Chapter 7
Antibody-Mediated Rejection and the Bronchiolitis Obliterans Syndrome

Henry W. Ainge-Allen and Allan R. Glanville

Abstract Why do apparently healthy, noninfected lungs fail after successful lung transplantation? Is there a unifying cause or do many insults and injuries lead to a stereotypic allograft response exacerbated by regional ischemia of the terminal bronchioles so that fibrogenesis dominates the histopathological result? These are basic questions that have troubled the lung transplant clinician since the first successful lung transplants were performed in the early 1980s. Perhaps we are closer to an understanding now, and the answer hinges, of course, on the concept of self and nonself and the recognition of the dichotomy that allows clonal expansion of B lymphocytes to mature into plasma cells that manufacture quantities of antibodies with allograft specificity ["the shock troops" of antibody-mediated rejection (AMR)]. The process is typically stealthy, however, and tends to remain clandestine until it is almost too late to undo or reverse the damage. If one does not seek, one will not find evidence that allograft damage is occurring due to AMR, and, as always, the tools that can be used to detect AMR are critical. We now have the tools, and the findings are quite overwhelming in their complexity. Therefore, some simplification is mandated. Hence, this chapter will attempt to clearly and succinctly explain how our understanding of the role and importance of antibodies to components of the pulmonary allograft has grown to the point where a seminal consensus can be reached about histopathological diagnosis that will help forge therapeutic endeavors with a novel uniformity of descriptive language, from whence adequate trials examining therapeutic efficacy will surely spring.

Keywords Antibodies • Capillaritis • Rejection • Chronic lung allograft dysfunction • Bronchiolitis obliterans syndrome

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Introduction

The nature of antibody-mediated rejection (AMR) after solid organ transplantation (SOT) remains a topic of debate among pathologists, immunologists, and clinicians and represents one of the great frontiers of research in transplantation medicine. While AMR is well recognized as a cause of acute graft loss in the immediate postoperative period, it is perhaps the ultimate cause of graft loss in the long term as well. In fact, there is a strong belief that pulmonary AMR is implicated in the pathogenesis of refractory "chronic rejection" after lung transplantation (LTX) and can manifest itself as the bronchiolitis obliterans syndrome (BOS). AMR is thought to be the major cause of late graft loss after kidney transplantation [1], and it would be naïve to surmise that the same factors would not be operational after LTX and capable of leading to chronic lung allograft dysfunction (CLAD).

AMR is driven by the humoral or B-cell arm of the immune system as opposed to T-cell-mediated rejection, which is often referred to as "cellular rejection" [2]. In AMR, the recipient's immune system recognizes the extracellular peptides on the cells of the donor organ as nonself and produces antibodies against them. The binding of these antibodies to the donor organ results in an inflammatory process that includes complement-mediated cell lysis [3] and antibody-dependent cell-mediated cytotoxicity [4]. In this chapter, our discussion surrounding AMR will focus on the antigens that elicit an antibody response and the extent to which they each contribute to graft dysfunction rather than the basic concepts of AMR.

AMR in SOT refers broadly to the formation of a circulating antibody to the donor organ; however, it has been described primarily in the setting of the formation of donor-specific antibodies (DSA) against mismatched human leukocyte antigens (HLA). While the potential for antibodies against additional targets has been raised in recent years, AMR in common parlance refers primarily to these anti-HLA DSA. The HLA represents the major histocompatibility complex (MHC) in humans, which are the most polymorphic genes known, with more than 200 alleles of class I and class II HLA genes that are codominantly expressed, such that most individuals are heterozygous at each gene locus. Six different HLA subtypes are expressed on cell surfaces, three class I (A, B, C) and three class II (Dp, Dq, Dr). With codominant expression, individuals may code for a maximum of 12 different HLA gene products [5]. As such, the probability of random complete matching of donors and recipients in the setting of lungs is diminishingly small. We will explore the various diagnostic tests utilized in the detection of anti-HLA DSA and other antibodies later in the chapter.

The effects of DSA have been documented most comprehensively in class I HLA. HLA class I molecules are constitutively expressed on all nucleated cells in the body, although to varying degrees, and hematopoietic cells express the greatest amount. HLA class II molecules are expressed constitutively on the surface of some hematopoietic cells and thymic stromal cells; however, they can be expressed by other cells following exposure to the cytokine, interferon-γ(gamma), and on bronchial epithelia [6]. Interferon-γ, an inflammatory mediator, is released from T helper

Table 7.1 Stages of antibody-mediated rejection

Stage	Circulating antibody[a]	Biopsy specimen	Graft dysfunction
Stage I	Yes	Normal	No
Stage II	Yes	Normal C4D positive	No
Stage III	Yes	Abnormal C4D positive	No
Stage IV	Yes	Abnormal C4D positive	Yes

[a]Presence of circulating antibody to human leukocyte antigen or other donor antigens

cells, cytotoxic T cells, and natural killer cells during an inflammatory response [7]. There is growing evidence that DSA against class II HLA can result in AMR, which is supported by the expression of HLA class II molecules in the donor organ. This aspect of AMR will be explored later in the chapter.

