from the detection level of older techniques such as complement-dependent cytotoxicity (CDC), which, on the other hand, indicate clinical significance.

Several groups have tried to define a cutoff for Luminex technology, allowing the prediction of a positive cross-match using either CDC or FCXM [19–22]. However, thus far, the complexity of the problem and the different experimental designs used have not allowed conclusions about either the cutoff or the best SPI to use.

In this work, we designed a simple experimental approach to facilitate the interpretation of this type of data. Sera from patients on a renal transplant waiting list who had known anti-HLA specificities (only class I HLA-A or -B antigens), defined by single antigen SPI, were studied. These sera were incubated with peripheral blood

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mononuclear cells (PBMC) from HLA-typed healthy donors that expressed only 1 of the class I antigens recognized by the serum to be assayed. We studied this reaction using flow cytometric assays. The resulting data were correlated with the SPI results (both phenotype and Luminex single antigen panels). We also compared data from FCXM and Luminex assay results using sera that reacted to more than 1 antigen in target cells. This comparison provided a closer approximation to clinical practice. We correlated data from FCXM with the level of sensitization present in each serum sample as defined by Luminex technology and quantified it using a summative strategy of individual Luminex values. Our results indicate a good correlation of FCXM with Luminex, mainly with the single antigen type of assay. We demonstrate that a cutoff of 6,500 mean fluorescence intensity (MFI) for Luminex single antigen predicts a positive FCXM with good sensitivity and specificity.

2. Subjects and methods

2.1. Experimental design and patients

To correlate the Luminex single antigen data (One Lambda, Canoga Park, CA) with data from FCXM and their value in clinical transplantation, we performed 169 FCXM with sera from patients with anti-HLA antibodies defined using the Luminex single antigen SPI.

First, we selected sera from 52 patients only with anti-HLA class I (anti HLA-A and -B) antibodies and PBMC from 27 healthy donors whose lymphocytes expressed only 1 of the HLA class I antigens recognized by the serum to be assayed. A total of 92 cross-matches were performed.

Then, we performed 77 FCXMs with 52 multispecific anti-HLA serum samples and with PBMC from 38 healthy donors. Each cell expressed several HLA antigens recognized by the serum used in each particular FCXM.

2.2. HLA typing

Healthy donor samples were typed for HLA-A and -B. Briefly, human PBMC were isolated from heparinized venous blood by centrifugation over a Ficoll–Paque gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden). HLA-A and -B were serologically typed using One Lambda monoclonal typing trays (One Lambda).

2.3. Anti-HLA antibody screening and characterization

Serum samples of patients from the renal transplant waiting list were screened for anti-HLA antibodies using the Luminex LABScreen mixed assay (One Lambda). Only those patients who had positive sera for anti-HLA class I antibodies and who were negative for class II were selected (52 patients). Anti-HLA class I antibodies were identified using the LABScreen single antigen class I (One Lambda) according to the manufacturer's instructions. Briefly, 20 μ L of patient serum was added to 5 μ L of antigen beads, incubated in the dark for 30 minutes, and washed 3 times with washing buffer. One hundred microliters of PE-conjugated goat antihuman IgG secondary antibody was added to the beads, incubated for 30 minutes in the dark, washed, and read on the LABScan 100 flow analyzer (One Lambda). Fluorescence intensity values of patient sera were normalized with the appropriate negative control. Normalized MFI values above 1,000 were defined as a positive reaction. Furthermore, the Quantiplex kit (Qplex; One Lambda) was used to standardize the MFI of the Luminex analysis. We considered MFI and Qplex values for each positive serum.

2.4. FCXM

Selected donor peripheral lymphocytes were incubated with sera of patients with anti-HLA antibodies recognizing only 1 HLA antigen of the cell. Altogether, 92 FCXMs were performed, matching 27 cells with 52 serum samples. Furthermore, 77 FCXMs were

performed using 52 serum samples and 38 PBMC samples in which more than 1 HLA antigen was recognized by each serum. Briefly, 2.5×10^5 cells were incubated with 20 μ L of patient and control sera for 20 minutes at 4°C and then centrifuged and washed 3 times with 0.15 M Dulbecco's phosphate-buffered saline (Gibco–BRL, Paisley, Scotland). Fluorescence-labeled antibodies (anti-CD45 PerCP, Becton Dickinson, San Jose, CA; anti-CD3 phycoerythrin, Becton Dickinson; and antihuman IgG F(ab)₂' FITC, Invitrogen, Camarillo, CA) were added. After 20 minutes of incubation in the dark, 2 washing steps with phosphate-buffered saline were performed, and lymphocytes were resuspended in 500 μ L of 1:4 diluted formalin (Sigma Chemical Company, St. Louis, MO). A total of 100,000 events were acquired using a FACSCalibur cytometer (Becton Dickinson). Analysis was performed with Cell Quest software (Becton Dickinson). A positive T-cell FCXM was defined as a median channel shift equal to or above 40.

