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Role of IgG and IgM antibodies against donor HLA antigens in organ transplant recipients.

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Introduction

Antibodies against donor HLA antigens were first evaluated by cross-matching of recipient serum against donor lymphocytes, which became a mandatory test when it was discovered that such antibodies could be associated with hyperacute allograft rejection [1, 2]. We now have tests that are at the same time more specific and much more sensitive and considerable progress has been made in describing and understanding the role of antibodies against HLA in the outcome of transplants. The specificity and magnitude of the antibody response can now be measured quite accurately. The same parameters for the cellular immune response against antigens of the allograft are quite difficult, if not impossible, to determine. Antibodies are made by cells and in one of the sections that follows we will describe an assay that has been found useful to measure antibody production in vitro by recipient peripheral blood B cells [3]. It should also be remembered that in some instances the presence of antibodies may be just an indicator that signals the development of an ongoing immune aggression, the effector arm of which could be antibodies, immune cells or both. The argument has been made that specific antibodies against donor HLA antigens may not be detected because of their sequestration in the transplanted organ. While undoubtedly small amounts of donor-specific antibodies are bound to the HLA antigens of the graft, it appears unlikely that sequestration can routinely outstrip the capacity of the immune system to produce detectable antibodies. Data obtained from the study of the course of antibodies after donor nephrectomies and our own studies showing absence of antibody production by B cells in vitro, suggest that in many cases absence of antibodies in serum is not due to sequestration.

An interesting question that has been debated for some time is whether for graft injury to develop from the effect of antibodies, the anti-HLA response has to be specific for the HLA antigens of the transplant. Older data has frequently shown an effect associated with
sensitization of the recipients as measured by the panel reactive antibodies (PRA). Some authors have concluded that sensitization, whether or not specific for the donor antigens may be associated with injury and loss of grafts. Our data in heart transplant recipients suggested that only antibodies that could bind to the donor tissue were associated with rejection [4]. We have now asked whether having antibodies that are cross-reactive with donor HLA antigens may also be associated with graft failure. Our results do not support that possibility.

IgM antibodies develop early in many immune responses to foreign antigens. IgM antibodies are good antigen binders and agglutinators, they fix complement and are known to execute other effector functions. However, it was widely believed that IgM antibodies are not harmful to the graft. Positive cross-matches due to antibodies of the IgM class have generally not been considered a contraindication for transplantation. In order to investigate the paradox of IgM antibodies being powerful effectors of immune reactions but not playing a role in allografts, we developed an assay for IgM anti-HLA using single antigen beads. Recent results of this assay in kidney and heart transplant recipients will be described. We now believe that IgM antibodies in many cases can predict rejection and testing for IgM anti-HLA may be important in the evaluation of the immune response in transplant recipients.

**How we test for HLA antibodies with single antigen beads.**

Testing for HLA antibodies moved slowly from assays with lymphocytes and other cells of the blood to immune assays using various preparations of purified antigens attached to solid supports. When whole cells were used, one had to deal with the possible confounding effects of other antigens present on the cell surface. Improvement in the resolution was offered by assays using solubilized membrane antigens purified with monoclonal antibodies against HLA class I or class II. But these tests still contained multiple antigens in each
preparation and specificity of antibodies could only be determined by statistical analysis of the binding results. A major improvement came with the development of single HLA antigens produced from transfected cells. These recombinant antigenic preparations could then be attached to solid phase supports. The most versatile assays were developed by attaching single HLA antigens to polystyrene microspheres, which allowed for the production of multiple arrays that could be read in the Luminex flow cytometer [5].

**a. Antigen density correction.** We used a monoclonal antibody against HLA class I antigens to determine the amount of HLA class I protein attached to each bead. What we found was that there were considerable differences. In some batches we observed as much as a 60-fold difference between the lowest and the highest antigen density beads. In order to apply a correction factor, we now test each lot of beads with ant-HLA class I, W6/32, anti-HLA-DR, L243, anti-HLA-DQ, clone SPVL3 (Beckman Coulter, Fullerton, CA), and anti-HLA-DP, SPM421 (Abcam, Cambridge, MA) monoclonal antibodies at appropriate dilutions. The antigen density factor (ADCF) is then determined as the 75th percentile of the fluorescence intensities for each of the beads. This ADCF is used to correct the mean fluorescence intensity (MFI) values for that bead in every experiment.

**b. IgG binding from normal sera.** In all immuno-assays, a small amount of IgG from normal sera will bind nonspecifically to the solid support used with the conjugated antigen. The amount of normal IgG that binds to each of the HLA conjugated beads varies considerably. It is therefore not feasible to use a single standard threshold to determine whether binding exceeds the normal range and should be considered a positive result. Instead, we determine the binding of 20 sera from non-sensitized male donors and the mean plus 3 Standard Deviations, multiplied by two (2X) is used as the negative cut-off, which is different for each bead.
c. Determination of weak-positive and strong-positive reactions for each bead.

