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Point-Counterpoint

Abolishing serum interference in detection of HLA antibodies: Who, How, When and Why?

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1. Introduction

This year marks the fiftieth anniversary of the landmark publication in the *New England Journal of Medicine* by Patel and Terasaki describing the association of the cytotoxic crossmatch (CDC-XM) with renal transplant outcomes [1]. In the following decades, development of flow cytometric crossmatching and multiplex solid phase antibody testing enabled even more sensitive and descriptive characterization of patient HLA antibody profiles, and has facilitated the modern era of virtual crossmatching. Solid phase tests for HLA antibodies are not quantitative, yet many laboratories rely on their mean fluorescence intensity (MFI) readout to estimate quantity or "strength" of HLA antibodies. The strength of the physical crossmatch generally correlates with the MFI readout [2], with thresholds specific to each laboratory.

Serum interference hindering detection of high titer HLA antibodies in solid phase assays was described by Zachary et al [3]. Since then, numerous laboratories have reported similar findings in which pre-treatment of serum with calcium chelators, heat or reducing agents, or dilution of serum, results in increased MFI of a subset of HLA antibodies. Moreover, serum interference accounted for more than half of failed virtual crossmatches at one center, causing discordant virtual and physical crossmatching [4]. This has prompted a community-wide concern about under-detection of high abundance HLA antibodies [5,6] which represents a challenge to daily laboratory virtual crossmatching, meta-analyses of literature describing the correlation of solid phase detected donor specific antibodies (DSA) with outcomes, and design of clinical trials in transplantation.

Results of the surveys reflecting variable practice across laboratories

To understand current laboratory practices in mitigation of serum interference, in 2018 the Section Editors sent out a survey to all ASHI-accredited HLA laboratories.

Of 68 respondents, 9 (13.2%) did not use sample pre-treatment, although two of these 9 were (as of June 2018) evaluating new methods. While cost was not a factor, one of the reasons cited for not pre-treating sera was insufficient or conflicting literature to support a need for it. Other concerns were loss of low titer antibodies, extra time involved in implementing some methods, and that the inhibition patterns might in fact be informative.

Among ASHI-accredited, North American laboratories who do pre-treat samples, the type of treatment varied: 29 (42.6%) use EDTA, 11 (16.2%) use DTT, 10 (14.7%) use heat inactivation, and 3 (4.4%) use Melon column (a replacement for hypotonic dialysis). The remaining minority reported use of AdsorbOut beads, FBS/FCS, freeze/thaw or centrifugation (Fig. 1**A**).

Moreover, 41 of 59 labs (only 65% of all labs) were treating every sample from every patient, while the remaining labs were pre-treating sera only from sensitized patients, only pre-transplant samples, or occasionally "as needed" on select patients.

Thus, there is wide variation in whether, when and how samples to be tested for HLA antibodies are pre-treated to mitigate inhibitory factors. One major gap identified by the survey was that the scientific literature did not yet support serum pre-treatment for every patient. A similar survey conducted in France through the SFHI's (Francophone Society for Histocompatibility and Immunogenetics) EPT provided similar conclusions (Fig. 1B).

We invited Rob Liwski (Halifax) and Jean-Luc Taupin (Paris) to debate their opinions on the mechanisms of serum interference (also referred to as complement interference [CI]), best practice to mitigate it, and its clinical significance. Both contributors have extensively published experimental characterization of the single antigen test and opinions on its limitations. Here, they share their expertise and identify key areas in need of further investigation.

2. Defining serum interference

Early reports [3] revealed occasional discordance between solid phase assays and physical crossmatching that could be improved by pre-treating serum prior to solid phase testing, which has been confirmed more recently [4,7,8]. Similarly, non-correlative results between IgG-MFI and complement binding assays (C1q/C3d) could be mostly resolved by serum treatment or dilution [9,10]. HLA antibodies in untreated serum become higher in MFI when serum is treated, or HLA antibodies in undiluted serum (whether treated or not) become paradoxically stronger when the serum is diluted. However,

