Chapter 2
The Histocompatibility Laboratory in Clinical Transplantation

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Our History

Histocompatibility testing in humans began in the 1950s with the discovery by Dausset, Payne, and others of leukoagglutinins in the serum of multiply transfused patients and multiparous women [1]. The science and clinical practice of histocompatibility testing, however, grew and matured in parallel with, and as a direct consequence of, the advent of clinical kidney transplantation. The major technological advances in the histocompatibility laboratory have been developed in a response to the need to better serve the transplant community, which for the first 25+ years was synonymous with serving renal transplantation. The histocompatibility laboratory is in a very interesting position, situated midway between the basic science laboratory and the clinic, and laboratories have been able to make significant contributions in both arenas from this enviable position.

The first successful kidney transplant was performed between identical twins, effectively circumventing the allogeneic immune response and eliminating the need for immunosuppression [2]. This accomplishment held huge potential for end-stage renal failure patients, but since most patients lack a twin to donate, broad clinical application of the new procedure meant that a means had to be found to either identify human leukocyte antigen (HLA) matched donor kidneys and/or to find a more effective means to suppress the allogeneic response elicited by unmatched kidneys (Fig. 2.1). It must be remembered that HLA-matched kidneys from close relatives means that the donor and recipient share chromosome 6, which carries the HLA complex of genes, but they do not necessarily share other chromosomes, indicating that they are not matched for the minor histocompatibility antigens (Fig. 2.2). With HLA-matched unrelated donors there is no guarantee that the donor and recipient share any chromosomes, including chromosome 6. Both of these situations require immunosuppression that was not needed in the transplant between identical twins. In the early years of histocompatibility typing, albeit in its infancy, permitted the search for HLA class I matched donors and thus aided in extending the availability of transplant to a broader segment of the end-stage renal failure patient population (Fig. 2.3).

The science of HLA typing began with the identification of antileukocyte antibodies that cause lymphocyte agglutination in vitro. The original leukoagglutination assays were analogous to
Fig. 2.1 Human leukocyte antigen (HLA) antigen matching between donor and recipient. A matched HLA antigen is considered any antigen which a donor and recipient share. Conversely, any antigen that is found in the donor phenotype that is not in the recipient phenotype is considered a mismatch, as the recipient can mount an immune response towards that antigen. In certain cases, a recipient or a donor may be homozygous at an HLA locus. In those cases, any antigen which a donor carries that a recipient does not is considered a single mismatch. Alternatively, if a donor is homozygous for an antigen carried by the recipient, a recipient has no “nonself” antigens to elicit a response and they are considered matched at that locus.

Fig. 2.2 Inheritance pattern of human leukocyte antigen (HLA) genes. HLA genes travel within conserved cluster called a haplotype. These haplotypes are passed on by traditional Mendelian inheritance patterns as shown in inset. Each haplotype is assigned a letter (father is arbitrarily given AB and mother CD). Haplotypes can be traced to the children with each child having one haplotype from the mother and one from the father. As a result, there are only four possible combinations a child may have. The exception to this is when pieces of the HLA region are exchanged between chromosomes during tetrad formation in meiosis. This exchange of DNA between chromosomes creates recombinant haplotypes.
The Histocompatibility Laboratory in Clinical Transplantation

The red cell agglutination assays that were being used in blood banks to identify red cell antigens. Unfortunately, since lymphocyte function requires cellular aggregation in order to form immunologic synapses, aggregation tends to occur naturally with these cells. This natural agglutination does not require antigen–antibody interaction as red cells agglutination does, but it is often mediated by other molecular interactions characteristic of intercellular communications necessary for immune activation. This non-antibody-mediated aggregation, or “stickiness,” made interpretation of leukoagglutination assays difficult and progress in identification of leukocyte antigens was slow until the development of the complement-dependent cytotoxicity (CDC) assay by Terasaki [3]. Although performing and analyzing the microcytotoxicity assay can arguably be considered at least equal part art and science, the availability of this assay single-handedly facilitated the discovery of the majority of the leukocyte antigens known today.

The CDC assay is exactly what its name implies, it is an assay were isolated lymphocyte are killed by complement activated by antibody bound to the cell surface. The assay is performed by mixing 1 μL each of cells and serum in a small multwell plate, a Terasaki plate. If anti-HLA antibodies are present in the serum, they will bind to any cell that carries the corresponding HLA
 antigen. Once the antibody is bound, rabbit complement is added. Rabbit complement is used because it is not easily inactivated by human decay accelerating factor (DAF), molecules that are natural controllers of complement activation in vivo but that can inactivate human complement in the CDC assay. If antibody is bound to the cells the rabbit complement is activated forming membrane attack complexes (MACs) compromising the integrity of the cell membrane, allowing the free flow of solutes across it and resulting in cell death. The dead cells are detected by the addition of a vital or fluorescent dye, dyes that are excluded by live cells but can penetrate dead cells. The cells are then observed microscopically, shiny small round cells are alive, not having bound antibody and complement, larger dull dark cells are dead and presumably bound antibody and were killed by complement activation. The percentage of dead cells in the well is estimated, up to 10% dead is scored a 1, 11–20% is a 2, 21–40% a 4, 41–80% a 6, and 81–100% is an 8. False-positive results are most commonly caused by spontaneous cell death or the presence of autoantibody and other non-HLA antibodies. False negative results can occur if the antibody is present in low titer or if the antibody is of an isotype that does not activate complement (Fig. 2.4).

Throughout the 1950s and 1960s a number of histocompatibility groups worked simultaneously in laboratories around the world using the CDC assay to identify HLA antigens. As with any scientific endeavor there were naturally efforts to be the first to define an antigen and explain the role of these antigens in eliciting an alloimmune response. In contrast to much basic science research, however, this effort also had immediate clinical application in the pressing need to identify well-matched kidney donors for a growing number of transplant facilities. Progress in developing an HLA typing system was being hampered by the fact that each group, as it defined a novel antigen–antibody combination, was assigning their own name to that antigen. For meaningful, widespread clinical application of HLA typing, especially for sharing of donors between transplant centers, it became clear that a universally accepted nomenclature was required. Fortunately, the histocompatibility community was able to put aside their scientific rivalries and come together at the first International Histocompatibility Workshop in 1964.

![Fig. 2.4](image-url) Examples of results from the complement-dependent cytotoxicity (CDC) assay used for human leucocyte antigen (HLA) typing, cytotoxic cross-matching and CDC antibody screening. The left figure shows a negative reaction by CDC (score = 1). The cells within the wells remain small, refractile and free of vital dye. Conversely, the well on the right shows a strong positive reaction (score = 8) with larger, dye filled cells that appear black under the microscope. The vital dye, which is normally membrane impermeable, can enter the lysed cells through the membrane attack complex created through the complement cascade.
At this workshop the representatives shared information and antisera with the goal of developing a universally applicable typing system and naming convention. In many regards the kind of cooperative effort exemplified by the Histocompatibility Workshops has been a hallmark of the histocompatibility community in general, a characteristic which has helped to advance many efforts within the transplant community. As the number of transplants increased and graft survival data was analyzed, the advantage of HLA matching of donors and recipients became clear [6]. At this time the number of deceased donors also began to increase, and finding well-matched recipients for these organs was difficult. The effort to put deceased donor organs into well-matched recipients naturally led to an agreement between regional histocompatibility laboratories in cooperation with their respective transplant centers to initiate the first organ-sharing organization, the South Eastern Organ Procurement Foundation (SEOPF) [7]. SEOPF was formed to permit sharing of matched or compatible grafts between centers in the southeast portion of the United States. Later this organization grew into a nationwide system for sharing organs, namely the United Network for Organ Sharing (UNOS). SEOPF initiated a system of serum sharing for sensitized recipients known as regional organ procurement cross-match trays (ROP trays) whereby sensitized patients in the region could be cross-matched with all regional deceased donors. If the cross-match on the ROP tray was negative the organ could be shared with that patient’s transplant center. This increased the accessibility to transplant for sensitized patients and continued to be a standard of practice in many areas until quite recently. Largely, this practice has now been replaced by virtual cross-matching, but for many years preliminary cross-matching on regional trays was central to management of sensitized candidates.