For scoring, the MFI for each bead is corrected first by subtracting the MFI value of the no-antigen bead and then corrected by the ADCF. Values for each bead that are between 2X and 10X of the mean plus 3SD are considered weak positive; values that are greater than 10X of the mean plus 3SD are considered strong positive. We have found that the multiplier, or X-factor, which is the ratio of the MFI obtained to the normal mean plus 3 SD, is a good measure of the strength of binding of antibodies to different beads.

This methodology for determining the positive and negative thresholds of single antigen bead tests provides accurate scoring of results which give comparable results in multiple assays, correlates with predictions of positive and negative cross-matches and offers a semi-quantitative estimate of antibody strength. Since MFI can vary considerably from bead to bead the multiplier of the mean plus 3SD of the normal serum Ig binding, or X-factor, provides a more robust parameter for estimating and comparing the strength of antibodies observed in different sera.

IgG antibodies against the HLA antigens of the donor

a. Kidney allografts. To determine the role of IgG antibodies against HLA antigens of donors in primary kidney transplants, we analyzed kidney transplants performed at our center between 1999 and 2007. During that period transplants were performed with negative T cell crossmatches, but without regard to whether the B cell crossmatch was positive or negative. We evaluated 148 patients, that were transplanted with a negative T cell flow cytometry crossmatch and either a positive (17) or negative (131) B cell, pronase treated, flow cytometry crossmatch. We tested these patients using a Luminex-based single antigen assay to determine if DSA were present. Overall, DSA were observed in 57/148 patients (38.5%). When compared to the 91 patients without DSA, there was a significant difference in outcome (p<0.002). Interestingly, among the primary kidney transplant recipients (Figure 1) 19 patients
had only antibodies against HLA class II antigens of the donor and rejection occurred more frequently in this group compared to patients without any anti-donor antibodies (p=0.004). Also, there was a statistically significant difference in outcome in patients with a negative or positive B cell crossmatches (p<0.003).

**b. Heart transplants.** In our center presence of IgG antibodies against HLA antigens of the donor, either pre-transplant, or developing de novo after transplantation, was associated with increased graft loss due to acute and chronic rejection, in adults and also in children [4]. Antibodies against HLA antigens, not present in the donor, appeared to have no effect on graft outcome.

**Sequestration of antibodies in grafts.**

Often specific antibodies against donor HLA antigens cannot be detected in the recipient serum. Because anti-HLA antibodies have sometimes been eluted from kidney allografts undergoing rejection and because many times high titers of HLA antibodies have been observed after graft nephrectomy is performed, it has been postulated that a major cause of the absence of antibodies against donor antigens in the serum is sequestration of antibodies in the graft [6]. A small amount of antibody probably does bind to the vessels in the allograft, but the arguments against this being a major mechanism for not finding antibodies in the blood are strong:

1. **The absorption would have to be complete,** it is too perfect. In many cases, anti-donor HLA antibodies cannot be detected even with the most sensitive techniques, such as single antigen beads read in the Luminex machine.

2. **There is a considerable delay in the appearance of antibodies after nephrectomy.** In one study it took an average 4 months for the antibodies to become detectable [7]. Sequestration would stop immediately when the graft is removed. The observed results suggested a memory recall mechanism, or perhaps even de novo production of antibodies after exposure associated with the nephrectomy procedure.
3. Not only donor-specific antibodies appear in the serum after nephrectomy. One finds cross-reactive antibodies, as well as antibodies against other HLA antigens not present in the graft [8]. How could they be adsorbed?

4. B cells did not make antibodies in vitro. We have cultured B cells from patients who had been immunized by a mismatched allograft and yet did not have anti-donor HLA antibodies in the serum [3]. These experiments will be discussed further below. We were interested in patients in whom antibodies against donor DQ antigens were found but DR antibodies were not detected. The B cells from several of these transplant recipients were found to similarly produce DQ antibodies and not DR antibodies in vitro. An example is shown in Figure 1. These results are not what one would expect if the cause of the absence of anti-DR antibodies in the serum were sequestration in the graft.

Antibodies against HLA antigens that are cross-reactive with HLA antigens of the donor.

