TRANSPLANTATION RÉNALE / KIDNEY TRANSPLANTATION
DONOR SPECIFIC ANTI-HLA ANTIBODIES DETECTION AND IMPACT IN KIDNEY TRANSPLANTATION

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ABSTRACT • Kidney transplantation is now accepted to be the best treatment for end-stage renal disease. Despite the improvement of immunosuppressive therapy, there is still actually substantial loss of allografts, in part due to uncontrolled humoral immunity. For many years, the primary technique for the detection of anti-HLA antibodies was the CDC (complement dependent cytotoxicity). The recent use of solid phase assays, mainly the Luminex technology allowed detection of antibodies at much lower levels, and it has been shown that these antibodies have negative impact on the graft survival. We herein review the principal techniques for anti-HLA detection and the different presentations of humoral rejection.

Keywords : Anti-HLA antibodies, CDC (complement dependent cytotoxicity), solid phase assays, Luminex, antibody-mediated rejection

RÉSUMÉ • Il est actuellement admis que la transplantation rénale est le meilleur traitement pour les patients en insuffisance terminale du rein. Malgré l'amélioration du traitement immunosuppresseur, la perte des greffons à long terme reste substantielle, en partie due à un contrôle insuffisant de l'immunité humorale. La lymphocytotoxicité a pendant longtemps été la technique principale pour la recherche des anticorps anti-HLA. L'utilisation plus récente de techniques en phase solide, notamment le Luminex, a permis la détection d'anticorps anti-HLA à des taux beaucoup plus faibles. L'impact négatif des anticorps anti-HLA spécifiques du donneur ou DSA sur la survie du greffon est actuellement établi. Nous résumons dans ce qui suit les principales techniques de recherche d'anticorps anti-HLA et les différentes présentations du rejet humoral.

INTRODUCTION

Kidney transplantation is now accepted to be the best treatment for patients with end-stage renal disease, conferring an increased survival and a better quality of life compared to dialysis [1-2]. There has been an improvement in allograft survival in the last decades [3-4] and mostly in the short-term outcomes, in part because we have a better understanding and a better control of the cellular immunity. But the long-term outcomes are still lacking behind, with a substantial long-term loss of kidney transplants, resulting in high morbidity, mortality and costs. It has long been considered that the late allograft failure was due to the toxicity of the calcineurin inhibitors, but this paradigm is now being challenged. The chronic allograft nephropathy term has been removed from the Bannf classification in favor of cellular or humoral induced rejection [5], and we now know that the insufficient control of the humoral reaction is one of the main causes of late allograft dysfunction [6-7]. One of the most important recent advances that contributed to a better understanding of the pathophysiology of the humoral immunity was the implementation of sensitive techniques for the detection of anti-HLA (human leucocytes antigen) antibodies. On the other hand, the discovery in 1991 of C4d, an inactive product of classical complement pathway activation that covalently binds to peritubular capillaries, permitted the detection of a "footprint" of the DSA's (donor specific anti-HLA antibodies, i.e. antibodies directed to the HLA of the donor) induced complement activation [8-10].

From a therapeutic point of view, the use of drugs that deplete DSA and target memory B cells and plasma cells i.e. plasmapheresis, intravenous immunoglobulins IVIg, rituximab and more recently proteazome inhibitors in the treatment of acute humoral rejection, but also in the prevention of this type of rejection in highly immunized patients allowed kidney transplantation in this population. Even if the allograft survival is lower, the patient's survival is still higher compared to dialysis patients on waiting lists [11-12].

We'll try in the following to review the different techniques of anti-HLA antibodies detection and identification, and the impact they have on the allograft as well as the latest consensus guidelines.

ANTI-HLA ANTIBODIES DETECTION TECHNIQUES

Immunization results from pregnancies, past transfusions or prior kidney transplantation. It is estimated that in the Unites States, 32% of the patients on waiting list for kidney transplantation are immunized against the HLA system. DSA can be historic (existing on a serum anterior to kidney transplantation) with an anamnestic

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response after the engraftment, and they can appear de novo after the surgery [13-14]. In a serie of 189 nonsensitized kidney transplant recipients, Everly *et al*. showed that, on sequential testing for DSA after transplantation, 11% of the patients developed de novo DSA during the first year, and 20% at 4 years [14].