Because the selection of immunosuppressive agents was limited in the early years, HLA matching assumed a central role in donor selection and increased the chances of graft and patient survival. When the histocompatibility laboratory refers to matching donors and recipients we are referring to HLA matching. ABO matching or at least ABO compatibility is absolutely required for successful transplant of solid organs in order to avoid hyperacute rejection due to the presence of natural anti-A and/or B antibodies (Fig. 2.5). Conversely, HLA mismatched organs can be transplanted without the risk of hyperacute rejection as long as there are no pre-formed anti-HLA antibodies present. In the absence of significant immunosuppression, which was not available in the early years of transplantation, HLA mismatched grafts were often lost to irreversible acute rejection. Although great strides were being made in standardizing HLA typing, it was obvious that the typing available was not sufficient to either explain or avoid many rejection reactions. We now know

<table>
<thead>
<tr>
<th>Patient ABO</th>
<th>Surface Sugar Molecules</th>
<th>Serum Antibody</th>
<th>Compatible Donors</th>
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<tbody>
<tr>
<td>O</td>
<td>None</td>
<td>anti-A, anti-B</td>
<td>O</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
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<td>O, A</td>
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<tr>
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<td>B</td>
<td>anti-A</td>
<td>O, B</td>
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<tr>
<td>AB</td>
<td>A, B</td>
<td>None</td>
<td>O, A, B, AB</td>
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Fig. 2.5 Blood group antigens are defined by carbohydrate residues found on the surface of cells. The two primary carbohydrates are the A group antigen, α-N-acetylgalactosamine, and the B group antigen, α-d-galactose. Typing for the blood group antigens is essential in transplantation as patients have antibodies to any blood group antigens not expressed on their own cells. These antibodies can lead to hyperacute rejection of the graft as the blood group antigens can also be expressed on graft tissues. Although some donor–recipient ABO combinations may be compatible but not identical, the sharing of cadaveric organs usually occurs within identical blood groups. This provides a more equitable distribution of organs and assures that no one blood group is disadvantaged.
that one reason for this was that early typing efforts were limited to detecting class I antigens. Histocompatibility laboratories were able to demonstrate that the mixed lymphocyte culture (MLC) could be used as an adjunct to serologic typing in identification of appropriate organ donors [8, 9]. The MLC identified an additional set of antigens, designated D region antigens, which elicited a strong proliferative response in some donor–recipient pairs that were previously thought to be well matched by the HLA typing that was available at that time [10, 11]. The MLC assay could help identify donors who were matched at the D region, the area which is now known to encompass the class II genes of the HLA gene complex. Use of the MLC had several significant drawbacks. First it took 5–6 days to run the assay limiting its use to living-donor transplants where there was adequate lead time to perform the testing and precluded its use in deceased donor situations where results were needed in a matter of hours as opposed to days. Second, as is now apparent, the lack of reactivity in an MLC does not always correlate well with graft survival [12]. Obviously, improved typing methods that were rapid and could reliably identify the antigens that drove the MLC were needed.

Serologic typing for class II antigens lagged behind class I antigens for a number of reasons, one of the foremost being the requirement to isolate a pure preparations of B lymphocytes. When screening for antibodies with peripheral blood mononuclear cells (PBMC), which is a mixed cell population, it became clear that not all the antibodies detected reacted with all of the cells equally. The class I antibodies were recognized first because they reacted well with all of the lymphocytes in the PBMC preparation. But over time it was noticed that there were often reactions with subpopulations of the cells. Although this low frequency cell death was at first thought to indicate the presence of a weak, low titer, antibody it was found through titration experiments that some of these antibodies were actually present at very high titers yet they failed to react with the majority of lymphocytes in the PBMC preparation. Ultimately, these antibodies were shown to be reacting with the B lymphocyte subpopulation and, as is now known, were defining HLA class II antigens. Methods were developed for the separation of T and B-cell populations to facilitate serologic testing for class II antigens, but the original process was tedious, time-consuming, and yielded limited numbers of viable B cells. Nonetheless, serologic typing for class II antigens permitted improved identification of matched grafts in a time frame that was compatible with placing deceased donor grafts [11, 13–15].

Although serologic class I and later class II typing identified what appeared to be well-matched donors, grafts continued to be lost immediately upon reperfusion, i.e., hyperacutely. It was shown by the seminal work of Patel and Teresaki that patients with positive CDC cross-matches were much more likely to lose their grafts in this fashion [16]. Following these findings, cross-matching every donor–recipient pair prior to transplant became routine. Unfortunately, as evidenced by the results in the Patel article, not all incidences of hyperacute rejection could be avoided by the methods available at that time, i.e., the basic cytotoxic cross-match. This was attributed to a lack of sensitivity in the assays and the members of the histocompatibility community worked diligently to develop methods that would improve their ability to detect relevant antibodies. These efforts led to the development of the Amos Wash and later the antihuman globulin (AHG) procedures [17]. Although anti-HLA antibodies are the primary cause of hyperacute and accelerated acute rejection, other antibodies have been shown to mediate these processes as well. In most instances these antibodies are not detectable using lymphocyte-based cross-match techniques [18–20]. Fortunately, these non-HLA antibodies are fairly uncommon and are not felt to constitute a major threat to graft survival in the majority of transplants.

Despite the best efforts of the histocompatibility laboratory to: (1) serologically type for both class I and class II; and (2) perform sensitive cross-matches, it was evident that these efforts neither guaranteed graft survival, nor extended the availability of transplant sufficiently to satisfy the demand. There simply were not...
enough well-matched kidneys available, even with national sharing. It was only with the advent of the first calcineurin inhibitor, Cyclosporin A, that transplant truly became broadly applicable not only for renal transplant but also for many other organs including the pancreas.

Where We Are Now

When cyclosporin and later tacrolimus, became available, the perceived need for HLA matching and therefore for HLA typing began to wane. Despite the fact that zero HLA-A,B,DR mismatched kidneys continue to enjoy significantly better graft survival, even in the calcineurin inhibitor era [21,22], the ability of the newer immunosuppressants to allow good graft survival for totally mismatched kidneys for the first time opened this life-altering therapy to all patients in need, whether or not they had matched donors available. With the advent of the newer immunosuppressants, the primary role of the histocompatibility laboratory in kidney or pancreas transplant began to change from one of finding HLA-matched and cross-match negative organs to one of helping the physicians assess the relative risk of any particular donor–recipient pair. Many factors have been shown to contribute to the relative risk of a transplant, including the level of sensitization of the recipient, the race and gender of the recipient, prior transplants or other sensitizing event the patient has experienced such as pregnancy or transfusions, the relationship of the donor to the recipient, the level of HLA matching, and whether the overall health of the patient allows full use of the ever-expanding collection of immunosuppressive medications [23–25]. Additionally, what constitutes risk at one transplant center may differ from risk at another center, depending upon the immunosuppressive protocols employed and the approach of the physicians. This has mandated that the histocompatibility laboratory establish a close relationship with their transplant programs so as to thoroughly understand the management philosophy of the physicians they serve.