2.5. Statistical analyses

Correlation coefficients were determined using comparisons of the Luminex single antigen MFI results with FCXM results. For categorical data, the χ^2 test and Fisher's exact test were used. All calculations were performed using SPSS for Windows, version 15.0 (SPSS, Inc., Chicago, IL).

3. Results

First, we performed 92 FCXMs by combining 52 sera from patients waiting for transplantation with cells from 27 healthy donors typed for HLA class I, so that each of the sera samples presented reactivity against only 1 of the HLA antigens expressed by lymphocytes. The specificity of the anti-HLA antibodies from sera had been previously evaluated by LABScreen mixed and LABScreen single antigen as described under Subjects and methods.

The strength of anti-HLA antibodies expressed as Luminex single antigen MFI values indicated a strong correlation with FCXM results ($r = 0.84$, $p < 0.01$; Fig. 1). Using a linear regression model, we calculated the MFI that theoretically predicts a positive T-FCXM (40-channel shift). This point corresponded to an MFI value of about 6,500.

Analyzing these data by intervals of Luminex single antigen MFI values (Table 1), no positive T-FCXM was observed when Luminex MFI was less than 3,000. In the range between 3,000 and 6,500, 7 of 33 samples (21%) were associated with a positive FCXM. In the range between 6,500 and 9,000, 71% of the FCXM samples were

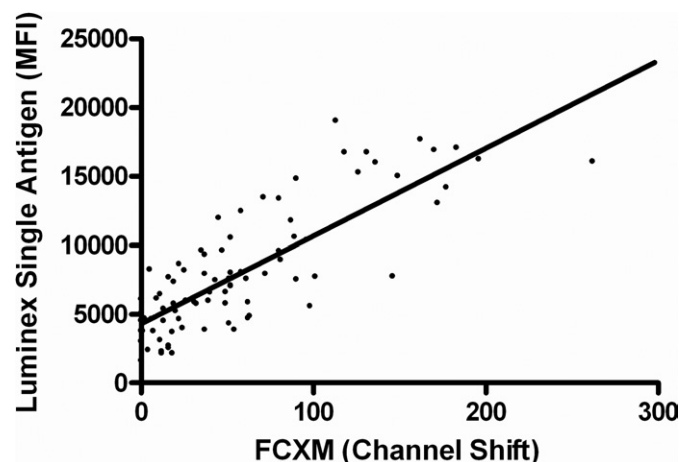


Fig. 1. Correlation between flow cytometry cross-match (FCXM) and Luminex single antigen results. Each dot represents the mean fluorescence intensity (MFI) value of the antibodies against a particular human leukocyte antigen (HLA) class I antigen measured by Luminex single antigen (vertical axis) and the result of FCXM measured in channel shift when this same serum was incubated with lymphocytes from a healthy donor expressing the same antigen (horizontal axis).

Table 1
FCXM results according to Luminex single antigen MFI intervals

Luminex single antigen	FCXM		<i>p</i>
	Negative	Positive	
Interval 1: MFI <3,000	10	0	ns ^a
Interval 2: MFI 3,000–6,500	26	7	<0.001 ^b
Interval 3: MFI 6,500–9,000	6	15	ns ^c
Interval 4: MFI >9,000	2	26	

FCXM, flow cytometry cross-match; MFI, mean fluorescence intensity.

^aWhen intervals 1 and 2 were compared.

^bWhen intervals 2 and 3 were compared.

^cWhen intervals 3 and 4 were compared.

positive (15 of 21 samples). These 2 intervals demonstrated significant differences for predicting a positive T-FCXM ($p < 0.001$). Finally, only 2 of 28 samples with MFI values greater than 9,000 had a negative T-FCXM.

Based on a theoretical cutoff of 6,500 MFI (Qplex MFSE units 145,000), the results were represented in a 2×2 table (Table 2). A total of 77 sera samples (84%) were in concordance by both methods (41 were identified as positive and 36 as negative by both techniques; $r = 0.67$, $p < 0.01$). However, 7 samples (16%) with a Luminex MFI value less than 6,500 were associated with a positive T-FCXM outcome. Eight sera (16%) were negative by T-FCXM but predicted to be positive by Luminex MFI. The cutoff value of 6,500 MFI for the Luminex single antigen had a sensitivity of 85% and a specificity of 82% for predicting a positive T-FCXM.

