Naturally occurring interference in Luminex® assays for HLA–specific antibodies: Characteristics and resolution

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ABSTRACT

Substances occurring naturally in the sera of patients can interfere with Luminex® antibody assays, causing increased background and changes in antibody specificity. We present data on the effectiveness of hypotonic dialysis (HD) or dithiothreitol (DTT) treatment in eliminating this interference. HD significantly increased reaction strength of positive control beads and reduced reaction strength of negative control beads. HD also improved specificity identification, determination of donor-specific antibody (DSA) strength, and crossmatch predictability compared with values in untreated serum. DTT also increased the reaction strength of positive control beads, but in most cases, further increased reactivity of negative control beads. DTT improved crossmatch predictability but to a lesser extent than did HD and may differ with specificities defined in other assays. Because interference is frequently observed in sera from highly sensitized patients, it is important to recognize and eliminate interference in Luminex® antibody assays for accurate and meaningful test interpretation.

1. Introduction

Two types of assays, differentiated by target, are used for the detection and identification of human leukocyte antigen (HLA)–specific antibodies: 1) the cytotoxicity and flow-cytometric assays that use lymphocyte targets; and 2) solid phase immunoassays (SPI) that use soluble HLA molecule targets. Many of the problems of cell-based assays, such as adequate access to cells, reduced cell viability, and lack of HLA specificity [1,2], have been resolved by SPI. In addition, SPI have superior sensitivity, require small serum volumes, and can be partially or fully automated [2–5]. The high degree of sensitivity and specificity and the ease with which antibody specificity can be determined permits differentiation of clinically relevant and irrelevant crossmatches, enhancing the clinical interpretation of test results.

However, SPI, like any immunologic assay, are not completely problem free [2,6,7]. We have observed in bead-based assays what appears to be nonspecific binding of some substance that results in high background reactivity and that may interfere with binding of HLA-specific antibodies. Based on the assumption that immunoglobulins of the M class (IgM) may be one substance that results in this effect, we tested the effectiveness of two serum treatments known to reduce or eliminate IgM in resolving this problem. Reducing agents such as dithiothreitol (DTT) are known to have a greater effect on immunoglobulins of the IgM class compared with those of the IgG class [8,9].

Hypotonic dialysis separates serum proteins into water soluble IgG-containing pseudoglobulins and water insoluble IgM-containing euglobulins [10]. We present here data showing the effect of interference on test outcome, the incidence of interference in SPI that is due to substances occurring naturally in sera, and the effects of two different serum treatments on that interference.

2. Subjects and methods

2.1. Sera

There were 42 sera from 31 transplant candidates and recipients which included 7 sera considered control sera because, without treatment, they demonstrated a normal range of reactivity with control beads and no change in specificity in any solid phase assay. The remaining 35 sera gave high negative controls without treatment, they demonstrated a normal range of reactivity with control beads and no change in specificity in any solid phase assay. The remaining 35 sera gave high negative controls values with the phenotype panel and/or specificity changes (SC) on either the phenotype or single antigen panel. All sera were tested untreated and after treatment with DTT and/or hypotonic dialysis. Tests for specificity and strength were performed by multianalyte bead assay on the Luminex® platform for all sera and by enzyme-linked immunoabsorbent assay (ELISA) on the DynaChip® platform for 23 sera. Twenty-three of the 42 sera were tested after IgG isolation by molecular size exclusion using spin columns, and the IgG portion of each was tested on the Luminex® platform. A total of 24 untreated sera were tested in crossmatch tests against donor cells. Six of the 24 underwent HD and were used in parallel crossmatch tests.
2.2. Antibody characterization

Sera were tested using phenotype panels on the Luminex® platform (Lifematch class I, class II, Tepnel LifeCodes, Stamford, CT) and on the DynaChip® platform (DynaChip® Antibody Analysis, Invitrogen Corporation, Carlsbad, CA) and/or single antigen panels (Single Antigen Beads, One Lambda, Canoga Park, CA) on the Luminex® platform, according to the manufacturers’ instructions. Results were expressed as specificity and as mean/median fluorescence intensity (MFI) or optical density values according to the manufacturers’ specifications. Specificity analysis was performed manually by two to three highly experienced individuals working independently. Raw values were used to assess reaction strength to avoid the impact of elevated negative control values of the untreated sera. All data presented in the tables are from tests performed on the Luminex® platform unless otherwise specified.