The histocompatibility lab performs risk assessment by gathering data from a variety of assays and evaluating the information in light of established outcomes data. First, labs continue to HLA type all recipients and donors. This is important since matching at DR still supplies points in the UNOS match run system and because zero antigen mismatch sharing remains available to highly sensitized patients. Zero antigen mismatched grafts have significantly better graft survival even in the current era of immunosuppression but sharing of zero antigen mismatched organs may soon be eliminated in most cases in an effort to reduce ischemia time and increase the number of African American and other ethnic minority patients that are transplanted. Nonetheless, knowing the match grade of the donor can help in decisions regarding the use of induction and immunosuppressive protocol selection and in virtual cross-matching. Serologic typing continues to be a mainstay in many laboratories but molecular typing techniques are rapidly becoming the method of choice for identification of HLA antigens [26,27].

There are a number of reasons for the migration to molecular techniques. First, the supply of good typing grade antiserum is limited, especially for rarer antigens. This can mean that serologic typing is unable to detect some antigens, and although these antigens are usually not common in the donor population it is important to detect their presence if the recipient is sensitized to them. The UNOS matching algorithm rules out potential recipients if they have antibody to an antigen present in the donor. If antigens are missed during donor typing, then time is spent cross-matching incompatible recipients, potentially contributing to increased ischemia time and needless shipping of organs. Molecular typing, usually referred to as DNA typing, detects the presence of the HLA genes on chromosome 6 and is much less likely to miss the presence of an antigen. Second, some HLA antigens are not well expressed on the cell surface, e.g., Cw and DP, making them difficult or impossible to detect in serologic assays [1]. Antigen expression can also be markedly altered in deceased donors as a result of medications administered during donor
management and the physiological effects of brain death [26]. It should be remembered that serologic typing methods use isolated lymphocytes and the level of antigen expression on lymphocytes is not necessarily equivalent to the level of expression of the same antigen on graft tissues. Therefore, missing an antigen in serologic typing and serologic cross-matches because of weak expression of the antigen on circulating lymphocytes can result in exposure of the graft to antibody-mediated injury due to higher antigen expression on the graft tissue. With DNA typing methods the genes for the antigens are readily identifiable although detection of the gene does not guarantee cellular expression in the graft, in the vast majority of cases the presence of the gene is synonymous with antigen expression. HLA null antigens, antigens that are not expressed due to mutations in the gene, have been identified [28]. The most common null antigens have been defined and most are identifiable by the molecular assays employed today. As mentioned, deceased donor management and the processes inherent to brain death can alter the cell surface expression of HLA antigens and complicate serologic detection of those antigens. Since the genes are not altered by patient management or brain death, DNA typing is unaffected by these circumstances. Lastly, there are a number of very closely related antigens referred to as splits such as A68 and A69, B57 and 58, antigens of the A19 group, etc. Splits are antigens that were initially thought to be a single antigen, but later, as antiserum became available, were recognized to be two or more different but closely related antigens [1]. Although these splits were originally identified serologically, they can be difficult to discern in CDC assays due to cross-reactivity of antibodies to the epitopes shared between these structurally related antigens. Additionally, shared epitopes are not limited to the splits, many HLA antigens share epitopes and will exhibit antibody cross-reactivity. Rodey was able to define groups of antigens that shared epitopes and which tended to cross-react in CDC assays. These Cross-reactive Groups are referred to as CREGs (Fig. 2.6) [1]. Cross-reactivity is generally not an issue with DNA typing since primers or probes can be constructed to react specifically with the nucleotide polymorphisms that result in the amino acid differences which define the epitopes that differentiate these antigens. In fact, DNA typing can identify not only the serologically difficult splits but can actually discern HLA alleles (Fig. 2.7) [29, 30]. For example, the antigen A68 has at least 48 different alleles, all of which are identifiable by molecular typing methods, whereas only the single antigen, A68, is identifiable in serologic typing. HLA typing at the allele level, that is, identifying exactly which allele is present for each HLA antigen, is referred to as high-resolution typing. High-resolution typing is required for successful bone marrow transplantation, but at the present time matching at this level has not been shown to be advantageous for the survival of solid organ grafts. High-resolution typing may have a place in solid organ transplant in the future, however.

With the new antibody detection assays it is possible to identify allele-specific antibodies, that is, antibodies that will react with only one allelic form of an HLA antigen. Frequently these allele-specific antibodies occur in a person whose HLA type contains a different allele of the same antigen. For instance a person whose HLA type is A1, A2, B7(Bw6), B8(Bw6), DR 1, DR4, may carry the allele A*0205. If they are exposed to the antigen A*0201 they can make antibodies specifically to the epitope(s) of that allele that are not present on the A*0205 allele. Those antibodies will not react with A*0205, the self-antigen, but they will react with any cell carrying the A*0201 antigen. Presently, when listing unacceptable antigens in UNOS, only the antigen A2 can be entered since very few allele level antigens are accepted by the UNOS system. For a person with anti-A*0201 the lab can only enter A2 as the unacceptable antigen. But if a candidate has A2 in their phenotype, as in the example above, A2 cannot be entered as an unacceptable antigen in UNOS because it would appear that the person had antibody to a self-antigen, which is not possible. Therefore, in the case above, despite the fact that the patient has antibody to A*0201, the most common A2 allele, that
ANTIBODY CANNOT BE ENTERED INTO UNOS BECAUSE THE PATIENT CARRIES THE ALTERNATIVE ALLELE OF THE A2 ANTIGEN. SINCE THE A2 CANNOT BE ENTERED AS AN UNACCEPTABLE ANTIGEN IT WILL NOT BE USED TO EXCLUDE DONORS IN MATCH RUNS AND THIS CANDIDATE WILL SHOW UP ON THE MATCH RUNS FOR ALL A2 DONORS. THESE DONORS WILL HAVE TO BE CROSS-MATCHED FOR THIS CANDIDATE EVEN THOUGH THE A*0201 ALLELE WILL BE PRESENT IN MORE THAN 90% OF THE A2-POSITIVE DONORS ENCOUNTERED. ALLELE-SPECIFIC ANTIBODIES ARE NOT TERRIBLY COMMON BUT THEY ARE SEEN MORE FREQUENTLY THAN ONE MIGHT PRESUME. AS A RESULT, THERE IS MOUNTING PRESSURE ON UNOS TO ALLOW THE ENTRANCE OF ALLELE LEVEL ANTIBODIES. ENTERING ANTIBODIES AT THAT LEVEL, HOWEVER, IS MOOT UNTIL DONORS ARE TYPED AT THE ALLELE LEVEL TO ALLOW THEM TO BE RULED OUT BASED ON ALLELE LEVEL ANTIBODIES. AT PRESENT, ALLELE LEVEL, HIGH-RESOLUTION TYPING TAKES TOO LONG TO BE APPROPRIATE FOR TYPING DECEASED DONORS; AND UNTIL A RAPID, RELIABLE METHOD OF ALLELE LEVEL TYPING IS DEVISED THE UNOS SYSTEM WILL MOST LIKELY CONTINUE TO FUNCTION USING HLA ANTIGEN LEVEL TYPING FOR LISTING OF PHENOTYPES AND ANTIBODY SPECIFICITIES. AS WITH ALL TECHNOLOGIES IN THIS FIELD, PROGRESS IS BEING MADE IN HIGH-RESOLUTION TYPING AND MORE RAPID METHODS USING BEAD TECHNOLOGY AND MICROARRAY CHIPS ARE CURRENTLY ON THE HORIZON. AS THESE PROCEDURES ARE DEVELOPED AND VALIDATED WE CAN EXPECT THAT ALLELE LEVEL TYPING OF DONORS WILL BECOME MORE COMMON AND EVENTUALLY MAY BE REQUIRED BY UNOS.

Fig. 2.6  Shared epitopes of the A2 CREG group—Due to the conserved nature of the human leukocyte antigen (HLA) molecules, different HLA molecules have shared antigenic epitopes. These shared epitopes can lead to antibody cross-reactivity between multiple HLA molecules. HLA antigens that share epitopes are grouped together in CREGs (cross-reactive groups). Each HLA molecule has a unique epitope which is found only on that molecule but some epitopes, either linear or conformational, can be found on multiple HLA molecules. A patient exposed to only a single mismatched antigen, through either transplant, transfusion or pregnancy, can make antibody that reacts to other HLA molecules to which they have never been exposed if the antibody is directed toward a shared epitope.