There are different anti-HLA antibodies detection techniques that can be divided in *cell-based techniques* like the CDC (complement dependent cytotoxicity) that has been for many years considered like the gold standard, and *solid-phase assays* like ELISA (enzyme linked immunosorbent assay) and Luminex. The level of sensitivity and specificity differs with the technique used and the clinical impact of a DSA differs accordingly. Donor specific IgG HLA antibody detected by CDC is currently considered as a contraindication to kidney transplantation [15] but DSA detected by solid-phase assay on a remote serum is considered as a risk for antibody-mediated rejection and inferior allograft outcome [16-18]. Conversely, there are data questioning the impact on allograft survival when it comes to DSA detected only by sensitive techniques but not by CDC [19-21].

Cell-based assays
● Complement dependent cytotoxicity
This technique consists of detecting anti-HLA antibodies by incubating the serum of the patient with a panel of typed lymphocytes expressing HLA on their surface membrane. Complement is then added as well as a fluorescent dye to detect lysed cells. A positive reaction translates the activation of the complement by the immune complex created by the reaction of the anti-HLA antibody with its antigen. The result is expressed as PRA or panel of reactive antibodies i.e. the percentage of positive cells [15]. The same technique is used for CDC cross match by using the lymphocytes of the donor. The main advantage of this technique is that it reflects the situation in vivo where the antigens are in their natural tridimensional conformation and a positive CDC cross match is still considered by many centers as a contraindication for kidney transplantation, because of a high risk of hyperacute and acute kidney rejection. Since its first use in 1969, there have been some technical improvements like the prolongation of the incubation times and the use of antihuman globulin to increase its sensitivity [22]. But there are many limitations like the low level of sensitivity compared to newer solid phase assays and the difficulty of determination of the antigenic specificity in highly immunized patients. More, it can miss antibodies directed against rare antigens not expressed in the panel. The detection of anti-HLA class 2 antibodies by CDC is complicated by the presence of class 1 and 2 HLA antigens on B lymphocytes and by the low presence of these cells in peripheral blood. CDC can also detect both IgG and IgM so that a positive reaction can be due to an IgM reactivity which turns to be autoreactive in many cases. To solve this issue, the serum can be treated with dithiothreitol (DDT) [23]. It should be also noted that therapies like plasmapheresis, IVIG, polyclonal anti-lymphocyte serum interfere with the results of this test [24]. Finally, a positive reaction can be due to non HLA antibodies.

Solid phase assays
● ELISA (enzyme-linked immunosorbent assay)
This technique consists of detecting anti-HLA antibodies directed against antigens coated onto wells in a cell independent technique. HLA molecules can be recombinant or purified. They are attached to the wells of microplates. Patient's serum is added, and then anti IgG antibody coupled with an enzyme, and finally the substrate of the enzyme. Antibody's reactivity is thus translated into a colorimetric reaction. This test can be qualitative, indicating a positive or negative reaction against class 1 or 2 HLA antibodies. It can also be semi quantitative and can be useful for the identification of the HLA specificities. It detects both complement-activating and non-complement activating antibodies [23]. Its sensitivity is increased compared to CDC [24-25]. Technological improvements have been made, notably with DYNACHIP, which is a completely automated high efficiency and high throughput platform for the detection and identification of anti-HLA antibodies based on ELISA [26].

● Luminex technology
Luminex technology is a high throughput platform for the detection of anti-HLA antibodies using a flow-based assay [27]. Flow PRA is equally a flow-based technology for detection and identification of anti-HLA, but for the sake of simplicity we will detail only Luminex technology. A set of polystyrene microsphere with different fluorochromes, coated with HLA molecules, are incubated with the patient's serum to allow the fixation of a potential anti-HLA antibody to its corresponding microsphere. An anti IgG labeled with phycoerythrin (PE) is then added and the mix of beads goes through the cytometer. The red laser excites the fluorochrome and the green laser excites the PE resulting in different fluorescence intensity detected, processed and analyzed by the Luminex platform. A positive control (bead coated with IgG) and a negative control (bead without HLA) are run in every assay. The negative control is used to determine the background for each bead. There are three types of panels: screening panels, phenotype panels and single antigen panels. In the screening test, the beads are coated with a wide number of purified class 1 and class 2 HLA antibodies (HLA A, B, C, DP, DQ, DR), and the result will be qualitative, i.e. positive or negative. To allow the determination of the specificities of HLA antibodies with the highest sensitivity, Luminex single antigen (SA) is used. In this test, each bead is coated with a single recombinant allelic antigen from transfected human cell lines corresponding to a class 1 or a class 2 HLA antigen. The phenotype panels resemble cell-based techniques in which every bead is coated with HLA antigens, either class 1 or class 2, from a cell line corresponding to one individual. Phenotype panels can be used to deter-

mine specificities but this needs a high quality of expertise in the interpretation of results [28-29].