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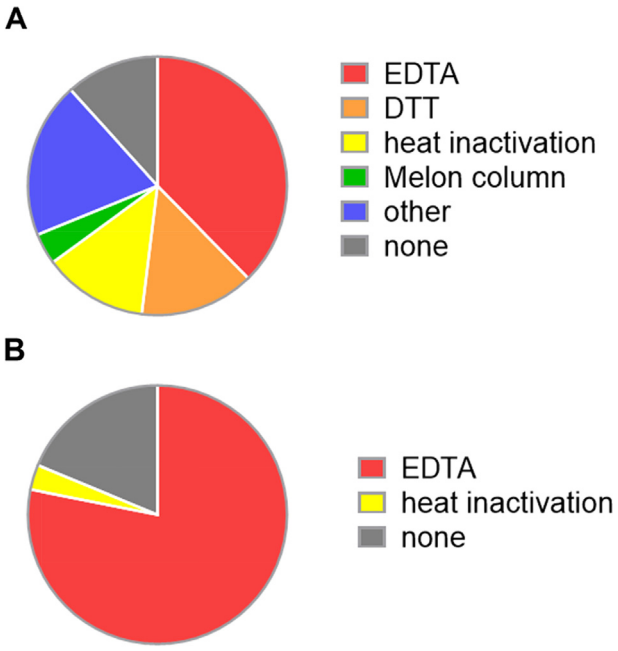

Fig. 1. Results from a survey of **(A)** North American ASHI-accredited laboratories (n = 68) and **(B)** Francophone SFHI-accredited laboratories (n = 32) showing respondents' use of serum pre-treatment prior to HLA antibody testing by single antigen beads.

the threshold for "stronger" differs across studies. What makes for a significant increase is not clear, particularly since the assay itself carries variability upwards of 25% CV even when rigorously standardized [11] and positive/negative cutoffs differ according to laboratory.

2.1. What is the most clinically relevant way to define "serum interference"?

Jollet & Taupin

How much MFI increase is required to conclude that interference phenomenon took place? I think we quite extensively answered this question [12]. Complement interference [CI] occurs for the strongest MFI, i.e. those above the 15,000 range, corresponding grossly to the value at which C1q binding can be observed. CI occurs when Ab density on the Ag crowded bead surface is sufficient to stably retain C1 through interactions between several of its Fc binding sites with Fc fragments located within the diameter of the C1 molecule, i.e. accessible to it. Therefore, IgG staining has to be high, and an MFI increase that does not reach the saturating value (in the 20,000 range) of the Luminex cannot be explained by CI, or at least solely by CI. Removal of the conjugate blockade will uncover the maximal binding of the serum, as this inhibition was caused by a very high level of the anti-HLA antibody. The more profound the inhibition by CI, the higher the MFI when CI is abrogated.

Nevertheless, it is hard to set a minimal MFI gain that would characterize CI because it would associate two parameters: 1) increase in a proportion above the local intra or interassay variability of the SAFB assay (if tests in both conditions are performed within or not the same batch, respectively, and in addition considering that interassay variability decreases when MFI increases), and 2) MFI after CI inhibition reaching at least the local C1q positivity threshold. Nonetheless, a situation might exist where the MFI value reached will remain below the expected minimum level, depending on the strategy used to abrogate CI involving IgM but we will describe it later. It is strange to observe that an IgG Ab at low level could strongly activate complement, while there are increasing data showing that IgG MFI is the main parameter conditioning complement activation and therefore CI occurrence. Subclass composition can play a role, but in this case the reverse should be observed, i.e. a strong MFI without complement binding, and this is rare given the high prevalence of the complement activating IgG isotypes IgG1 and IgG3 among HLA Abs, as concordant articles tend to confirm.

Greenshields & Liwski

As with many aspects of HLA antibody testing and interpretation, there is significant variability in how serum interference is defined in the literature. In the first published study describing the phenomenon of serum interference in Luminex based solid phase assays, the authors did not provide a specific definition with respect to the MFI increase with serum treatment (DTT or hypotonic dialysis) [3]. Rather, they referred to "changes in antibody specificity" and "large differences in reaction strength". An early definition described prozone as a >2 fold increase in MFI, following serum treatment [13]. This definition was adopted by several subsequent studies [14–16], however, the experimental evidence to support its validity is lacking, and application of this rule could lead to false assignment of prozone positive and negative specificities. For example, as the coefficient of variation for specificities <2000 MFI can exceed 40% in the SAB assay, doubling of fluorescence in this MFI range can occur simply as a result of run to run variation. In addition, as the SAB assay saturates around 20,000–25,000 MFI, specificities >10,000 MFI are unlikely to double their fluorescence upon treatment, even in the presence of significant prozone. In a recent study, Guidicelli and colleagues used a novel, data driven approach to define the prozone effect [17]. Specifically, the authors determined the mean and 3SD MFI values (obtained by testing positive control sera on multiple SAB assay runs) and performed linear regression calculations to assign bead specific MFI cutoffs and identify serum interference. The bead specific cutoff-based definition is scientifically sound, but the approach is labor intensive, laboratory specific, and may be difficult to apply in routine practice.