We examined the possible effect of cross-reactive antibodies in 193 heart transplant recipients who were tested for HLA antibodies with class I and class II single antigen beads pre-transplant and at the time they first developed TCAD, or at an average time point for those who did not have TCAD. Overall 54 patients of the 193 (28%) were found to have antibodies against donor HLA antigens and of these 23 (43%) developed TCAD (p<0.005). In contrast, only 9 of the 193 (5%) patients had antibodies only against antigens known to be cross-reactive with HLA antigens of the donor. As shown in Table 1, in this group 2/9 (22%) developed TCAD (p=ns). Thus, it appears that antibodies against cross-reactive HLA, in the absence of antibodies against the donor, were very rare. It was observed in only 9 patients of almost 200. The frequency of TCAD in this group was 22%, which is less than the overall frequency of TCAD in these patients (54/193=28%). Therefore presence of
cross-reactive antibodies without reactivity to donor HLA did not appear to increase the risk of developing TCAD.

**IgM antibodies against HLA antigens of the donor**

There is a fairly widespread belief that antibodies of the IgM type detected during cross-matching are often autoantibodies, are harmless and should not be considered for transplantation [9]. It has also been claimed that IgM antibodies may be beneficial [10]. The effect of IgM anti-donor HLA is not known. With the availability of beads conjugated with single HLA antigens, antibodies can be precisely identified. We obtained and validated a detection antibody specific for the heavy chain of human IgM and used it to investigate the possible role of IgM antibodies against donor HLA in transplant rejection. We selected 34 transplant recipients who received a kidney from a deceased donor and were known not to have IgG antibodies against donor HLA prior to transplantation. They were tested for IgM antibodies against HLA with single antigen beads and immunologic rejection was determined from biopsy findings. IgM antibodies against donor HLA were detected in pre-transplant serum in 5 of the 8 (62%) patients with immunologic rejection and in only 4 of 26 (15%) with good function (p<0.02). In addition, we studied a group of heart transplant patients: 18 who developed transplant-related coronary artery disease (TCAD) and 20 without TCAD. Before transplantation, IgM anti-donor HLA were found in 8 of the 18 (44%) patients that developed TCAD and this increased to 16 out of 18 (89%), when tested just before diagnosis of TCAD was established (Table 2). In contrast, only 2 out of 20 (10%) patients without TCAD had such antibodies. The association of IgM anti-donor HLA with development of TCAD was statistically highly significant (p<0.0000). None of these patients developed IgG antibodies prior to the onset of TCAD. We conclude that presence of specific IgM against donor HLA prior to transplant predicted rejection of kidney allografts and development of TCAD in heart transplants. We did not observe a switch from IgM to IgG before development of significant chronic rejection in heart transplants. The role of IgM anti-HLA
antibodies should be further evaluated in a larger study and may be important in donor
selection and transplant monitoring.

**Antibodies against transplant donor HLA produced in vitro by circulating B cells.**

In an attempt to learn more about HLA antibodies we investigated antibody production by
recipient B lymphocytes in vitro [3]. Peripheral blood B cells were obtained from 36
subjects, including 16 allograft recipients, 12 sensitized patients, 3 multiparous women with
serum HLA antibodies, and 5 healthy non-transfused male subjects. Purified B cells were
cultured with a cell line expressing CD40 ligand. Culture supernatants were screened for
HLA antibodies and positive samples were analyzed using single antigen beads to determine
antibody specificity. HLA antibody-producing B cells were detected in persons known to be
sensitized. In 13 out of 16 allograft recipients, IgG antibodies against mismatched donor
HLA antigens were observed and sometimes donor-specific antibodies were produced in B
cell cultures when serum reactions were negative. In two patients tested, the majority of
antibody producing B cells developed from CD27+ memory B cells. Our results suggest that
analysis of B cells producing antibodies specific for donor antigens may be a useful tool for
identifying and monitoring the humoral immune response in organ transplant recipients.

IgM antibodies appear in the early stages of the immune response. Their significance in the
postoperative course is not known. We wanted to determine if donor-specific HLA antibodies
of the IgM type can be produced by recipient B lymphocytes.

IgM antibodies against HLA were detected in B cell cultures from 7 of the 15 allograft
recipients and 9 of the 16 patients on the waiting list that we tested. None was found in the
5 unimmunized controls. Among the 7 allograft recipients who had positive B cell cultures,
IgM antibodies against mismatched donor HLA antigens were identified in 6 and, in 3
patients we found donor-specific IgM antibodies in B cell cultures when serum reactions
were negative. B cells from one recipient, who was transplanted 6 months earlier, were found to produce only IgM donor-specific antibodies.

Detection of donor-specific IgM antibodies in B cell cultures from peripheral blood of allograft recipients suggests the development of de novo immune responses against the transplanted organs. This assay may provide a useful tool for identifying and monitoring the humoral immune response in organ transplant recipients. The clinical significance of IgM antibodies against donor HLA during the long-term post-transplant course is being investigated.