2.3. Crossmatch testing

Cytotoxicity tests were performed with positively selected T and B lymphocytes using antoglobulin-enhanced and one wash procedures, respectively [1]. Three color flow cytometric crossmatches were performed on a Becton-Dickinson FACSCalibur as described previously [4]. The second antibody used was an IgG heavy chain-specific, mouse monoclonal antibody (BD Biosciences, San Jose, CA). Results were expressed as a ratio of the median channel fluorescence of the test serum to that of the negative control.

2.4. Hypotonic dialysis

Hypotonic dialysis (HD) of serum was performed using Pierce Slide-A-Lyzer Dialysis Cassettes #66383 with a size cutoff of 10,000 Kd (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer’s instructions. Briefly, hydrated cassettes were loaded with 500 µl of the serum of interest and dialyzed against 4 L of deionized water, overnight (14–16 hours) in the cold (2–8°C), with stirring. After overnight dialysis and when a precipitate was visible, the contents of the cassette were removed and centrifuged for 1 hour at 15–20 K at 2–8°C to collect the precipitated fraction. The precipitated euglobulin fraction was resolubilized in isotonic, buffered saline. The retrieved soluble pseudoglobulin fraction was removed and placed into a new dialysis cassette and back dialyzed against two changes of 4 L of phosphate-buffered saline for 4–6 hours at room temperature to restore isotonicity. At the end of the second dialysis, the contents were removed from the cassette and reserved for antibody testing. The back dialysis is necessary for cell-based assays but not for solid phase assays.

2.5. DTT treatment

DTT inactivation of IgM was performed by adding 10 µl of a 0.05-mol/l solution of dithiothreitol to 90 µl of serum and, after mixing, incubated at 37°C for 30 minutes. Treated sera were centrifuged for 10 minutes at 1400 g before solid phase testing. The centrifugation step is necessary to prevent clogging in the Luminex® Fluorometer.

2.6. Spin column immunoglobulin fractionation

Separation of IgG and IgM immunoglobulins was performed by molecular size exclusion using the 300K Nanosep® Centrifugal Device (Pall Corporation, East Hills, NY). Briefly, 50 µl of serum was loaded into the sample reservoir, covered, and spun at 14,000 g in a fixed angle centrifuge for 12 minutes. The IgG portion of the serum was recovered from the lower chamber of the device.

3. Results

All results of antibody screening tests were performed on the Luminex® platform unless otherwise specified.

3.1. Effect of DTT treatment and HD on reactivity with control beads

3.1.1. Phenotype panels

Both the phenotype and single antigen panels have a positive control bead. The phenotype panel has three negative control beads and the single antigen panel has one. We evaluated the lowest and highest scoring of the negative control beads (Table 1). Comparison of the results obtained with these control beads for untreated and treated sera tested with phenotype panels are given in Table 1. The results show that for all sera tested, scores with positive control beads had higher values with both HD- and DTT-treated sera than with the untreated sera. The differences for all comparisons were highly significant. For the two negative control beads, values were lower with HD-treated sera than with untreated sera, but were higher with DTT-treated sera than with either untreated or HD-treated sera. However, none of these differences were significant.