In two recent studies from our laboratory we defined prozone as an increase in MFI of $\geq$3000 with EDTA treatment and further classified the prozone effect based on the extent of MFI increase into mild (ΔMFI 3000–4999), moderate (ΔMFI 5000–9999) and marked (ΔMFI $\geq$10,000) categories [18,19]. This classification was initially derived based on our experience and was admittedly arbitrary. However, we have demonstrated that these different prozone categories correlated very well with the complement fixing potential of individual specificities as assessed by C3d deposition in the SAB assay (Fig. 2). In fact C3d positivity (C3d-PE MFI $\geq$ 4,000) could be used to accurately predict the prozone effect (sensitivity = 95.2%, specificity = 97.2%), validating our approach to classification of prozone [19]. This definition of prozone is simple and practical. Furthermore, it accurately predicts prozone and classifies the specificities based on the extent of complement mediated interference.

3. Causes of serum interference

It is the observation that antibodies change from negative or weak to higher in MFI upon dilution that likely prompted the term "Prozone." However, the Prozone phenomenon impacting other immunoassays is defined in terms of precipitation, where false negative results are observed in the presence of high titer antibodies in excess of antigen. Indeed, the proposed mechanisms of serum interference in solid phase detection of HLA antibodies are entirely different than the mechanism that causes true Prozone [20]. The majority

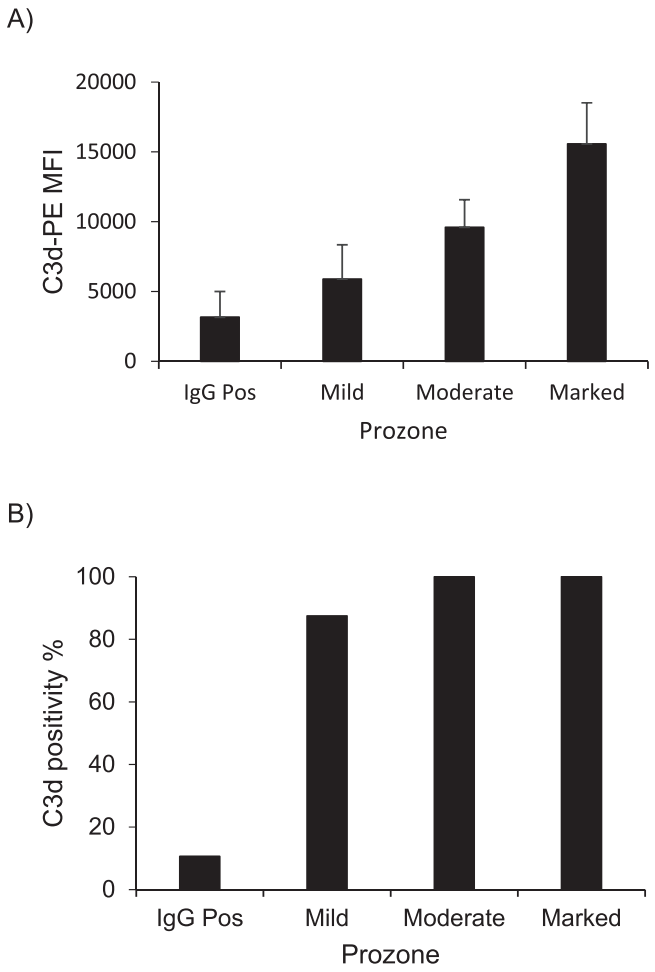

Fig. 2. C3d positivity is strongly associated with the strength of the prozone effect. Sera from 30 highly sensitized patients were tested by the SAB assay +/− EDTA treatment. Prozone was defined as an increase in MFI of ≥3000 with EDTA treatment compared to untreated sera. Prozone was further classified into 3 categories based on the extent of the MFI increase in the EDTA treated serum: (1) mild (ΔMFI 3000–4999), moderate (ΔMFI 5000–9999) and marked (ΔMFI ≥10000). The same sera were then tested with a modified SAB assay using anti-C3d-PE secondary antibody to detect C3d deposition. Association between (A) C3d-PE MFI or (B) or C3d positivity (%) and prozone negative (IgG Pos) and prozone positive reactivities is shown.

of studies cite activation of endogenous complement in the patient's serum. Another hypothesis is that IgM is the interfering factor responsible, blocking the secondary antibody [21]. Finally, true antibody excess bound at high density to the bead may sterically hinder recognition by the secondary antibody [22,23]. It is possible that these or other yet unidentified mechanisms are responsible for inhibition of IgG recognition in solid phase HLA antibody assays, depending case-by-case on the patient and the patient's immunological status. The cause is important because it will guide the most effective and reproducible way to eliminate it.