Conclusions

Our results suggest that precise definition of the specificities, which is possible with beads that carry single HLA antigens, is now defining the immune response against HLA that is frequently associated with loss of grafts. The strength of the antibody response can best be estimated in relation to the binding of normal immunoglobulins to the beads, which can be quite variable. In other words, by dividing the observed MFI by the upper limit of normal IgG or IgM binding, given by the mean plus 3SD obtained from testing a population of sera from normal unsensitized persons. This ratio is a convenient semiquantitative measure of the strength of antibody binding on single-antigen beads. Our study in heart transplant recipients clearly shows that antibodies that are cross-reactive but without reactivity to the HLA of the donor did not increase the risk of TCAD. The emphasis on sequestration as the cause of absence of anti-donor antibodies in the serum of transplant recipients may not be the best interpretation of the facts. IgM antibodies against donor HLA, which are produced both early and late in the course, appear to be associated with graft loss and may be testimony of the potential for renewal of the HLA specific immune response.
References


Legends for Figures.

Figure 1. Effect of pre-transplant antibodies on the survival of 137 primary kidney allografts. Between 1999 and 2007 at our center transplants of kidney were performed with only a negative T-cell flow-cytometry cross-match. The corresponding pronase-treated B cell flow-cytometry cross-matches were performed retrospectively. This resulted in 137 first kidney allografts with complete testing and follow-up of at least one-year. The profile of serum antibodies present just before transplant was obtained by analysis with single-antigen Luminex beads and the presence of antibodies against donor HLA-A, B, C, DR or DQ was recorded. Because of the selection for transplant of only those patients that had a negative flow-cytometry against donor T cells, only 28 of these patients had anti-donor HLA class I antibodies and only 3 of them bound more than 10X the threshold of the mean plus 3SD of normal sera and were therefore considered strong. HLA class II antibodies were found in 33 of these recipients and 22 of the antibodies detected were considered strong because the MFI was greater than 10-times the cutoff. Interestingly, in 11 recipients the only antibodies identified were directed against donor HLA-DQ antigens and in 6 additional patients the only antibodies observed were against donor HLA-DRB3, DRB4 or DRB5 and 2 had antibodies against donor HLA-DRB1. The presence of antibodies against donor HLA class II antigens in these 19 recipients of primary kidney transplants was associated with low graft survival compared to the 94 patients without antibodies against donor HLA (p<0.004). Graft survival was also significantly impaired in patients with antibodies against HLA class I and class II of the donor (p<0.02). The 14 patients with anti-donor HLA class I antibodies only appeared to do better. Survival of grafts in this group of patients was no different from the controls without any antibodies against donor HLA antigens (p>0.8). We believe that the fact that HLA class I antibodies in this group of patients were not associated with a decrease in graft survival was due to the selection of recipients with a negative T cell cross-match against the donors. It appears that the single-antigen bead assay is more sensitive than the crossmatch by flow-cytometry and that the low titer antibodies detected had little effect on
outcome under the treatment regimen used in our center. Few of these antibodies were strong and the evolution of these immune responses after transplantation still needs to be evaluated.

**Figure 2.** Absence of antibodies against HLA-DR in patient BL was not due to sequestration of these antibodies in the graft. Shown are the results of testing for antibodies against HLA antibodies with single-antigen beads. On the left side of the graph the recipient antigens are shown, they are HLA-A2, B15, B61, Cw3, CW7, DR13 and DQ6. No antibodies against these antigens could be detected in the serum or in the supernatants of B-cell cultures. To the right side of the graph are shown the donor HLA antigens. They are HLA-A2, B44, Cw5, DR7, DQ2, and DRB4. Of interest is that in the serum we found antibodies against donor DQ2 but not against DR7. The absence of antibodies against HLA-DR7 could be due to sequestration of antibodies produced by HLA-DR7 antigens present at high levels in the graft. Alternatively the failure to detect antibodies against HLA-DR7 could be due to specific inhibition of the response to this antigen. Results of testing supernatants of B cell cultures from this patient are shown in the lower half of the figure. It can be seen that B cells made antibodies against HLA-DQ2, and DRB4, but antibodies against HLA-DR7 were not found in the cultures. These results suggest that the immune response against HLA-DR7 was inhibited and that the absence of these antibodies was not explained simply by sequestration of antibodies in the graft.
Effect of Pre-Transplant Antibodies on Survival of 137 Primary Kidney Allografts

Graft Survival (%)

Time after Transplantation (Years)

No Anti-Donor HLA (N=94)

Anti-Donor HLA Class I (N=14)

p<0.004

Anti-Donor HLA Class II (N=19)

p<0.02

Anti-Donor HLA Class I+II (N=14)
Table 1. IgG Antibodies to cross-reactive HLA antigens without reaction with donor HLA

<table>
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<th>Patient</th>
<th>HLA Antibody</th>
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</tr>
<tr>
<td>RC</td>
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Table 2. IgM anti-HLA pre-transplant and post-transplant at the time of diagnosis of TCAD

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