<table>
<thead>
<tr>
<th>Control bead group</th>
<th>Untreated n = 40</th>
<th>HD n = 40</th>
<th>DTT n = 9</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD vs. DTT</td>
<td>UN1 vs. HD</td>
<td>UN1 vs. DTT</td>
<td>HD vs. DTT</td>
<td></td>
</tr>
<tr>
<td>PC, all</td>
<td>14695.9 ± 2259</td>
<td>18614.4 ± 1350</td>
<td>17213.9 ± 490</td>
<td>3 × 10^-13</td>
</tr>
<tr>
<td>PC-HI, all</td>
<td>184.2 ± 152</td>
<td>119.2 ± 91</td>
<td>277.2 ± 282</td>
<td>9 × 10^-8</td>
</tr>
<tr>
<td>PC-LC, all</td>
<td>502.9 ± 456</td>
<td>336.5 ± 320</td>
<td>789.9 ± 762</td>
<td>1 × 10^-5</td>
</tr>
<tr>
<td>PC-H1 vs. H1</td>
<td>2.2 × 10^-6</td>
<td>1.2 × 10^-6</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Control sera</td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 1</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>14649 ± 2259</td>
<td>18021 ± 698</td>
<td>ND</td>
<td>0.02</td>
</tr>
<tr>
<td>PC-LC, all</td>
<td>898.8 ± 22</td>
<td>55.1 ± 10</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>PC-HI, all</td>
<td>166.4 ± 50</td>
<td>160 ± 95</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>PC-SU, all</td>
<td>152774.7 ± 2086</td>
<td>18315.9 ± 1376</td>
<td>16910.8 ± 323</td>
<td>9.3 × 10^-6</td>
</tr>
<tr>
<td>PC-SC, all</td>
<td>137531.4 ± 2015</td>
<td>18897.4 ± 1408</td>
<td>17329.3 ± 454</td>
<td>6.6 × 10^-6</td>
</tr>
<tr>
<td>SC-P vs. SC-S</td>
<td>0.08</td>
<td>0.02</td>
<td>0.0003</td>
<td>0.008</td>
</tr>
<tr>
<td>SC-N-CO, SU</td>
<td>140.5 ± 75</td>
<td>90.8 ± 42</td>
<td>135.5 ± 26</td>
<td>0.018</td>
</tr>
<tr>
<td>SC-N-CO, SC</td>
<td>318.9 ± 173</td>
<td>201.4 ± 105</td>
<td>483.0 ± 327</td>
<td>0.027</td>
</tr>
<tr>
<td>SC-P vs. SC-S</td>
<td>0.05</td>
<td>0.007</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SC-N-CO, SU</td>
<td>390.2 ± 315</td>
<td>196.2 ± 112</td>
<td>488.2 ± 324</td>
<td>0.016</td>
</tr>
<tr>
<td>SC-N-CO, SC</td>
<td>904.8 ± 485</td>
<td>661.6 ± 580</td>
<td>1291.3 ± 903.9</td>
<td>0.027</td>
</tr>
<tr>
<td>SC-P vs. SC-S</td>
<td>0.007</td>
<td>0.0015</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

HD, hypotonic dialysis; DTT, dithiothreitol; PC, positive control; PC-LC, low negative control; SC, specificity changed; SU, specificity unchanged. Comparisons of controls for specificity unchanged group excluded the control sera. SU groups for untreated and HD groups: n = 13; SU for DTT group: n = 4; SC groups for untreated and HD sera: n = 20; SC for DTT group: n = 4.
specificity, and patterns of reactivity were comparable between treated and untreated sera. Surprisingly, with the control sera, treatment increased the strength of the positive control bead reactions and decreased that of the negative control beads. These differences were significant for both the positive and low negative control beads. Overall, the effect of HD or DTT on control beads, for all 42 sera, was not uniform. However HD tended to lower the strength of negative control beads and increase the strength of positive control beads with higher incidence than did DTT as follows: reduced strength of low negative control HD = 34/40 (85.0%), DTT = 3/9 (33.3%); reduced strength of high negative control HD = 32/40 (80.0%), DTT = 4/9 (44.4%); increased strength of positive control HD = 37/40 (92.5%), DTT = 8/9 (88.9%). The differences were not significant for any comparison.