3.1. In your opinion, what is the major cause of serum interference?

Greenshields & Liwski

Initial studies describing serum interference in the SAB assay attributed the inhibitory effect to IgM [3,13,24]. This hypothesis was based on data showing that treatment with DTT, hypotonic dialysis, or immunoglobulin fractionation, which reduce or eliminate IgM, prevented serum interference. In addition, significant association between the presence of HLA-specific IgM and prozone positivity was identified [24]. However, most prozone positive specificities had IgM MFI below 500, while others were IgM negative, which could not account for the degree of inhibition observed in the study.

In 2011, new data implicated complement activation, rather than IgM, as the cause of serum interference [14]. Serum treatment with EDTA, which chelates calcium required for complement activation, abolished interference in all cases previously identified as prozone positive through serum dilution. In addition, treatment with a C1 inhibitor or heat (56 °C) also prevented prozone. Furthermore, all prozone positive specificities bound high levels of C1q. While interference was also inhibited by DTT treatment, the authors speculated that this was mediated through the reduction of C1 disulfide bonds, rather than IgM. Subsequent studies using methylamine and cobra venom to interfere with C3 and C4 [15], and the depletion of specific complement components from sera [12], helped clarify the mechanism behind the prozone effect and confirmed the critical role of complement in this process. In the current model, prozone occurs as a result of classical complement activation and subsequent deposition of C4 and C3 activation products on HLA-bound IgG, this in turn interferes with anti-IgG-PE antibody binding and HLA specific antibody detection [12].

Based on these studies, the majority of HLA laboratories in North America pre-emptively treat sera with EDTA, and recent reports support the use of EDTA over DTT for the prevention of prozone [25,26]. However, recent studies show that IgM dependent interference is possible [21]. In elegant experiments using monoclonal IgG and IgM antibodies, IgM was shown to interfere with IgG binding or detection. A recent study from our laboratory, using EDTA treatment to uncover serum interference, showed that complement dependent prozone is common, affecting 80% of highly sensitized patients in our center [19]. Interestingly, a small subset of prozone positive specificities (7.9%) exhibited an additional increase in MFI upon serum dilution. Most of these specificities had a substantial IgM component, suggesting that IgM may have contributed to the interference. Notably, these specificities were identified in patients with a history of transplantation, suggesting IgM dependent prozone may be common post-transplant. Further studies are warranted to determine the prevalence and impact of this phenomenon.

Jollet & Taupin

Actually, serum interference has several causes, and CI is only one of them. IgG binding is impaired by the presence of IgM targeting the same epitope or an epitope of the same antigen with steric hindrance due to IgM size. We actually described this situation with two patients displaying de novo IgG DSA which appeared low in raw serum, increased to medium range MFI with EDTA, and increased further to saturating values with DTT. EDTA abrogated C activation due to IgG and IgM binding, but had no effect on IgG/IgM competition, while DTT could act on both parameters. These sera showed a strong IgM binding in the presence of an anti-IgM conjugate [21]. This hypothesis had been proposed a few years earlier [27], but without the experimental data we provided. We believe this very same phenomenon occurred in the article by Tambur et al. [16], and we commented on that. Regarding the findings reported by Rob Liwski's group recently [19], IgM might not explain all the complement independent interference patterns that might exist. Indeed, in this article, these authors report on non-complement binding situations where significant MFI increase occurs upon EDTA treatment, without detectable IgM. Such cases are a minority, but remain unexplained.

3.2. Does the current literature support that antibodies to only certain loci or class I vs. class II exhibit interference?

Jollet & Taupin

No HLA class or locus should be exempt from risk of CI. DQ and A were the most frequently involved in our previous work [12]. This could be due to the high prevalence of these antibodies in patients, and to their presence at high MFI values. Cw is seldom involved, possibly because MFI levels are lower, therefore the Ab density on the bead surface is less, but for these antigens, the W6/32 staining performed by the provider suggests that Ag amount is not as high as for A and B molecules, suggesting again that Ag density might not be optimal to trigger C1 stable binding. Alternatively, and actually both explanations can occur at the same time, denatured antigens if in a too high proportion, will take away epitopes from well conformed antigens, creating conditions less favorable to C1q binding to anti-native HLA Abs through a lowered density of target antigens. Overall, many situations do exist, depending on parameters involving allele, beads and sera, and each case should be considered specifically.