<table>
<thead>
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<th>Reference sequence</th>
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<tr>
<td>A*0102</td>
<td>5 nucleotide differences</td>
</tr>
<tr>
<td>3 silent mutations (no amino acid change)</td>
<td></td>
</tr>
<tr>
<td>2 amino acid substitutions at residues 9 and 17</td>
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</tr>
<tr>
<td>A*0103</td>
<td>2 nucleotide differences</td>
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<tr>
<td>1 silent mutation</td>
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</tr>
<tr>
<td>1 amino acid substitution at residue 97</td>
<td></td>
</tr>
<tr>
<td>A*0104N (null)</td>
<td>1 nucleotide insertion</td>
</tr>
<tr>
<td>causes a frameshift mutation and lack of expressed HLA molecule</td>
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</table>

Fig. 2.7  Single human leukocyte antigen (HLA) antigens can have multiple alleles detected at the DNA sequence levels that can range from silent mutations that lead to no difference in expressed proteins to the complete lack of expression in other cases. This example shows four different alleles of the HLA-A1 antigen. Changes in certain nucleotides can lead to amino acid substitutions as in the case of A*0102 and A*0103 or the creation of a molecule that is no longer expressed on the cell surface as in the A*0104N (null) allele.
antibody both pre- and posttransplant [31–34]. Pretransplant antibody testing is a major risk assessment tool since previously sensitized patients are at risk for hyperacute, accelerated acute, antibody-mediated, and chronic rejection if the graft carries antigens that correspond to the recipient’s circulating antibodies. Circulating antibody is also a marker for the presence of both T and B memory cells [35–37]. The presence of HLA-specific memory cells is a useful measure of risk since the immunosuppressive agents currently at our disposal are not optimally effective at controlling memory cell activity and have limited activity against B cells and plasma cells [36–38]. Whereas memory B cells can lead to accelerated antibody production, memory T cells, once reactivated, will accelerate and augment the entire immune response. This accelerated immune response can develop before optimal levels of immunosuppression can be attained. Patients with low levels of donor-specific antibody, who are not at risk for hyperacute rejection but have evidence of memory cells, are often induced with antilymphocyte therapy to help control the memory T-cell responses until therapeutic levels of other immunosuppressive agents can be attained [39–41].

Just as in HLA typing, the CDC assay was the first assay used for detection of circulating antibody. In the CDC antibody screening assay, a panel of cells with known HLA phenotypes is used to screen patient sera. The percent of panel cells that the serum reacts with is called the panel reactive antibody or PRA. Because the HLA types of the panel cells are known, the pattern of reactivity indicates the HLA specificity of the antibodies present in the serum. However, if the antibody does not react strongly or consistently, or if there is reactivity with a high percentage of the cells, the specificity of the antibody cannot easily be determined by this technique. For instance, a serum has a PRA of 98% and can be shown in a cell-based assay to contain antibodies to A2, B7, B8, and B44. Those antibodies can explain about 80% of the reactivity, but clearly there are other antibodies present. The specificity of those additional antibodies often cannot be determined because there are so few cells left in the panel that are negative for A2, B7, B8, and B44 that no pattern of reactivity for any other antigen can be determined. This can sometimes be overcome by titering the serum and testing each titer on the same cell panel. However, this process is time-consuming and labor intensive and will often fail to identify low-titer antibodies that are masked by antibodies to more common antigens that are present in higher titer.

As the term complement-dependent cytotoxicity implies, this assay uses cytotoxicity, i.e., cell death, as the means of evaluating if antibody is present. Any assay that uses the death of live cells as a readout of reactivity is prone to being confounded by spontaneous cell death. First, human T and B lymphocytes have a fairly short life span once they are purified, and the spontaneous death of these cells over time can complicate the final analysis since it is impossible to differentiate between spontaneous cell death and antibody- and complement-mediated cell death. In these assays a dead cell is a dead cell and the cause of death is not always discernable. Additionally, cytotoxic assays often have limited sensitivity due to the nature of the antibody complement interaction. For the complement cascade to be initiated by the antibody-mediated pathway, complement factor C1q has to bind two adjacent antibody molecules and remain bound to those molecules long enough to generate an adequate amount of active complement factor 2a to sustain the necessary sequential reactions to produce a sufficient number of membrane attack complexes to compromise the integrity of the cell. If there are insufficient antibody molecules attached to the surface of any one cell, due either to a low titer of antibody in the serum or a scarcity of antigen on the cell surface, there is often too much distance between the antibody molecules for C1q to contact two antibody molecules simultaneously. In these cases the complement cascade cannot be initiated or sustained and cell death will not occur. This often leads to the erroneous assumption that little or no anti-HLA antibody is present, which would imply that there are few if any memory cells present and that a particular donor–recipient pair carries minimal risk when there is actually a substantial risk of
early antibody-mediated rejection and of memory cell responses. Misinterpretation of antibody screening can also occur if the anti-HLA antibody is of an isotype that inefficiently binds complement, such as IgG2, IgG4, or IgA [42]. In that instance a substantial quantity of antibody can be present but goes undetected in complement-dependent assays because of the absence of complement binding and activation. Over the years a number of modifications of the cytotoxic assay have been devised to overcome these shortfalls, the most effective of which is the antihuman globulin (AHG) augmented assay [1, 17]. In this assay an antibody is added that will react specifically with the anti-HLA antibody that is bound to the cell surface, forming a complex of two antibodies in close enough proximity for 

\[ \text{C}1q \text{ to bind and be activated} [43]. \]

The AHG assay permits detection of both lower concentrations of antibody bound to the cell surface and of noncomplement binding isotypes, antibodies that are referred to as cytotoxicity negative absorption positive or CYNAP antibodies [43, 44]. For many years this was the most sensitive assay available and much effort went into refining the performance of the assay in individual laboratories to give the most sensitivity possible. This is not a simple assay to perform, however, and there remains large variation in the sensitivity of the AHG assay between laboratories, depending upon both the techniques being used and the reagents employed. Even with the most sensitive AHG assay, however, antibodies continue to be missed and the degree of sensitization is often underestimated when a negative cytotoxic screen is the only result available.

Although CDC assays for antibody monitoring are still used and continue to provide useful information in risk assessment, newer more sensitive and specific methods are now available. The first innovation in detection of circulating antibody was a cell-based flow cytometric assay [45]. This assay uses a pool of isolated T lymphocytes with a variety of phenotypes such that all of the CREGs are represented. Since flow techniques are very good at detecting cross-reactive antibody reactivity, this assay can detect a wide array of preformed antibody despite the limited number of HLA antigens represented. This flow antibody screen is more sensitive than even the CDC-AHG assay and is capable of detecting low titer and CYNAP antibodies that are not detectable in CDC assays but which can produce positive flow cross-matches. This flow screening procedure was the first to have the sensitivity capable of giving a more accurate estimation of flow cross-match reactivity and proved helpful for laboratories that were using flow cross-matches to determine whether or not to proceed to transplant. One advantage of a cell-based assay using flow cytometry is that they do not rely upon cell death but detect anti-HLA antibody bound to the cell surface with a secondary antihuman IgG antibody that is fluorescently tagged. Not relying on cell death as a readout eliminates a number of the disadvantages encountered in the CDC assays. There are at least two disadvantages however, of using a pool of T cells as a screen for the presence of circulating antibody: (1) it does not yield any specificity information; and (2) it cannot detect anti-class II antibody. The histocompatibility laboratory at Emory University developed a system where multiple pools of cells were employed with the cells in each pool expressing a limited number of different CREG antigens [46]. This at least allowed determination of which CREGs needed to be avoided, but identification of individual unacceptable antigens was usually not possible. Further, since this assay is cell based it is still plagued by false-positive results due to the presence of autoantibodies or other non-HLA specific antibodies that do not need to be avoided for a successful transplant. Additionally, maintaining a supply of multiple cell pools can be time-consuming among other issues. The advantages of a screening technique with the sensitivity of the flow cytometer were obvious, but the shortcomings needed to be rectified for the technique to demonstrate its full potential. In response to this need several manufacturers have developed new antibody screening techniques collectively referred to as solid phase assays.