The initiation of the classical pathway of the complement cascade by antibodies at the donor organ interface is a central event in the process of AMR. This consists of a series of enzyme cleavage reactions following the binding of C1q to the antigen–antibody complex that result in pathogen opsonization and peptide-mediated local inflammation. During this process, complement breakdown products are deposited on the endothelium and on the basement membranes of inflamed tissue [3]. Most significant from the standpoint of AMR is C4d, a breakdown product of activated C4b, which is deposited in the donor graft during AMR. C4d deposition is one of the best known markers for AMR, and positive staining for C4d in a graft biopsy concurrently with the detection of a circulating DSA is considered diagnostic of AMR [2].

Staging of AMR varies slightly from organ to organ (Table 7.1). In the kidney, AMR is a well-established phenomenon, and staging follows the 2005 Banff diagnostic criteria [2]. The general principles of staging, however, remain constant. Staging is dependent on the presence or absence of clinical graft dysfunction, histopathological changes, positive C4D staining, and circulating antibody, irrespective of whether the antibody specificity is anti-HLA or to an alternate donor antigen. These criteria are summarized below.

The presence of positive C4D staining in the absence of a detectable antibody is suspicious for AMR, but this is not included within the renal diagnostic criteria [1]. However, building evidence suggests that autoantibodies may play a significant role in AMR of the pulmonary allograft.

AMR has varied clinical presentations that extend beyond the realm of chronic graft dysfunction. These presentations appear to be dependent upon the mechanism of antibody production, the strength of the antibody response, and the timing of the AMR relative to transplantation. Hyperacute rejection (within the first 24 h) can result from high titers of pre-transplant antibodies, particularly anti-HLA DSA in the so-called "sensitized patient." This process is rapidly progressive and usually

results in graft loss. Acute AMR may occur within the first week due to an anamnestic response that leads to a vigorous increase of a previously low-level or even undetectable pre-transplant antibody. Clinically, this is similar to hyperacute rejection and leads to rapid graft loss. In less severe cases, which sometimes occur in patients who were desensitized prior to transplantation, the graft is not lost, but long-term graft damage frequently occurs due to the acute event [8].

Recipients who are not pre-sensitized to donor antibodies can develop a de novo DSA response resulting in AMR. When this occurs acutely in the weeks and months after transplantation, it is typically aggressive, but it may be responsive to treatment. The development of late de novo AMR is often indolent, with a lengthy silent period that eventually manifests clinically as slowly progressive graft dysfunction [8]. It is suspected that this late-onset AMR plays a large part in chronic rejection processes and graft dysfunction. The extent to which these processes contribute to BOS after lung transplantation remains a focus for research.

There is also an emerging body of evidence to support the concept of non-HLA AMR as a potential cause of BOS. The lung allograft sustains injuries from a variety of sources that include ischemia reperfusion injury, alloimmunity, and external pathogens. Individually, collectively, and severally, these can lead to the release of inflammatory mediators and growth factors, thereby producing an environment that is conducive not only to alloimmune processes but autoimmune processes as well. While multiple autoantigens have been suggested as potential targets for this process, the two for which the strongest research evidence exists are collagen type V (Col-V) and K-α(alpha)1 tubulin. K-α1 tubulin is an antigen expressed on the surface of airway epithelial cells (AEC) [9]. Col-V is a minor collagen, intercalated within fibrils of collagen type I, and Col-V is considered a sequestered antigen in the normal lung due to its location within peribronchial and perivascular spaces. When the transplanted lung becomes inflamed, however, this antigen can be exposed and become a target for both cellular [10] and humoral immune responses [11].

AMR in Other SOT

AMR remains an area of research interest after lung transplantation and is beginning to assume a greater clinical relevance in day-to-day practice. Indeed, it is already recognized as a core clinical problem in transplantation of other solid organs. This relative paucity of data is largely due to the fact that we do not yet have large, long-term studies that focus on AMR in lung transplantation. Not surprisingly, the bulk of evidence for AMR comes from transplantation of the kidney, the organ in which it was first described. Williams et al. reported on hyperacute rejection in seven renal transplant recipients with preexisting circulating anti-HLA antibodies in 1968 [12]. Since that initial report, AMR has been shown convincingly to cause hyperacute, acute, and chronic rejection in the kidney as well as other solid organs [13]. The depth of evidence for AMR in renal transplantation relies on the numerical superiority of renal transplants that have been performed around the world. A total

of 17,682 renal transplants in the United States in 2009 alone, which was 3 times the next most common transplanted organ and more than tenfold the number of lung transplants performed over the same period [14].