Greenshields & Liwski

To our knowledge there has only been one published paper addressing this question systematically [17]. In this study the authors showed that antibodies targeting any HLA locus can be affected by the prozone effect, however, the degree of susceptibility for different loci varies. For example, serum interference was more common for class II than for class I HLA specificities. Furthermore, within class II, HLA-DQ specificities were affected more frequently than DR or DP, while for class I, HLA-C specificities were the least affected. Our recent study [19] confirms that prozone can affect any HLA locus. However, in contrast to the previous report [17], we found prozone to be more common for class I (70%) compared to class II (30%) HLA. This may be related to the differences in the extent of patient sensitization, antibody composition and how prozone was defined in these studies. After adjusting for the total number of IgG positive beads per locus we found the frequency of prozone for each locus to be as follows: HLA-A (31%), HLA-B (29%), HLA-DQ (26%), HLA-DP (17%), HLA-C (16%) and HLA-DR (5%). Therefore, while class I HLA-A/B and class II HLA-DQ loci are most commonly involved, prozone can affect antibodies targeting any HLA locus.

3.3. What evidence do we have that antibodies causing serum interference are indeed higher in titer in the serum than those that are not?

Jollet & Taupin

Association between Ab titer/MFI/concentration and CI should not be questioned anymore. Schaub et al clearly demonstrated it in 2014 [28], other articles confirming it [10], and the final hit was brought in by Yell et al. [29] by showing that concentrating/diluting sera could on its own decide whether C would be activated or not.

Greenshields & Liwski

Intuitively, the notion that prozone positive antibodies are higher in titer than prozone negative antibodies makes sense, however, the evidence for this is only circumstantial. The study by Tambur and colleagues demonstrated that antibodies exhibiting the prozone effect are high in titer, although the authors did not directly compare titers between prozone positive and prozone negative specificities to demonstrate the difference [16]. Interestingly, they also showed that antibody titer strongly correlated with the complement binding potential of an antibody as assessed by the C1q assay and that C1q MFI correlated best with the peak IgG MFI obtained with serial dilutions rather than neat IgG MFI. Others have found a strong correlation between IgG-MFI and C1q positivity when sera were treated with EDTA [28], DTT [29], and EDTA or dilution to 1:20 [27]. Several studies published to date investigating serum interference showed that prozone positive specificities are significantly more likely to bind C1q compared to prozone negative antibodies [14,15,26,27]. Therefore, if C1q binding potential is used as a surrogate for serum interference, it is likely that prozone positive specificities on average exhibit higher titer than those that are not.

4. Can we abolish serum interference?

The literature to date is complicated by a myriad of reported methods of serum treatment to abolish serum interference that also reflect the discordance in practice across laboratories. Very few studies have directly compared different methods, and none have compared all. EDTA treatment of a few sera in two studies [25,26] resulted in the highest MFI values, but whether that MFI value is more clinically relevant has never been addressed. Moreover, there is conflicting evidence suggesting that EDTA treatment may be insufficient to identify antibodies with serum interference, and only still revealed by dilution. Of note, plasma containing anti-coagulants and calcium chelators, which may inhibit complement activation, is rarely used due to incompatibility with crossmatch techniques. The majority (57 of 63) of ASHI laboratories responding to our survey test serum, while a minority (5 of 63) use plasma (always or occasionally).

4.1. In your opinion, is there any method currently reported in the peer-reviewed literature that a laboratory can adopt and be 100% certain serum interference has been overcome?

Jollet & Taupin

To me there is absolutely **no doubt that EDTA is a reliable strategy for CI**. It has been used since the end of 2012 in Bordeaux and mid September 2015 in Paris, the two labs I have been working in since I have been involved in HLA, and I can certify that prediction of CDC-XM positivity has been dramatically improved, and CDC positive XM not predicted at the virtual XM step have become exceptional (250 XM a year run in Bordeaux and >3000 a year in Paris). The unexpected CDC positive XM have actually been limited to hypersensitized long-waiters for whom an immunological risk is taken, patients needing an emergency transplant without any history in the lab, patients with a strong increase in HLA Abs since the last sample due to recent sensitizing event, and non HLA positive XM. Although several at risk situations still exist, these cannot be anticipated by the HLA laboratory, by contrast to the situation prior to EDTA treatment.