In solid phase assays recombinant or purified HLA antigens are attached to a plastic carrier, currently this is either an ELISA plate
or plastic beads but other solid phase platforms such as microarray chips are in development [34, 47–49]. The serum to be screened for antibody is added to the well or beads and incubated to allow any antibody present to bind to the HLA antigens. The serum is then washed off and a secondary antibody specific for human IgG is added. The use of an IgG-specific secondary antibody avoids interference from Fc autoantibody. The secondary antibody is tagged with either an enzyme or a fluorescent marker. If human anti-HLA antibody is present the secondary antibody will bind to the excess secondary antibody is washed off and the bound, tagged antibody is detected by substrate development or by fluorescence in a flow cytometer or Luminexxx machine (Fig. 2.8). These solid phase assays are more sensitive than CDC assays and are able to detect antibodies that fail to cause positive CDC results but are capable of producing a positive flow cross-match. An additional advantage of these assays is that they eliminate interference from antibody specific for cell surface molecules other than HLA molecules because the only target for the antibody on the solid substrate is the purified HLA molecules.

There are now solid phase assays available that have a single HLA antigen bound per bead or per well [50, 51]. These assays very precisely define the HLA specificity of any antibodies that are present (Fig. 2.9). This has introduced a new era of antibody identification where the antibody specificities can be determined even in sera with very high PRA values where historically it has been very difficult to determine all of the specificities due to some antibodies being masked by the presence of other antibodies. With the single antigen solid phase assays it is now possible to determine all of the specificities present with the only limitation being the absence of an antigen.

**Fig. 2.8** Assays for flow cytometric antibody screening contain plastic beads coated with human leukocyte antigen (HLA) molecules derived from cell lysates or recombinant HLA molecules. These HLA molecules are added to a percentage of the beads that represent the percent of donors in the general population that carry that HLA molecule in their phenotype. If the patient’s serum contains anti-HLA antibodies they will bind to the HLA molecules on the beads and are detected by adding a fluorescently labeled secondary antibody. If no anti-HLA antibody is present the secondary antibody will not bind and the bead population remains on the left side of the scale, equivalent to the negative control population. If anti-HLA antibody is present the secondary antibody will bind and the bead population will shift to the right. The further to the right the bead population moves the more secondary antibody bound per bead giving a semiquantitative measure of the amount of anti-HLA antibody present in the serum. The percentage of beads above the positive cutoff value gives an estimation of the percentage of donors a recipient may be expected to have a positive cross-match with. This percent is known as the panel reactive antibody (PRA).
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The Luminex platform has largely overcome the restriction on the number of antigens that can be represented in a single assay. The Luminex platform can use up to 100 different beads, each with a unique fluorescent “address” that can be identified by the machine. Each bead can carry a single HLA antigen. By testing the class I and II antigens separately 200 different antigens can be tested in two wells of a 96-well plate. Additionally, these systems not only detect the traditionally defined anti-A,B,DR and DQ antibodies, but also allow clear definition of anti Cw and DP antibodies which were not detected in the cell-based assays [52].

Unfortunately, no assay is perfect and the solid phase assays also have disadvantages. An issue that is often encountered is the presence of antibody that reacts to the solid phase carrier itself [53]. These antibodies are reacting with the

![Flow single antigen beads contain multiple beads that can be identified by a fluorescent “address.” Each “address” contains a group of beads that is coated with a single recombinant human leukocyte antigen (HLA) antigen. Addition of patient serum to these beads can determine the antibody specificities present in that serum.](image)

![Each single bead group can be investigated individually to determine the strength of the antibody specificity using a MESF conversion. In this figure, the patient has antibodies specific for HLA-A24, A25 and A26, the strongest being HLA-A25, as the fluorescent shift to the right is the greatest for the bead carrying the HLA-A25 molecules.](image)
plastic or latex present in the plate or beads and they represent no threat to a graft. However, it can be difficult to determine if these nonspecific antibodies are obscuring the detection of anti-HLA antibodies. This can be overcome to some extent by employing multiple assays that use different compounds to make the carrier. Often antibodies that will react with the carrier in one assay will not react with the carrier in other assays. The need to develop and validate multiple assays does increase the complexity of testing for each laboratory, however. A second disadvantage of the solid phase assays is that the purified HLA proteins may not reassemble fully, and these incomplete or denatured proteins are bound to the beads or plate. The problem with this is that antibody may not bind well to the denatured antigens or the antibody may bind to sites on denatured molecules that are not accessible in the intact molecules that are expressed on the surface of cells. In this case the bead reactivity is not accurately representing the reaction expected in a cell-based cross-match or a transplant. The manufacturers are aware of this issue and have been working to improve the quality of the molecules represented in their assays. A third, shortcoming of the solid phase assays as well as flow cytometric assays is that because of the IgG-specific secondary antibodies used they cannot detect IgM anti-HLA antibodies. It is extremely rare for a person to make anti-HLA antibody of only the IgM isotype, but it does occur occasionally. IgM anti-HLA antibody can activate complement and cause damage to the graft and therefore should be avoided when encountered. This is one reason why it can be advantageous to perform a CDC final cross-match with serum that is not treated to remove IgM. When the information from this CDC cross-match is combined with a thorough alloantibody and autoantibody history of the patient it is possible to identifying an IgM anti-HLA antibody. The final shortcoming is that it is difficult to determine what level of reaction represents the presence of weak antibody and what level simply represents the background reactivity of any normal serum with the beads. The cutoff between negative and positive can change from patient to patient and most certainly changes from laboratory to laboratory. Each laboratory must determine the level of sensitivity that is appropriate for identifying patients that will have a positive cross-match in their hands and would therefore be ruled out for transplant. The cutoff determined however is not an absolute and transplant centers will find that some patients with very low levels of antibody pretransplant, levels that yield negative cross-matches, will still result in memory responses and antibody-mediated rejection. It is evident that the amount of antibody present pretransplant does not directly correlate with the risk of antibody-mediated rejection. What does correlate with the risk of rejection is the reactivity of the particular patient’s immune system, but to date there is no means to measure individual reactivity pretransplant and the best we can do is to use the surrogate measure of antibody level. Despite these shortcomings the solid phase assays have greatly improved the ability to identify the presence and especially the specificity of anti-HLA antibodies.

The ability to identify antibody specificities precisely using solid phase assays has introduced a new approach to PRA assessment, and based on this UNOS implemented a new system of PRA assignment in the latter part of 2009. In this system the PRA values are calculated based on an algorithm that uses the antibody specificities entered into the system and the frequency of those antigens in the UNOS donor pool over the last several years. The introduction of the calculated PRA (CPRA) requires antibody specificities to be entered on every patient in order for their PRA to be calculated. If no specificities are entered the patient will have a PRA of zero, if several specificities are entered and the PRA calculates to >80% the patient will get the extra points allocated for the match run. For each specificity entered, the patient will automatically be eliminated from match runs on donors that carry the corresponding antigen. Depending on the philosophy of the transplant center, any HLA antigen can be listed as an antigen to avoid whether or not the patient actually has circulating antibody to that antigen. This allows previous
mismatched antigens to be listed if that is the policy of the transplant center. As long as the antigen is listed in UNOS it will be used to calculate the PRA value. This permits the CPRA to more accurately reflect what percentage of the donor population is unacceptable for each transplant candidate.