Many sera had changes in specificity following treatment, therefore we compared the effect of HD and DTT on control bead reactions for sera with specificity unchanged (SU), excluding the control sera, with those for sera with specificity changes (SC) (Table 1). The positive control bead had higher scores with the SU group than with the SC for untreated sera (difference trended toward but was not significant), whereas there was no difference between SU and SC groups for HD and DTT treated sera. The scores for both negative controls were higher in the SC group compared to the SU group and the differences were highly significant for both untreated and HD treated sera. Differences for the DTT-treated sera were substantial but did not reach significance, most likely because of the small numbers.

3.1.2. Single antigen panels

Because we had observed significant differences in the strength of reactivities with the control beads of phenotype panels, we divided the single antigen control beads into two groups: those with no change in antibody specificity (excluding the control sera described above), and those with changes in specificity (Table 2). There were large differences in the reaction strengths of the positive control between untreated sera and both HD and DTT treated sera. The differences were very highly significant between untreated and treated sera, regardless of whether the specificity changed. For the negative control sera with no change in specificity, both HD and DTT significantly reduced reaction strength. Among sera with specificity changes, HD also significantly reduced reaction strength of the negative control; however, DTT treatment resulted in a significant increase in strength. Comparing controls between groups defined by specificity change, there was only one significant difference which was for the negative control of the DTT treated group ($p = 0.007$).

3.2. Effect of treatment on specificity

Changes in the specificity of the strongest antibodies were evaluated for both HD and DTT treated sera tested on phenotype panels. Specificity changes occurred in 61% of sera and were reflected in 61% of HD-treated and 50% of DTT-treated sera. A few of the specificity changes were minor such as the addition of DR11 to a serum reacting with DR12. However there were many drastic changes in specificity such as B15C to B7C; 19C to 7C; 19C to A2C; DR4, 7, 8 to DR1, 8, 52; and DR53 to DR52, among others, where the letter C represents crossreactive group. For single antigen panels, the specificities of the first five beads, ranked by strength, were compared and any specificity difference was discounted if the difference was accounted for in the next five beads. The incidence of specificity changes for single antigen panels was similar to those for phenotype panels and were 47%, 52%, and 48% for the untreated, HD, and DTT groups, respectively. As with phenotype panels, some of the differences were limited but in other cases, there were multiple differences (Table 3). Considering both phenotype and single antigen panels, 35% of sera exhibited specificity changes in both class I–specific and class II–specific antibodies, and 65% had changes in only one specificity class, with an equal distribution between class I and class II.

There were cases of sera with changes in specificity, in which the specificities differed between HD and DTT treated sera. In an attempt to resolve which treatment yielded the correct specificity, we made two types of comparisons. In the first comparison, we ran 23 of the sera in this study on the DynaChip® assay, a multianalyte assay with class I and class II phenotypes on glass microchips. The controls of all the DynaChip® assays were within normal ranges, with the means of the positive and negative controls being 0.795 ± 0.09 and −0.0087 ± 0.005 optical density units, respectively. Comparing specificities of untreated and HD sera, the specificity on the DynaChip® assay agreed with that of the HD sera 10 of 10 times and for differences between HD and DTT sera, DynaChip® agreed with HD three of four times. In the second comparison, we examined sera tested on both phenotype and single antigen panels for consistency of specificities between the two panels. The specificities determined by the two assays were in agreement for six of six HD sera and for none of three DTT sera. One of the three DTT-treated sera differed with specificities defined by both the untreated and the HD-treated sera.