EDTA will not address the interference caused by IgM, of course, but eliminating IgM (e.g. with DTT) would give a result for IgG that would not represent the reality of the patient's serum when both species do exist. The question becomes another one, i.e. do IgM DSA represent a threat for the transplant when IgG coexist, meaning should we be able to detect them in the routine, either by comparing the IgG SAFB assays with and without DTT (which would also inactivate complement), or run an IgG and an IgM assay in parallel. But this is out of the scope of this article. Plasma - could represent a convenient alternative in order to manage CI, but the anticoagulant used has to be carefully chosen because in many laboratories the same blood sample is also used for biobanking until the cytotoxicity crossmatch has to be performed. As cation chelating agents inhibit both clotting and complement, a strategy has to be validated to fully reverse this inhibition in all cases.

Greenshields & Liwski

In short, no. While serial dilution/titration of sera [16] may seem to be a foolproof method to uncover prozone, this approach is too costly and time intensive to be widely implemented for routine HLA antibody screening. Testing all sera at a dilution such as 1:10 [14] should in principle eliminate most of the complement mediated interference (by virtue of diluting out complement), however, it may compromise the detection of weak HLA antibodies and would alter the overall MFI of prozone negative reactivities as well as some specificities that exhibit mild prozone. In addition, it is unlikely that a single dilution such as 1:10 would be effective at uncovering IgM mediated prozone in all cases given that the optimal dilution would likely depend on the relative titers of IgG and the interfering IgM. While treatment with **EDTA** is very effective in abolishing complement dependent prozone [12,14,17,25,26] and is the most common treatment used by HLA laboratories in North America, it **cannot block IgM mediated interference** [21]. In addition, we have recently demonstrated that EDTA can have off-target effects that may impact the MFI and compromise the detection of some prozone negative specificities [18]. Heat (56 °C) treatment, which appears to be as effective as EDTA [10,14], would suffer from the same limitation in the face of IgM mediated prozone and may also affect the detection of low level antibodies by increasing the background and/or degrading IgG. While DTT can in principle block both complement- and IgM-dependent mechanisms, its efficacy at inhibiting interference due to complement was shown to be inferior when compared to either EDTA [25,26] or heat [26]. Interestingly, both of these studies used a single (5 μM) concentration of DTT; therefore, it may be useful to determine if higher doses of DTT could be more effective.

An interesting approach of simultaneously treating sera with EDTA and DTT to inhibit both complement and IgM mediated interference was recently used in a study by Visentin and colleagues [21]. The combination treatment appeared to be effective at blocking both types of interference in the same sample. However, only two patient sera were tested and the potential negative impact of the combination treatment on prozone negative specificities was not investigated. Therefore, while this approach appears to be promising, more research is needed to determine its usefulness. Other modalities which remove both IgM and complement interference from sera and that were shown to inhibit prozone include hypotonic dialysis and IgG purification spin columns [3]. A recent study from the Johns Hopkins group presented at the ASHI annual meeting in 2016 suggests that the Melon™ Gel IgG spin purification kit and hypotonic dialysis are superior at removing serum interference when compared to DTT or EDTA [Kielek 2016, S132]. The disadvantages of using these approaches is the added cost and time required to treat. In addition, the study tested a small number of sera and the impact of these treatments on prozone negative specificities was not assessed. **Further research will be required before these approaches can be widely implemented into routine clinical testing**.

Although serum treatment techniques such as EDTA, DTT and heat are in many cases effective at minimizing the prozone effect, there is some concern that these treatments could have off-target effects and may compromise the detection of low level IgG. To this end we have recently developed a dual antibody rapid test (DART) protocol, which uses a combination of anti-IgG-PE and anti-IgG-C3d-PE reporter antibodies to detect HLA antibodies both in the presence and absence of complement mediated interference [18]. Indeed, DART protocol increased the MFI values of all specificities exhibiting complement dependent prozone, albeit the MFI increase was less pronounced (by 25–30%) compared to the treatment with EDTA. However, the MFI correlation for prozone negative specificities was significantly better between the DART protocol and untreated sera than for EDTA treated samples. Therefore, the DART protocol can overcome the prozone effect without compromising the detection of HLA antibodies.

5. Are we ready to standardize methods?

There are important implications of serum interference for routine clinical laboratory practice, analysis of the clinical literature and the endpoints relied upon by multi-center clinical trials in solid organ transplantation. Taken together, numerous studies [3,10,13,15,16,25,30,31] prompted the Sensitization in Transplantation: Assessment of Risk (STAR) workgroup and other national guidelines that "measures to remove inhibition must be put in place" [5,6] in order to prevent under-estimation of HLA antibodies, yet no specific method was recommended in these consensus documents.