An additional advantage of the calculated PRA system is that both class I and II antibodies will be used to calculate a single PRA value. Previously, either the class I PRA or the class II PRA could be entered as the current PRA, but since no single assay gave a PRA value that reflected reactivity with both class I and II antigens a combined value was not available. Again, this was designed to allow the CPRA value to accurately represent the probability of encountering an unacceptable donor in the donor population. If two patients at different centers have the same unacceptable antigens listed they will have the exact same CPRA. The goal of using the CPRA is to produce equitable PRA values between transplant programs and it has succeeded to some extent. Variation in PRA will continue to occur depending upon what assays are used to define unacceptable antigens and upon how the assay is interpreted at a particular center.

As explained, the CDC PRA was determined by testing the reactivity of a patient’s serum with a panel of lymphocytes. The same serum could give vastly different percent PRA at different centers depending upon the cells used to make up the panel. More recently the solid phase antibody detection systems have made the panel of antigens being tested more uniform since all the laboratories buying a single manufacturer’s product will have the same panel of antigens represented. However, even using the same panel of antigens, laboratories continue to get a range of PRA values depending upon how sensitive the assay is in their hands and upon what level of reactivity is considered “positive” by the laboratory. Different labs use different positive/negative cutoffs in the solid phase assays based upon what level of antibody they find produces a positive cross-match using the cross-matching techniques available in that specific laboratory and the policies of the transplant centers they service [34]. Centers that require a flow cytometric cross-matches for final cross-matching might call weakly reactive antigens positive, whereas a center that uses an AHG final cross-match might consider the weak reactivity to be too sensitive and would therefore not call those antigens unacceptable. The choice of final cross-match techniques is usually an issue agreed upon between the laboratory and the transplant centers it services and depends on the amount of risk the physicians are willing to accept for their patients and the immunosuppressive protocols employed.

Transplant centers in some areas of the United States have complained that the racial makeup of their donor population differs markedly from the racial makeup of the national donor pool and that the CPRA does not accurately represent the probability of a positive cross-match with their local donor population. This could be disadvantaging their transplant candidates in match runs by falsely reducing their CPRA and eliminating points from their scores. As the CPRA system is employed to allocate organs the transplant community will be able to assess the merits of these complaints and inequities can be addressed by further refinement of the system. With the implementation of the CPRA system UNOS has mandated that at least one solid phase assay be used for antibody identification. CDC screening methods may be used, and these continue to provide useful information for patient management, but for sensitized patients these methods must now be augmented with results from some form of solid phase testing.

One of the most important aspects of the advent of solid phase testing is the ability to define all of the antibodies present without fear that some are being masked by other antibodies. This has permitted a whole new approach to donor allocation, the “virtual cross-match” [54–58]. The use of virtual cross-matching is not just limited to renal and renal–pancreas transplant, it can be used with transplant candidates for any organ as long as the antibodies of the candidate have been clearly defined, usually with a solid phase single antigen assay, and the HLA type of the donor is known. This approach has helped expand access to donors especially
for sensitized candidates. A virtual cross-match is performed by comparing the donor’s HLA type with the patient’s list of unacceptable antigens as defined by a solid phase assay. If an antibody has been defined to an antigen present in the donor type, then the virtual cross-match would predict a positive cross-match. If there are no antibodies identified to any of the donor antigens, then the virtual cross-match would predict a negative cross-match. If a donor is not typed for all the antigens to which the patient has antibody, then the virtual cross-match is incomplete and the outcome of a final cross-match cannot be accurately assessed. This often happens when allele-specific antibody is present or when there is antibody to HLA-DP or to the alpha chain of the DQ molecule. These antibodies can now be identified by some solid phase assays, but deceased donors are not routinely typed for these antigens. One caveat that must be remembered when using the virtual cross-match is that the prediction is only valid for the state of the patient at the point in time when the serum sample was drawn and tested for antibody. If several months have elapsed since the patient was last tested for antibody they may have experience sensitizing events in the interim and the antibody profile used for the virtual cross-match may not accurately reflect the current status of the patient. It is imperative that physicians ascertain if the patient has had any sensitizing events since the date of the last tested serum sample. Often transplant candidates fail to recall sensitizing events, or they do not fully understand what a sensitizing event is making it difficult to get an accurate history. Therefore, a virtual cross-match does not preclude the need for a final prospective cross-match. With virtual cross-matching, however, organs should only be offered to patients who are expected to have a negative cross-match and who can potentially receive the graft. This should avoid needless cross-matching of multiple patients before a cross-match negative patient is identified and needless shipping of organs occurs.

Since the primary antilymphocyte antibodies that have been shown to be relevant to graft survival are anti-HLA antibodies, the antibodies detected in the solid phase assays are antibodies of relevance. Other antibodies that react with lymphocytes in cell-based assays, such as autoantibodies, have previously been a source of confusion because they do not cause graft injury nor decrease graft survival but appear to indicate increased risk associated with a transplant because of the positive cross-match. Indeed they can obscure more dangerous anti-HLA antibody and make it extremely difficult to predict the risk associated with a particular donor–recipient pair. Unfortunately, it is not easy to determine in cell-based assays exactly what the antibody is that is causing the cell death. With the solid phase assays, using purified HLA molecules, if the antibody reacts with the antigen on the plastic then it is an IgG anti-HLA antibody and should be avoided. If, on the other hand, the antibody does not react with the HLA antigen in the solid phase assay then it is probably not necessary to avoid the transplant even if the antibody was cytotoxic in the CDC assay. The solid phase assays allow us to discern if there is anti-HLA antibody present even in the presence of autoantibody because the autoantibody will not bind to the HLA coated beads. Use of solid phase assays therefore permit increased confidence in risk assessment.

With the increased awareness of the frequency of antibody-mediated rejection (AMR) post-transplant monitoring for donor-specific antibody has also increased. The Banff criteria for diagnosis of AMR includes demonstrating the presence of circulating antibody to the donor [59, 60]. Solid phase antibody testing has proved quite helpful in posttransplant monitoring. The fact that these assays are semiquantitative permits a relative assessment of the amount of antibody present at any time point. Since there is generally more inter-assay than intra-assay variation any estimation of antibody quantity usually requires concurrent analysis of multiple samples collected over a period of time pre and posttransplant. Simultaneous analysis permits a relative estimation of whether the antibody concentration is increasing or decreasing over time. It can be helpful if serum samples are obtained periodically posttransplant and stored frozen for analysis when AMR is suspected. Solid phase testing
is also helpful for posttransplant monitoring in patients who have received induction or rescue therapy with therapeutic antibodies. Many of the therapeutic antibodies interfere with cell-based assays because the cells express the antigens targeted by the therapeutic antibody such as CD3 or CD52. Some of these therapeutic antibodies can remain in the circulation for long periods of time, for example alemtuzumab and rituximab can be detected for months after administration. Most of these antibodies do not interfere with the solid phase assays because the target antigen is not present on the bead or plate and therefore does not interfere with detection of anti-HLA antibody. It should be noted that rabbit antithymocyte globulin may contain anti-HLA antibody and the secondary antibody used in the solid phase assays, antihuman IgG, cross-reacts with rabbit immunoglobulin. Therefore, any serum samples that contain rabbit antithymocyte globulin may need to have the rabbit immunoglobulin absorbed out before testing, even in solid phase assays [61].

There is an additional solid phase assay that can be used for posttransplant monitoring. This assay, the DSA assay, is designed to specifically identify the presence of donor-specific antibody by binding HLA molecules extracted from donor cells to the beads [62, 63]. One advantage of this is that only donor-specific antibody is detected and that all donor-specific antibody is detected including allele-specific antibody where the specific HLA allele may not be represented in the other solid phase antibody screening assays.