Tests of sequential sera on a patient further substantiated the validity of HD treatment. This patient had antibodies for several years before transplantation that included specificity for donor mismatches, B7, DR1, and DR52. As predicted from the MFI values, the flow-cytometric crossmatch immediately before transplantation was positive with moderately low strength. In the week after transplantation, we observed increasing strength of the negative control sera with no change in specificity. The specificity changes were major such as B15C to B7C; 19C to 7C; 19C to A2C; DR4, 7, 8 to DR1, 8, 52; and DR53 to DR52, among others, where the letter C represents crossreactive group. For single antigen panels, the specificities of the first five beads, ranked by strength, were compared and any specificity difference was discounted if the difference was accounted for in the next five beads. The incidence of specificity changes for single antigen panels was similar to those for phenotype panels and were 47%, 52%, and 48% for the untreated, HD, and DTT groups, respectively. As with phenotype panels, some of the differences were limited but in other cases, there were multiple differences (Table 3). Considering both phenotype and single antigen panels, 35% of sera exhibited specificity changes in both class I–specific and class II–specific antibodies, and 65% had changes in only one specificity class, with an equal distribution between class I and class II.

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### Table 2

<table>
<thead>
<tr>
<th>Control bead</th>
<th>Effect of treatment on specificity</th>
<th>Untreated</th>
<th>HD</th>
<th>DTT</th>
<th>$p$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$n = 34$</td>
<td>$n = 27$</td>
<td>$n = 21$</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>No change</td>
<td>8600 ± 3077</td>
<td>13083 ± 3527</td>
<td>14327 ± 3163</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>Changed</td>
<td>9092 ± 2582</td>
<td>12349 ± 2303</td>
<td>14010 ± 2242</td>
<td>0.002</td>
</tr>
<tr>
<td>Negative</td>
<td>No change</td>
<td>251 ± 247</td>
<td>92.8 ± 43</td>
<td>119.7 ± 144.4</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Changed</td>
<td>333 ± 222</td>
<td>91.5 ± 44</td>
<td>382 ± 9.23</td>
<td>0.003</td>
</tr>
</tbody>
</table>

All comparisons of no change to changed specificity groups were not significantly significant except negative control of DDT-treated sera where $p = 0.007$.

Number of sera with specificity unchanged: untreated, $n = 18$; HD, $n = 13$; DTT, $n = 11$; number of sera with changed specificity: untreated, $n = 16$; HD, $n = 14$; DTT, $n = 10$. 

HD, hypotonic dialysis; DTT, dithiothreitol.
We examined whether reactivity strength in the negative controls of untreated sera could predict if serum treatment results in a change in specificity. We found that six of 25 (24%) sera with negative control values <275 MFI on the phenotype panel had changes in specificity, whereas six of seven (86%) sera with control values ≥275 MFI had a change in specificity (p = 0.005). For the single antigen panel, specificity changes occurred in four of 16 (25%) sera with a negative control value <150 MFI and in 12 of 18 (67%) sera with a negative control value ≥150 MFI (p = 0.016) (Fig. 1). We also compared the distribution and mean PRA values of sera with and without changes in specificity. The mean PRA values for sera with and without specificity changes were 96.2% and 46.5%, respectively (p = 0.002). The distribution of PRAs among these two groups is shown in Fig. 2.

3.3. Effect on correlation between solid phase and crossmatch results

We have established correlations between MFI values and crossmatch strength based on numerous comparisons and can predict crossmatch outcome with 93% accuracy (11). Our investigation was undertaken, in part, because of cases in which the strength of reactions in the antibody identification tests could not be reconciled with those of the crossmatch tests. Two sets of crossmatch data were used to evaluate the effect of HD and DTT on the correlation with crossmatch outcome.

### Table 3

<table>
<thead>
<tr>
<th>Serum</th>
<th>HD</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6802, B2708, B6601, A3402, A6801, 13156, 11751, 11682, 11117, 10261</td>
<td>0.0219, 19948, 18482</td>
<td>0.0219, 19948, 18482</td>
</tr>
<tr>
<td>DOQ0501, DQQ0304, DOQ303, DOQ302, DR1001</td>
<td>13471, 12506, 10917, 20903, 4915</td>
<td>13301, 15750, 15988, 15582</td>
</tr>
<tr>
<td>BD401, B3901, B1402, A3001, Cw1402 12278, 12253, 12195, 12109, 11835</td>
<td>0.0219, 19948, 18482</td>
<td>0.0219, 19948, 18482</td>
</tr>
</tbody>
</table>

Numbers shown below HLA alleles are MFI values. For serum number 1, the A2 alleles were among the 10 lowest reacting beads for the untreated serum.