Luminex technology has revolutionized the evaluation of complex sera from highly immunized patients [30]. It is more sensitive than the CDC technique and flow cytometry [28]. Moreover, its comprehensive array of common and rare HLA antigens permits the detection of antibodies against HLA Cw, HLA DPA, and HLA DPB which role in kidney transplantation is now increasingly reported [31]. Luminex SA also enables detection of non HLA antibodies like MICA (MHC class I polypeptide-related sequences A) as well as different classes and isotypes of immunoglobulin (complement fixing and C1q). The test is rapid and semiquantitative permitting determination of the antibody levels. In fact, the level of HLA specific antibody binding is expressed as the mean fluorescence intensity (MFI) of the reporter signal. Numerous reports show correlation between MFI, antibody level, cross match results and clinical outcome [32-33] but standardized cutoff values for positivity have not yet been established. Moreover, the test might be too sensitive and can detect antibodies whose presence does not correlate with worse graft outcome. Besides, there have been reports describing anti-HLA antibodies in non-immunized males [34]. These tests results do not correlate with flow cytometry cross match results and may be the result of detection of antibodies to denaturated HLA on the beads.

Without going into the technical details of the Luminex, it just should be noted that while rendering the interpretation of a Luminex result, the laboratory should take into account: a) the concordance of different results using different technologies, b) the history of immunizing events of the patient, c) the HLA of the patient to ensure that an antibody to a self-epitope is not included in the results.

IMPACT OF DSA
ANTIBODY-MEDIATED REJECTION

The pathophysiology of antibody-mediated rejection is still not fully understood but different patterns of injury or accommodation are now recognized. The binding of DSA to its specific HLA antigen on the endothelial cells results in different reactions with a probable role of the isotype of the heavy chain. If the DSA is complement activating (IgG1 and IgG3), there is activation of the classical complement pathway, resulting in hyperacute and acute antibody-mediated rejection as defined by the Banff classification [35]. DSA can also bind to endothelial cell and activate antibody dependent-cell mediated cytotoxicity (ADCC) where the effector cells are the natural killer cells (NK) and this usually translates into a sub-acute form of AMR evolving to chronic glomerulopathy [36-37]. Finally, accommodation results from DSA binding to HLA molecules and incompletely activating the complement [38].

The spectrum of antibody-mediated rejection goes from hyperacute rejection, to acute AMR, subclinical AMR and chronic AMR. With the use of a positive CDC as a contraindication to kidney transplantation, the advent of hyperacute rejection has practically disappeared. Reports from kidney transplantations in the early 60's described a rapid onset of edema and thrombosis of the allograft in the minutes following the implantation. Histopathology showed massive immunoglobulin coating with endothelial swelling and thrombosis.

Acute antibody-mediated rejection is most frequently encountered in high immunological risk patients who have a positive DSA on a remote serum before transplantation, and in patients with DSA who have been desensitized. It presents as an acute allograft dysfunction with allograft biopsy showing glomerulitis, peritubular capillaritis, acute tubular necrosis or vasculitis [5]. The presence of a positive C4d by immunofluorescence or immunoperoxidase staining is required as diagnostic criteria by the Banff classification, though there is actually some convincing data that C4d negative AMR rejection does exist [39-40]. One possible explanation might be a low sensitivity of C4d staining. There also might be a complement independent antibody-mediated injury.

Chronic antibody-mediated rejection was first described in 2001 [41]. It results from the different pathogenic effects of DSA and leads to chronic irreversible lesions described as peritubular capillary multilamellation, transplant glomerulopathy, interstitial fibrosis and tubular atrophy. Decreased allograft survival invariably ensues. Again, the signature of a DSA-mediated injury is found via the detection of C4d on peritubular capillaries.