A second approach to antibody detection is the cross-match which is typically performed prospectively for all kidney and/or pancreas transplants. Cross-matches have been used since the earliest days in the history of transplantation once it was appreciated that most hyperacute rejection could be avoided with the information a cross-match provides. There has never been a single cross-match technique that can absolutely guarantee that hyperacute rejection will not occur. There are a number of reasons for this. First, no single cross-match can detect all of the anti-HLA antibodies that can mediate hyperacute rejection hence, histocompatibility laboratories usually use a battery of cross-match techniques to increase the probability of detecting as many deleterious antibodies as possible. Second, part of the sensitivity of the cross-match technique depends upon the level of expression of the HLA antigens on the cell surface. Cells can vary in the relative level of antigen expression altering the ability of the assay to detect the presence of the antibody. Third, antibodies other than anti-HLA antibodies have been shown to produce hyperacute rejection and the lymphocytotoxin-based assays used in the histocompatibility lab cannot detect these antibodies, i.e., anti-MICA and antiendothelial cell antibodies just to name a few, and different antibody isotypes are detected in some assays and not in others. Years of experience with cross-matching has shown that the single most important indicator of risk in kidney or kidney–pancreas transplantation is a positive T-cell CDC cross-match, but a negative T-cell CDC cross-match does not necessarily indicate the absence of risk.

To be effective a cross-match has to be rapid, specific, and sensitive. The complement-dependendent lymphocytotoxicity assay, with a variety of modifications to improve sensitivity, such as added washes, extended incubation times, and AHG, has been the gold standard. However, as when using the CDC assay for antibody identification, CDC cross-matches are prone to artifacts such as spontaneous cell death, autoantibodies, and failure to activate complement. Because of its strong correlation with hyperacute rejection, however, many centers continue to use the CDC cross-match in the final decision to transplant. The CDC cross-match has been augmented in the past 20–25 years by flow cytometric cross-matching techniques [64–66]. Each of these assays has advantages and disadvantages, but when run in combination, often the strengths of one assay will compensate for the weaknesses of the other. Using the information gleaned from a combination of CDC and flow cross-matches has proved helpful in improving both patient and graft survival [67–70].

Flow cytometric cross-matches detect antibody bound to T and B lymphocytes with a fluorescently labeled secondary antibody specific for
human IgG (Fig. 2.10). This assay offers several benefits over the cytotoxic assays, including the fact that spontaneous cell death does not confound assay interpretation since dead cells can be excluded from analysis based on their light scatter properties. Additionally, the use of an IgG-specific secondary antibody eliminates interference from IgM antibodies, which are most often autoantibodies. Although IgG autoantibodies can remain an issue, this too can be eliminated to some extent by pronase treatment of the cells prior to testing. One other benefit of the flow cytometric assay is that it is semiquantitative and largely eliminates the necessity for serial dilution analysis (Fig. 2.11). As with the CDC assays the cell-based flow assays are subject to interference from many of the therapeutic antibody preparations that are used for induction or treatment of rejection.

A common complaint of the flow cytometric cross-match is that they may be too sensitive, detecting levels of antibody that do not represent the presence of a dangerous amount of antibody or a significant number of memory cells and hence do not represent a significant risk of graft injury [71–74]. The ability to discern what level of sensitivity is clinically relevant is confounded by the fact that not all human immune systems respond the same. Some people will consistently mount a vigorous immune response with a minimum of antigenic stimulation, while others can encounter repeated antigenic stimuli yet respond minimally if at all. Whereas low levels of antibody may be an indicator of very significant risk for antibody-mediated graft injury in some people, the same low levels may not be at all relevant to graft injury in another patient. To date there are no means of differentiating these two types of responders pretransplant and thus no means of predicting the risk entailed in transplanting across a weak antibody. Generally, the best practice seems to be to
CONSIDER LOW LEVELS OF ANTIBODY TO REPRESENT LIMITED RISK IN A FIRST TRANSPLANT CANDIDATE BUT TO REPRESENT A SIGNIFICANT DETERRENCE TO TRANSPLANT IN A PATIENT WHO HAS PREVIOUSLY REJECTED A GRAFT. PATIENTS WITH WEAK POSITIVE CROSS MATCHES ARE FREQUENTLY EARMARKED FOR INDUCTION THERAPY, WHICH HAS BEEN SHOWN TO HELP REDUCE THE INCIDENCE OF AMR. POSTTRANSPLANT MONITORING FOR DONOR-SPECIFIC ANTIBODY HAS PROVEN USEFUL IN FOLLOWING PATIENTS TRANSPLANTED ACROSS A POSITIVE CROSS MATCH SINCE THIS CAN IDENTIFY PATIENTS WHO ARE MOUNTING A MEMORY RESPONSE AND ALLOW EARLY INTERVENTIONS WHICH HAVE BEEN SHOWN TO BE EFFECTIVE IN IMPROVING GRAFT SURVIVAL [75–79]. LONG-TERM EFFECTS OF AN EARLY AMR HOWEVER HAVE SHOWN POORER LONG-TERM FUNCTION AND HIGHER INCIDENCES OF CHRONIC GRAFT REJECTION.

B-CELL CROSS-MATCHES HAVE PROVEN TO BE A POINT OF CONTROVERSY IN THE ASSESSMENT OF RISK IN RENAL AND PANCREAS TRANSPLANT. B CELLS ARE HELPFUL IN THAT THEY GENERALLY CARRY A HIGHER DENSITY OF HLA ANTIGEN ON THEIR SURFACE MAKING THEM MORE SENSITIVE AND CAPABLE OF DETECTING LOWER TITERS OF ANTI-HLA ANTIBODY IN THE SERUM [80]. A CLASSIC EXAMPLE OF THIS IS IN THE CASE OF A PATIENT WITH ONLY LOW TITER ANTI-CLASS I ANTIBODY. FREQUENTLY, THESE PATIENTS WILL HAVE A NEGATIVE T-CELL CDC AND/OR FLOW CROSS-MATCH WITH A POSITIVE B-CELL CROSS-MATCH. IN THESE CASES IT MIGHT BE THOUGHT THAT IF NO ANTI-CLASS II ANTIBODY HAS BEEN DETECTED IN THE PATIENT’S SERUM THE B-CELL CROSS-MATCH IS FALSE-POSITIVE AND INCONSEQUENTIAL. HOWEVER, SINCE B CELLS CARRY MORE CLASS I ANTIGENS ON A PER-CELL BASIS THAN DO T CELLS A POSITIVE B-CELL CROSS-MATCH CAN BE AN INDICATOR OF INCREASED RISK FOR AMR DUE TO ANTI-CLASS I ANTIBODY. IN ADDITION, A B-CELL CROSS-MATCH IS THE ONLY CELL-BASED ASSAY AVAILABLE THAT CAN DETECT THE PRESENCE OF DONOR-SPECIFIC ANTI-CLASS II ANTIBODY SINCE HUMAN T CELLS DO NOT EXPRESS CLASS II ANTIGENS UNDER NORMAL CIRCUMSTANCES. SINCE HYPERACUTE REJECTION DUE TO PREFORMED ANTI-CLASS II ANTIBODY HAS BEEN REPORTED THE RESULTS OF A B-CELL CROSS-MATCH PRETRANSPLANT PROVIDES IMPORTANT, RELEVANT INFORMATION [81, 82]. ALTHOUGH HLA CLASS II ANTIGEN IS NORMALLY ONLY EXPRESSED ON ANTIGEN-PRESENTING CELLS, INFLAMMATION CAN UPREGULATE ITS EXPRESSION ON MOST HUMAN CELLS INCLUDING T CELLS. GRAFT TISSUE CAN EXPRESS HLA CLASS II ANTIGEN UNDER A VARIETY OF CIRCUMSTANCES, INCLUDING FOLLOWING REPERFUSION INJURY, SURGICAL TRAUMA, REJECTION EPISODES, AND INFECTION.