Thirty flow cytometric and/or cytotoxicity crossmatches were performed with 24 untreated sera from this study and with six of the 24 after HD treatment. The results of solid phase assays were available for 24 and 8 HD and DTT treated sera, respectively. Crossmatch outcomes predicted from the strengths of donor-specific antibodies (DSA) of untreated and treated sera were compared to the actual crossmatch outcome and the results are provided in Table 5. The differences in the predicted vs. actual crossmatch outcomes were statistically significant (p = 0.017). The agreement between predicted and actual outcomes was 58.3% for untreated sera, 87.5% for HD sera, and 70% for DTT sera. Of the six crossmatches performed with HD sera, the outcomes of four were unchanged and for the other two, one CDC and one flow cytometric crossmatch were converted from positive to negative. Substituting these crossmatch results for those performed with untreated sera changes the agreement between predicted and actual outcomes to 37.5%, 95.8%, and 60% for untreated, HD, and DTT sera, respectively. The 24 HD and eight DTT treated sera were also tested by SPI and evaluated for changes in DSA strength. Increased, decreased and unchanged DSA strength occurred with 66.7%, 25%, and 8.3% of HD sera, respectively, and with 50%, 12.5%, and 37.5% of DTT sera, respectively.

It is our practice to include, as a reference, the original serum tested in all subsequent crossmatches between a recipient and the same donor. We examined the initial and subsequent outcomes of 86 repeated flow cytometric crossmatches, 52 of which tested positive in an initial crossmatch. Twenty-six of the 52 converted to negative in the repeat test, possibly because of deterioration of IgM during storage. There was increased background and/or decreased positive control reactivity in solid phase assays of 24 of the 26.

![Fig. 1. Relationship of negative control MFI to change in specificity following hypotonic dialysis treatment. Untreated sera were tested prior to IgM depletion by hypotonic dialysis to determine if negative control values were predictive of whether there would be a change in antigen specificity after IgM removal. Sera are grouped as either “no change in specificity” or as “specificity change.” The solid lines indicate median values for each group and the dashed lines are cutoff values. For the phenotype panel, 87% (6/7) of sera with MFI values >275 had a specificity change, whereas only 24% (6/25) of sera with MFI <275 had a change. With the single antigen panels, 25% (4/16) sera with MFI <150 had a change in specificity compared to 67% (12/18) with MFI >150.](image-url)
untreated sera used in the crossmatch tests. We HD treated seven of
the sera used in the original crossmatch and rested the treated sera
in SPI and all results agreed with the outcomes of the repeat cross-
match tests.

3.4. Additional tests of HD treated sera

One serum had both the water soluble and insoluble fractions
tested on phenotype panels. No pattern of specificity could be
found in tests of the euglobulin fraction but specificity, identical to
historic sera that had demonstrated normal control bead reaction,
was seen with the pseudoglobulin (IgG) fraction.

We also tested a serum untreated, after HD treatment, and after
the two dialyzed portions were recombined. The serum had an
extremely high mean negative control value (2570 MFI) before
treatment which was lowered to 566 MFI after HD treatment. The
negative control of the recombined specimen showed a back-
ground (1439 MFI) intermediate between the untreated and HD
serum. Clear specificities could not be determined for any of
the three sera because of the broad reactivity. However, when the
antigen-bearing beads were ranked in descending order by
strength, none of the top five beads in the untreated serum were
within even the top ten positions with the HD specimen, while four
of the five top beads with the recombined serum were among the
top five beads of the untreated serum, indicating that the order
of beads in the untreated specimen had been restored, in part, when
the insoluble fraction and soluble fractions were recombined. The
same experiment with a different serum only partially restored the
reactivity pattern and negative control values of the untreated
serum, most likely due to deterioration of the IgM.