5.1. Once a clearly superior method has been identified, is it reasonable or feasible to propose that all laboratories should be using the same standardized approach?

Greenshields & Liwski

As discussed above, several serum treatment/dilution options are currently available to overcome the prozone effect. Each method has its advantages and disadvantages, some of which may be laboratory specific. It is **unlikely that a single method will be developed** that is clearly superior and effective in all cases. Thus, while it would be helpful to develop standards mandating that each laboratory develop an approach to recognizing and handling the prozone effect, it is probably **best to leave it up to individual laboratories** to make an informed decision with regards to the method(s) of choice. Establishing proficiency testing exercises and/or serum exchanges in order to evaluate a laboratory's ability to detect prozone positive specificities and facilitate inter-laboratory comparison of different serum treatment modalities may be helpful in this regard.

Jollet & Taupin

We yearly survey the laboratory practices in francophone countries affiliated with the Francophone Society for Histocompatibility and Immunogenetics (SFHI)'s external quality control program, currently used in France and North Africa. In May 2019, among the 32 respondents, 6 did not use any pretreatment (a percentage identical to the one reported for ASHI at the beginning of this article), while 25 used EDTA and 1 used heating (Fig. 1**B**). In 2018, among the 26 respondents, only three did not use any pretreatment. However, the SFHI recommendations established at the end of 2016 clearly recommend a systematic pre-treatment of all sera to circumvent the complement interference phenomenon. **EDTA is not officially recommended but it is the only one to be cited as an example with a concentration**.

5.2. Given the variability across laboratories, are there any implications for proficiency testing?

Greenshields & Liwski

The impact of serum treatment variability on proficiency testing (PT) would depend on several factors such as the source and type of sera used, the proportion of participants using serum treatment (including types of treatment) vs. no treatment, the type of data reported (i.e. pos/neg vs MFI) and how the survey is scored. With this in mind, the impact on a laboratory's performance in a PT survey such as the ASHI AC survey would be negligible given that: 1) most sera come from unsensitized or only moderately sensitized individuals, which are not likely to exhibit significant prozone, 2) the specificities are reported as positive or negative and MFI values are not reported, thus only antibodies exhibiting a profound prozone, which is relatively uncommon, could potentially be missed, 3) the survey uses consensus based scoring (90%) and the passing grade is awarded as long as 80% of the consensus specificities are reported by a laboratory. The HLA antibody PT survey available from the College of the American Pathologists (CAP) is even less stringent in terms of grading. Therefore, **PT surveys that are currently available to histocompatibility labs in North America are not designed to assess a laboratory's ability to handle the prozone effect or the impact of different serum treatments**.

Jollet & Taupin

Actually, we developed the SFHI proficiency testing partly because the available programs do not address all questions, including this very one of CI. As Rob says, it is not easy to find adequate samples, and we so far have only found sera that display a partial CI only leading to moderate or strong MFI decrease but not to signal extinction in the absence of an adequate pre-treatment. Therefore, when the performance of the labs is established from the percentage of antigens found positive, these discrepancies do not come into light, as few antigens are concerned and keep an MFI above the positivity threshold. Therefore, the most appropriate way to highlight the risk is to proceed to MFI data collection and analysis as we perform it, and this clearly helps sensing what local practices are and how they evolve. Among the 12 sera tested in May 2019, a moderate but significant CI occurred for 2 of them. Proficiency testing programs have to adapt to the questions the professionals cope with, and this will help the professionals to move in the right directions faster thanks to the challenging power of inter-laboratory comparisons.

6. Clinical significance

Physical crossmatching has decades of literature to support the detection of clinically relevant outcomes in transplantation. How does serum interference relate to outcomes?

6.1. Is serum interference a significant problem, or does it only affect a minority of patients?

Greenshields & Liwski

The most comprehensive study published to date performed in an unselected patient cohort including both sensitized and unsensitized individuals, estimated the frequency of prozone at 29.5% for class I and 45.9% for class II HLA [17]. Not surprisingly, the rate of prozone reported by Tambur and colleagues [16] in a sensitized patient cohort was higher (71%), however, the inclusion criteria were somewhat ambiguous and did not include the type of sensitization. Our recent study [19] performed in a cohort of 30 highly sensitized patients (cPRA $\geq$95%) estimated the frequency of prozone at 80% and suggested that patients with a history of previous transplant are at a particularly increased risk (87%) of serum interference. Furthermore, in 33% of patients with a history of previous transplant, some antibodies were missed (using a 2000 MFI cutoff) in untreated sera due to prozone. Therefore, serum interference is common and is a significant problem especially in highly sensitized individuals.