UNFORTUNATELY, B-CELL CROSS-MATCHES HAVE ALSO PROVEN TO BE A SOURCE OF SOME CONFUSION AND CONSTERNATION. B CELLS HISTORICALLY WERE DIFFICULT TO ISOLATE AND THE ISOLATION TECHNIQUES USED, I.E., NYLON WOOL SEPARATION, WAS HARD ON THE CELLS, CAUSING SPONTANEOUS CELL DEATH AND

**Fig. 2.11** Standard curve for determining the molecular equivalent of soluble fluorochrome (MESP) values. Commercial beads with known quantities of fluorochrome molecules per bead are run on the flow cytometer to create a standard curve with the x-axis being the channel value read from the flow cytometer and the y-axis being the number of fluorescent molecules that correspond to that channel value. The median channel value of a population of beads or cells can then be converted to MESP using the standard curve. These values are used as a quantitative reading for antibody strength.
loss of antigen expression on the remaining live cells. This meant that the B-cell cross-match took longer and was more difficult to perform. Fortunately, this is encountered less frequently today since newer cell isolation techniques have been developed that are more rapid and less harsh on the cells. Additionally, B cells are very frequently the target of autoantibodies. Therefore, without a thorough antibody history and knowledge of the autoantibody status of the candidate the risk attributable to a positive B-cell cross-match can be difficult to determine. Even the more modern B-cell cross-match techniques such as flow cytometric cross-matches can be difficult to interpret due to the presence of Fc receptors which can nonspecifically bind immunoglobulin molecules to the B-cell surface. These issues have resulted in a longstanding debate in the literature as to the relevance of B-cell cross-match results in risk assessment. Although they can be controversial and difficult to interpret, this assay still provides unique, relevant information about the presence of donor-specific antibody. The usefulness of the B-cell cross-match is improved by the availability of the solid phase antibody detection systems and by pronase treatment of the cells used for flow cross-matches to remove the Fc receptors.

It has been known for some time that antibodies to cellular antigens other than HLA can be deleterious to graft function and survival. With the availability of C4d staining and the sensitive, specific solid phase antibody assays it may be found that the non-HLA antibodies are a more frequent cause of graft injury than previously thought. The specificity of some of these antibodies has been determined but many antibodies remain to be identified. Often these antibodies are seen in conjunction with anti-HLA antibody, making it difficult to tease out their relative effects, but there are clear instances where C4d deposition is evident in the absence of any detectable donor-specific anti-HLA antibody. Unfortunately, assays to detect many of these antibodies are not generally available at this time. There is an anti-MICA antibody detection assay available that uses the Luminex platform and an assay has recently been introduced that detects antiendothelial cell antibodies, the XM-ONE assay [83]. XM-ONE can be run rapidly using the flow cytometer similar to a lymphocyte-based flow cross-match. The XM-ONE assay isolates precursor endothelial cells from donor peripheral blood using micro-magnetic beads. Once isolated these cells are used as targets to detect antiendothelial cell antibody in patient serum. Preliminary work with this assay has shown that patients with a positive XM-ONE assay have increased incidence of rejection early posttransplant. As other antibodies to non-HLA antigens are identified and their relevance to graft survival is assessed, assays to detect these antibodies should become available.

Due to the shortage of donor organs and the difficulty in finding compatible grafts for highly sensitized patients, there have been a number of techniques developed for the removal of anti-HLA or natural anti-ABO antibody in order to permit transplant across previously positive cross-matches or of ABO incompatible grafts. Techniques employed include: (1) immunosorption, (2) splenectomy, (3) high- and low-dose IVIg, (4) plasmapheresis, (5) rituximab or other depleting antibodies, and most recently (6) bortezomib [84–90]. Usually the protocols use a combination of these techniques to lower the antibody concentration to a point considered safe. A number of transplant centers have had very good results with these protocols. These same techniques have been shown to be advantageous when used posttransplant to treat AMR. There are disadvantages to desensitization protocols, including the increased incidence of antibody-mediated rejection, increased susceptibility to infection following plasmapheresis, and the high cost of treatment. However, these treatments have permitted transplant of patients where transplant would previously have been precluded.

Whether these approaches are employed pretransplant or posttransplant to remove anti-HLA antibody the histocompatibility laboratory usually has an active role in monitoring the levels of antibody throughout treatment and often for
periods of time posttreatment to determine if antibody titers return [91–93]. The success of desensitization protocols depends on good communication between the laboratory, physicians and apheresis teams. Often solid phase assays are used to monitor antibody levels during and following desensitization procedures. In some cases high serum levels of IVIg can interfere with solid phase antibody testing, so timing of sample collection can be crucial. CDC assays for monitoring the effects of IVIg in desensitization have been developed by the histocompatibility laboratory at Cedars-Sinai when their protocol for desensitization using high-dose IVIg was being investigated [94].

Ironically, despite the fact that the role of T cells in graft rejection has been understood since early in the practice of transplantation, there are no methods for assessing the antidonor reactivity of T lymphocytes prior to transplant. The MLC was first thought to reflect the capacity of recipient T cells to respond to donor tissue, but it has been found over time that the reactivity in MLC assays does not correlate well with outcomes. This, in addition to the length of time required to perform an MLC, have made this assay of minimal use for patient management. More recently an ELISPOT assay which measures gamma interferon (IFNγ) production by T cells stimulated with allogeneic B cells has been reported by Heeger and colleagues [95–97]. The procedure uses a panel of 20 B cells from different donors, analogous to the panel of cells used for PRA analysis. The B cells in the panel are chosen to give a broad representation of HLA phenotypes. These B cells are cultured on CD40 transfected feeder cells, so the B cells are not virally transformed and there is no viral antigen present to contribute to the T-cell stimulation. Recipient T cells are stimulated for 18 h with each of the 20 different cells and IFNγ production is detected by ELISPOT. The percent of the B-cell panel that stimulates increased IFNγ production is called the panel reactive T cells (PRT). This assay is still in the research stage, but is showing promise in identifying patients who have memory T cells that could be at risk for increased reactivity posttransplant.

One other assay that measures T-cell activation is the ImmuKnow assay. This assay measures ATP production during polyclonal CD4+ T cell activation with the mitogen phytohemagglutinin (PHA). The ATP levels are divided into three zones, low, moderate, and high reactivity. Clinical correlation of the ATP production with patient status has demonstrated that patients who fall into the high activity range may be under immunosuppressed and are at increased risk for acute rejection, and patients in the low activity range are frequently experiencing viral infections [98–101]. Since the cells are stimulated with a polyclonal mitogen, there is no allospecificity to this assay and it does not yield any information of donor-specific responsiveness.

Our Future

The cooperative relationship between the renal/renal–pancreas transplant community and the histocompatibility laboratory has been a long and productive one. Laboratories, transplant physicians, and transplant surgeons have worked together consistently and diligently to evaluate the relationship between laboratory results and transplant outcomes in an effort to expand our understanding of transplantation and the alloimmune response. Laboratories have and will continue to work to develop means of assessing the risk associated with any specific donor–recipient pair and to help translate this information into clinical usage. Research is beginning to identify biomarkers that will help with the diagnosis of rejection and hopefully biomarkers of pre-transplant risk will begin to be identified. Many histocompatibility laboratories have large archives of transplant candidate and recipient sera that are being stored frozen. These sera should prove to be a resource for identity of pre and posttransplant markers. The goal of the laboratory is to serve the patients and physicians of the transplant centers they serve and to work in conjunction with the transplant community to further the success of transplantation as a whole.
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