3.5. IgM depletion with spin columns

In an attempt to shorten the procedure time, we evaluated spin
columns that provide separation of IgG and IgM fractions by mo-
lecular size exclusion. The IgM-depleted fractions of HD and spin
column treated sera obtained from separate aliquots of 23 sera
were tested in SPI using phenotype panels. The mean values of the
negative controls were not significantly different. However, the
positive control values were significantly different between the HD
treated (18666.4) and spin column treated (17048.2) sera (p =
0.001). In all cases, the HLA-specific reactivity of the IgM-depleted
fraction obtained by dialysis was stronger than that of the compa-
able fraction obtained by spin column separation. Also, there were
differences, between the two treatments, in the specificities iden-
tified in single antigen panel tests.

3.6. Frequency of sera requiring treatment

We assessed the frequency with which sera appeared to require
treatment based on high negative control values for either panel and/
or low positive control values for the single phenotype panel. Single samples from 1496 unique, consecutive patients tested over an
8 month period were evaluated. Of these sera, 496 (27%) were
positive on a solid phase assay. A total of 130 (26%) required treat-
ment to accurately assess strength and/or specificity.

4. Discussion

The development of solid phase immunoassays has been a boon
to histocompatibility testing providing greatly improved sensitiv-
ity and specificity [2–5]. In turn, this has resulted in improved
monitoring of the strength of donor-specific antibodies, even
among broadly reactive sera and of sera containing lymphocyte
reactive, non-HLA specific antibodies, and of crossmatch predict-
ability, the virtual crossmatch [2,12–19]. These features have great
clinical relevance [12], as accurate measurement of DSA strength
provides an evaluation of the effectiveness of desensitization pro-
tocols [19], allows pre-emptive and rapid treatment of patients
experiencing antibody-mediated rejection [19,20], improves the
immunologic evaluation needed to predict compatibility with de-
ceased donors [15–18], and broadens the geographic area from
which organs with short permissible ischemia times can be recov-
ered [21]. However, achieving this requires a thorough and accu-
rate assessment of the test results [2,14]. Naturally occurring inter-
ference, i.e., interference not caused by the presence of therapeutic
agents, can greatly affect the solid phase test results leading to an
incorrect and accurate interpretation. We have presented here the
impact of such interference and the effect of two serum treatments,
hypotonic dialysis (HD) and dithiothreitol (DTT) on resolving this
interference.

The impact of naturally occurring interference is an increase in
the strength of negative controls of both phenotype panel and
single antigen panel assays or a decrease in the strength of the
positive control of single antigen panel assays that exceed values
that permit accurate interpretation of antibody strength. Sera with
these characteristics can also have changes in antibody specificity.
Both HD and DTT increased the strength of positive controls, the
extent of increase was always significant for HD but not for DTT. For
all comparisons with untreated sera, HD significantly reduced the
strength of the negative control, however DTT increased the nega-
tive control in three of four comparisons and the increase was
highly significant in one of those comparisons. The incidence with
which the strength of the negative control was reduced was appreci-
cably different between the two treatments with a greater effect of
HD on both the mean strength values and incidence of reduction. It
was interesting that the seven control sera which had appropriate
reaction strength with the control beads, also had increases in the
strength of positive controls and decreases in the strength of neg-
ative controls. We interpreted this to mean that, if not most,
sera contain some level of interfering factors.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Predicted vs. actual crossmatch outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Same</td>
</tr>
<tr>
<td>Untreated</td>
<td>10</td>
</tr>
<tr>
<td>HD</td>
<td>21</td>
</tr>
<tr>
<td>DTT</td>
<td>7</td>
</tr>
<tr>
<td>HD</td>
<td>23</td>
</tr>
<tr>
<td>DTT</td>
<td>6*</td>
</tr>
</tbody>
</table>

HD, hypotonic dialysis; DTT, dithiothreitol.