Recently, Loupy *et al.* suggested that antibody-mediated rejection must be considered as spectrum of diseases associated with DSA. They used the term indolent, or subclinical AMR, for rejection that occurs usually as a consequence of insufficient immunosuppression, diagnosed because of the appearance of DSA detected by sensitive techniques, with variable degrees of glomerulitis, peritubular capillaritis on allograft biopsy with C4d positivity, without acute dysfunction or proteinuria. They also considered that AMR should also be considered as a continuous process with fluctuation of the DSA level and the pathological features as shown by protocol biopsies [42].

CONSENSUS GUIDELINES ON THE TESTING
& MANAGEMENT ANTI-HLA ANTIBODIES

Consensus guidelines on the interpretation and management of anti-HLA antibodies in transplantation have been recently published [43]. Three groups of experts debated and elaborated recommendations or suggestions according to the level of evidence. They focused on the technical aspects and on the impact of anti-HLA antibodies pre and post transplantation. We will try in the following to give a brief summary of these recommendations.

Concerning the laboratory testing:
- ✓ At least one solid phase assay should be used to detect anti-HLA class 1 or 2 antibodies. If the test is positive it should be followed by a single antigen bead assay to detect specificities, particularly to detect eventual anti Cw, DQA, DPA, DPB anti-HLA antibodies.
- ✓ Cross matching should be done using cell-based (CDC) and bead-based assay (flow cytometry).
- ✓ Always take into account the history of immunization of the recipient.
- ✓ HLA typing of the donor and the recipient should be done at a level required to accurate antibody interpretation.

Before transplantation:
- ✓ DSA detected by CDC for antibody screening and cross matching on the most recent serum must be avoided because of a high risk of AMR and graft loss.
- ✓ If DSA is present, but CDC cross match against donor T and B cells is negative, this should be considered as a risk but not a contraindication for kidney transplantation.
- ✓ Avoid blood transfusions to limit immunizations in potential transplant recipients and test sera after a sensitizing event, proinflammatory events and at regular intervals.
- ✓ In case of immunization, a complete characterization of the HLA antibodies using sensitive technique and HLA typing of the donor should be done,
- ✓ ABO incompatible transplantation can be performed if the level of isoagglutinins is lowered to acceptable levels.

Post transplantation:
The post transplantation group categorized recipients according to their immunologic risk as very high, high, moderate and low risk patients.
- ✓ *Very high risk patients*: are at high risk of early AMR (clinical and subclinical), like desensitized patients. Testing for DSA and allograft biopsy should be done during the first 3 months and if rejection is detected it should be treated aiming improvement of kidney function and lowering the level of DSA. The situation is not clear if the DSA level increases without evidence of rejection of allograft biopsy and some experts felt these patients should be treated to lower the level of the DSA.
- ✓ *High risk patients*: DSA positive but cross match negative. Like very high risk patients, testing for DSA and biopsy should be done by 3 months. The management is also the same. Note that if DSA is positive but the biopsy is negative, lowering of immunosuppression should be avoided.
- ✓ *Intermediate risk patients*: DSA positive on past serum but negative on most recent serum, and patients with history of immunization and at least one positive serum on anti-HLA screening. DSA

should be monitored during the first month, and if positive a biopsy should be done. If negative, continue screening and do not decrease immunosuppression.
- ✓ *Low risk patients*: non sensitized, first transplantation. Check for DSA once between 3 and 12 months and whenever there is a change of immunosuppression, suspicion of non-compliance or graft dysfunction. If DSA is positive, the same algorithm is followed.

After 12 months post transplantation, all risk groups should be tested once every year for DSA. Testing should also be performed if there is change of immunosuppression, suspected non-compliance or graft dysfunction. Again, if DSA is positive, biopsy should be done and the patient treated accordingly.

CONCLUSION

In conclusion, the advent of sensitive techniques for HLA antibodies detection in the recent years played a major role in our understanding of humoral immunity and the impact it has on allograft survival. Solid phase assays are actually increasingly used in histocompatibility laboratories because of their higher sensitivity, with the risk of being sometimes too sensitive and detecting irrelevant antibodies. While interpreting a result of HLA antibodies, the history of immunizing events should always be taken into account as well as the concordance of the results between different techniques and different sera. Much is yet to be done from a technological point of view, and the task now is to standardize results of sensitive anti-HLA detection techniques to allow proper interpretation and to accurately assess the immunological risk.

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