Our recent study [19] suggests that the degree of patient sensitization (cPRA $\geq$95%) and type of sensitizing event (eg. previous transplant) are associated with a high risk of prozone. However, it is unlikely that these or other clinical factors could be used to accurately identify all patients who are at risk. We have recently shown [19] that C3d positivity (MFI $\geq$4000) can predict prozone with good accuracy (sensitivity = 95.2%, specificity = 97.2%), however, additional testing using the C3d conjugate would be required to identify patients at risk using this approach. Guidicelli and colleagues [17] demonstrated that the SAB assay parameters such as the number of positive beads or the highest bead MFI cannot be used to reliably predict prozone. In addition, data from our laboratory shows that neither the positive nor negative control bead MFI can identify sera impacted by complement dependent interference. Taken together, these data suggest that **upfront treatment of all sera prior to HLA antibody testing may be the simplest and safest approach to avoid the prozone effect**.

Certainly in our center we have seen a few patients with high titer post-transplant DSA, which could be missed or underestimated if untreated sera were used in the SAB assay. However, to our knowledge there are no published data addressing these questions.

Jollet & Taupin

Clearly nothing can warn you of possible interference when looking at the MFI profile of one serum for a patient you are testing for the first time. Therefore, **systematic pre-treatment is the safest way to master this risk**. However, for pre and post-transplant, when considering a patient's evolution during time, one may detect a strong decrease in MFI for a subset of alleles that used to be very high, and this profound drop, if it occurs over a quite short period of time, is hardly explainable by IgG's known half-life of 3 weeks.

As IgG can increase much faster than it is supposed to decrease in blood, this must warn us, but this is a quite extreme and, as such, rare case. Even for some patients with very stable profiles you might have been dealing with a strong CI since the beginning without having ever suspected it once. Another scenario that may occur post-transplant is a DSA found in a biopsy eluate but which is absent in serum. In some cases this can be due to CI, especially when the *in situ* DSA is high, given that CI does not occur with eluates as serum complement has been washed out with the elution procedure. But you must admit that this is a quite complex strategy to screen for CI prone patients. . .. Indeed, such cases have not been reported although they may have perfectly illustrated and therefore convinced people earlier that a proper action was required.

6.2. What evidence (if any) demonstrates that the increased MFI obtained upon treatment of samples compared with untreated is actually clinically significant?

Greenshields & Liwski

In our experience, this level of complement fixing activity would be predicted to result in a positive cytotoxicity crossmatch suggesting that most prozone positive antibodies would be highly relevant clinically. Misclassifying such antibodies as negative or weak based on untreated serum MFI would compromise virtual crossmatching and could have serious clinical consequences in cases where prospective physical crossmatching is not performed.

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This is all about the definition of interference based on MFI change after treatment, whatever it is. One additional important point to me is that weak MFI changes might be due to interassay variability and/or extra dilution of the serum sample with the treating agent(s), so all parameters have to be carefully considered in the calculation.

Editor's note: Additional evidence relating serum interference to clinical outcomes was by proxy, where antibodies that were detectable or increased at 1:160 dilution correlated with positive surrogate cytotoxic crossmatches, and a small number of patients with pre-transplant DSA detectable at dilution experienced AMR [8].

6.3. Are there any important consequences of serum interference for widespread reliance on virtual crossmatching without a prospective physical crossmatch?

Greenshields & Liwski

Based on our data [19] the frequency of prozone is especially high in highly sensitized patients (80%). Furthermore, in a significant proportion of these individuals (30%) at least one specificity would have been missed due to the prozone effect if untreated sera were used for HLA antibody testing. Therefore, prozone would be predicted to have a significant negative impact on virtual crossmatching in highly sensitized patients.

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The higher and wider the sensitization, the higher the risk of interference, grossly. Hypersensitized recipients are therefore the most at risk, but they are not the only individuals at risk, of course. If the interference is present but not for a donor antigen or an epitope present in the donor HLA typing, there is supposedly no impact. As a clinical laboratory specialist and a skilled professional, you cannot be satisfied with your own work if you missed something the size of an elephant in the small tube of serum. Therefore, in all ways, there are consequences, either for the patient or for yourself, or both.

Declaration of Competing Interest

NV is an honorarium recipient from One Lambda/ThermoFisher (2018). RL, JLT and IJ have no disclosures.

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