*Results obtained when six crossmatches performed with HD treated sera were
substituted for those obtained with untreated sera.
Serum treatment resulted in specificity changes in 50% of cases, considering both panel types used. However, the changed specificity was not always in agreement between the two treatment types. When specificities were assessed by ELISA performed on a glass microchip matrix, there was greater agreement with HD than with DTT. There are threshold negative control values of untreated sera that provide increased probability of a specificity change. However, the data show that a change in specificity can occur with any inflated negative control value. We found a significant association between PRA value and changes in specificity. This is not likely to reflect an inability to identify antibody specificities accurately since the specificity changes were confirmed by single antigen panel tests. Importantly, the strength of DSA changes in many cases, which affects the predictability of crossmatch outcomes. For crossmatch predictions, there were significant differences in comparisons of predicted versus actual crossmatch outcomes among untreated, HD treated, and DTT treated sera. Again, HD permitted greater accuracy than did DTT. Notably, we found that approximately one of every four sera containing HLA-specific antibodies required an initial test of the first serum tested be performed untreated to confirm the presence of interference. Subsequent sera from patients whose sera demonstrate high background in Luminex® can be presumed to also have high background unless the patient has undergone plasmapheresis which removes IgM effectively.

We have not attempted to identify the factor(s) interfering with test outcomes. The most likely candidates are high IgM levels, immune complexes, and nonspecific binding to beads. The contribution of IgM is suggested by the series of flow crossmatch outcomes that converted from positive to negative since the stored sera used in the repeat crossmatches are likely to have undergone some deterioration of IgM. That IgM is a component of the interference is further supported by the reconstitution experiments in which resolubilized IgM precipitant partially restored the blocking activity to IgM depleted sera. Immune complexes might explain the differences between the effect of HD compared with that of DTT, as some of these complexes might be removed by HD but would remain following DTT treatment. The lack of impact on the ELISA performed on a glass matrix might suggest nonspecific binding to the Luminex® beads, although, in cases not presented here, we have seen that increased background can occur in the DynaChip® assay. Interestingly, we have observed cases in which the interference occurs on either the phenotype panel or the single antigen panel assay but not both, and cases in which interference occurs with class I–specific tests or class II–specific assays but not both. Possible explanations of the former are differences in the amount of antigen saturation of the beads of the two assays. A possible explanation of the latter is the presence of IgM antibodies specific for only one class of HLA antigen. However, this explanation does not account for the reactivity with negative control beads. Clearly, nonspecific binding to beads of some interfering substance would account for both increased negative control values and specificity changes but not, necessarily decreased positive control strength. We note that the data suggest that the IgG–specific conjugates, used both in Luminex® assays and flow cytometric crossmatches, also appeared to bind to IgM.

The question of which treatment to use remains. DTT takes a much shorter amount of performance time than does HD allowing same-day crossmatch testing. However, HD affects a greater improvement in control results, specificity identification, and crossmatch prediction and may offer an improved evaluation of antibody strength. Similarly, spin column separation of immunoglobulins is rapid and does not require the use of toxic substances. However, the strengths of both control and donor specific antibodies were significantly less than for HD treated sera. Thus, correlations with crossmatch outcomes would have to be re-established with the spin column extracts. In our opinion, the outcome achieved by HD is preferable to that of either DTT or spin columns. A compromise might be to use HD when time permits and DTT or spin columns when time does not.

In conclusion, we have shown that interference can occur that affects the outcome of solid phase antibody assays which can lead to an incorrect interpretation of the test results. There was a surprisingly high frequency with which this was observed in our population –one in four sensitized patients. We demonstrated that at least three methods eliminate or reduce this interference. However and importantly, deriving clinical benefit from SRI requires a thorough understanding of the assays, precise interpretation of test results, and recognition of when interference has occurred.

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