We performed 77 additional cross-matches to address the issue of anti-HLA sera recognizing more than 1 HLA antigen in target cells. We correlated Luminex single antigen data for these serum samples ($n = 52$) with the results from T-cell FCXM using PBMC from 38 healthy donors. In 25 T-cell FCXMs, 1 of the HLA antigens was targeted by an antibody with Luminex MFI > 6,500. In this situation, 22 of 25 (88%) FCXMs were positive. When none of the anti-HLA antibodies from the sera was Luminex MFI > 6,500 and their total sum was less than Luminex MFI 6,500, the majority of FCXMs were negative. If the sum was greater than Luminex MFI 6,500, the majority of FCXMs were positive (Table 3). In this model, the cutoff value of 6,500 MFI for the Luminex single antigen had a sensitivity of 89% and a specificity of 88% for predicting a positive T-FCXM.

Concerning the Luminex LABScreen mixed panel, we also obtained a significant correlation between results with that assay and those from T-FCXM ($r = 0.43$, $p < 0.01$), although it was not as good as the correlation between the Luminex LABScreen single antigen panel and T-FCXM.

Fig. 2 illustrates the correlation between the LABScreen mixed and LABScreen single antigen panels. We observed a good correlation between both techniques ($r = 0.80$, $p < 0.01$).

4. Discussion

Here we demonstrate that a cutoff of 6,500 MFI for Luminex single antigen predicts a positive FCXM with good sensitivity and

Table 2
FCXM results according to the Luminex single antigen cutoff of 6,500 MFI when the target cell expressed only 1 HLA antigen recognized by the serum

Luminex single antigen ^a	FCXM		<i>r</i>
	Negative	Positive	
MFI <6,500	36	7	0.67
MFI >6,500	8	41	

Sensitivity, 85%; specificity, 82%; positive predictive value, 84%; negative predictive value, 84%; $p < 0.01$.

FCXM, flow cytometry cross-match; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; *r*, correlation coefficient.

^aValue refers to the implicated HLA antigen.

Table 3
FCXM results for sera with multispecific anti-HLA when target cells expressed several antigens recognized by each particular serum.

Luminex single antigen ^a	FCXM		<i>r</i>
	Negative	Positive	
MFI <6,500	30	2	0.75
MFI >6,500	4	16	

Sensitivity, 89%; specificity, 88%; positive predictive value, 80%; negative predictive value, 94%; $p < 0.01$. Each individual Luminex MFI was always under 6,500, and the sum of all was > or <6,500 MFI.

FCXM, flow cytometry cross-match; MFI, mean fluorescence intensity; *r*, correlation coefficient.

^aValues refer to the sum of Luminex MFI individual data.

specificity. These data add to what has been published in the field of the detection of anti-HLA antibodies by Luminex technology and its correlation with other laboratory data with clinical predictive value [19–21].

A clear goal in this field has been the prediction of cross-match results with data from SPI [23,24]. CDC has been for many years the only technique available for detecting antidonor antibodies. More recently, flow cytometry has added sensitivity in this field. Although the clinical relevance of the data offered by this technique is controversial, enough evidence is available to allow consideration of FCXM as an indicator of clinical outcome [25]. Technical differences between different types of SPIs have posed problems for efforts to correlate these assays with cross-match data. In the phenotype panel, each bead contains different HLA antigens from different cells. The principal characteristic of the single antigen panel is that only 1 particular HLA antigen is bound to each bead. Thus, the phenotype panel is a technique for screening for HLA sensitization, and the single antigen technique identifies the specificity of this sensitization. For this reason, the best SPI to correlate with cross-match data should be the single antigen assay. Both techniques have been correlated with the results of cross-match using both CDC and FCXM [26]. Furthermore, a particular transplant receptor can produce antibodies to different HLA antigens, making the correlation of SPI techniques with the results of cross-match more difficult.

In this study, we focused on T-cell FCXM and its correlation with the presence of anti-HLA class I antibodies detected by Luminex. We understand that this approach is in some ways restrictive be-

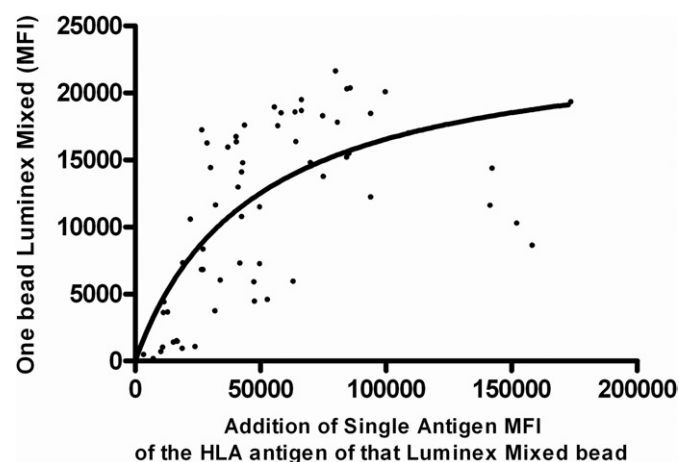


Fig. 2. Correlation between LABScreen mixed and LABScreen single antigen values. We illustrate on the vertical axis the strength of human leukocyte antigen (HLA) antibodies of each particular serum measure using Luminex mixed. The horizontal axis illustrates the sum of single antigen mean fluorescence intensity (MFI) values of the particular HLA antigens that expressed the corresponding Luminex mixed bead for each particular serum.

cause the study did not include HLA-C antigens, B-cell cross-match, and anti-HLA class II antibodies. The compatibility for HLA-C antigens is not a part of renal transplantation protocols, and for that reason, patients are not typed for this locus. Thus, the clinical relevance of anti-HLA-C antibodies is generally unexplored. Because HLA-C specificities have been excluded in this work, our cutoff applies only to sera containing HLA-A and -B antibodies. B-cell cross-match is not an immunological marker with indication in solid organ transplantation. Moreover, the possible presence of anti-HLA class II antibodies makes interpretation of results much more difficult. Additional work is necessary to clarify these aspects, and the resulting information will undoubtedly help in the understanding of the prognostic role of immunological factors and their influence on transplantation outcome.

One goal of the correlation between SPI and FCXM is to determine a cutoff for SPI to define the level above which the cross-match will be positive. Despite the above-mentioned technical inconvenience that likely applies to the Luminex single antigen assay [17], at least in our experimental design, this type of assay in which only 1 HLA antigen is present in the Luminex bead proved to be the most appropriate for correlation with FCXM.

First, we studied the correlation between FCXM data and Luminex single antigen assay of sera with known anti-HLA antibodies and cells from healthy donors expressing only 1 of the HLA antigens recognized by the sera. In this way, we tried to avoid the cumulative effect of different antigen–antibody interactions. This approach has been justified as a valid tool to determine what can be extended to more complicated systems in which more than 1 HLA antigen is recognized by the sera [20].

We determined that values for the Luminex single antigen panel above 6,500 MFI predicted FCXM positivity with 85% sensitivity and 82% specificity. On the other hand, almost 100% concordance between the 2 techniques was identified for MFI values less than 3,000 and above 9,000. Our identified cutoff value for the Luminex single antigen is similar to what has been published for correlation with FCXM [19]. Batal et al. [20] reported the same level for HLA class I antigens but, surprisingly, using CDC cross-match. Because CDC sensitivity for detecting HLA antibodies is much lower than the sensitivity achieved with FCXM, the MFI value of Luminex to correlate with this level of antibodies should be higher.

Afterward, we studied the model of multiple HLA antigens recognized by a single serum in the target cell. In this work, we have demonstrated that Luminex single antigen correlated well with FCXM when we studied sera with several HLA specificities expressed by target cells. In this situation, the Luminex single antigen cutoff previously defined in the model where only 1 antigen is recognized in the target cell was also valid, and this cutoff worked when dealing with sera with several low-level antibodies. In these cases, a summative strategy allowed definition of a positive FCXM when the serum indicated a value higher than the Luminex 6,500 MFI cutoff.

As explained above, we observed a significant correlation of FCXM data with the Luminex single antigen MFI value. As could be expected given the experimental design, we identified a lower correlation value when FCXM data were compared with the results of the phenotype panel (Luminex mixed) assay. However, Zachary et al. [19] demonstrated a better correlation of FCXM with the phenotype panel (Luminex mixed) assay. They argued that Luminex single antigen is not standardized enough to correlate with other techniques that detect anti-HLA antibodies. The summative strategy they used to validate sera with several anti-HLA specificities was, they suggest, not adequate.

Despite that difference, their paper [19] and ours indicated that SPI correlates with FCXM and that it is possible to determine a cutoff for SPI to differentiate the level of HLA sensitization with clinical significance. In the current work, this level of antibodies

(MFI 6,500) had a reasonably good positive predictive value for identifying positive FCXM. Some sera identified as negative by Luminex single antigen were positive on FCXM, presumably because of the presence of autoantibodies and/or the absence of some alleles in Luminex beads. On the other hand, we detected positive sera by Luminex that were FCXM negative, probably because of nonnative HLA epitopes exposed as a result of the binding of HLA antigens to the solid phase [17,19]. Moreover, low levels of anti-HLA antibodies detected by the Luminex assay likely have no pathological significance [27,28] but may be beneficial to the graft [29,30]. This possibility has also been discussed recently [4].

Overall, although SPI techniques must be improved for their use as a measure of HLA sensitization with clinical relevance, our data indicate that Luminex single antigen correlates with data from FCXM and can predict a positive FCXM, thus adding clinical value to laboratory data. This type of assay can serve as a surrogate for cross-match tests when the latter are not feasible or reliable because of inadequate access to donor cells or the presence of interfering factors. Thus, this option can facilitate the allocation of grafts and a better transplant outcome.

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