Though the strongest evidence for AMR lies in the settings of hyperacute and acute rejection, we will focus our discussions on the evidence that AMR represents a cause of chronic rejection and graft dysfunction in SOT, as this provides the most accurate parallel to CLAD in lung transplantation. The first evidence for the role of AMR in chronic rejection came in 1969, when Morris et al. detected HLA antibodies in 11/29 (38 %) of patients who had rejected their renal transplant after a minimum of 2 months post-transplant [15], and this percentage steadily rose with the development of more sophisticated investigations. In 2002, Lee et al. [16] reported that HLA antibodies were detected by enzyme-linked immunosorbent assay (ELISA) both pre- and post-transplantation for all 29 recipients who subsequently developed chronic rejection. This stood in contrast to an 11 % rate of HLA antibody detection in those who did not develop chronic rejection ($n=129$, $p<0.01$). However, a significant difference in graft survival times between those who did vs. those who did not develop HLA antibodies was not observed, and similar findings have been reflected in subsequent reports [17–20]. It was noted in some studies that class II HLA antibodies in particular were present prior to the onset of chronic rejection [18, 20].

More recently, anti-HLA DSA have been utilized to predict AMR. In 2003 Worthington et al. [21] reported on a study group in which 50.9 % of patients who progressed to graft failure within the 5-year follow-up period ($n=112$) demonstrated evidence of de novo DSA by ELISA as compared to 1.6 % in the control group ($n=123$). While this was highly significant ($p<0.01$), the antibodies were not detected in 36 % until after the onset of graft failure, and these results have been supported by subsequent work [19, 22]. The detection of DSA does not necessarily represent a timely or sensitive screening method for the prediction of chronic graft dysfunction in isolation, which is potentially due to adsorption of circulating antibodies by the graft. However, when the presence of de novo DSA is combined with other criteria that define clinical AMR, the combination provides a more accurate clinical picture.

C4d staining of grafts, a recognized part of the diagnostic criteria for AMR in renal transplantation, was initially demonstrated as an independent marker for acute rejection [2, 23]. In a retrospective review of 265 patients, Nickeleit et al. [24] did not find an association between C4d staining alone and chronic rejection. Subsequent studies have supported this [25]; however, when C4d is used in conjunction with other markers like DSA or transplant glomerulopathy, it is highly predictive of graft loss. Similar results have been published in the heart transplant literature. Rodriguez et al. [26] reported in 2005 that C4d deposition was detected in 16 patients from a consecutive series of 165 recipients who underwent right ventricular endomyocardial, biopsies but only 5 of the 16 recipients went on to develop AMR as determined by the combination of immunofluorescence criteria and clinical graft dysfunction. Of these five, three had circulating DSA by flow cytometry.

In 2009 Einecke et al. [19] reported on one of the major problems with the current diagnostic criteria for AMR. While 17 of 27 kidney failures after 1 year could be attributed to AMR if defined by microcirculation changes on biopsy and anti-HLA DSA, only seven fit the current definition for clinical AMR due to the requirements for positive C4d staining ($n=173$). By multivariate analysis, C4d staining was not a significant factor. Indeed, there is a subset of recipients with DSA who develop AMR in the absence of C4d staining, and evidence indicates that C4d, though it represents an excellent marker for acute graft rejection, is less sensitive in predicting chronic rejection.

Though DSA antibodies have more of a role in the hyperacute and acute setting, it is worth briefly mentioning the impact of recipient pre-sensitization to DSA on graft loss. In 2008 Lefaucheur et al. [27] analyzed the significance of pre-sensitization with DSA prior to transplantation and found an 8-year graft survival of 67.95 % in those with preformed DSA vs. 77.3 % in those without preformed DSA ($p=0.03$). The incidence of AMR in those with pre-transplant DSA was 34.9 %, and these recipients had an 8-year graft survival of 43.6 %. Although the episodes of AMR associated with graft dysfunction occurred mainly in the acute setting with a median onset at 16 days post-transplant, the group continued to experience more graft loss out to 8 years.

Pulmonary AMR

Given that AMR represents a major cause of progressive chronic allograft dysfunction in other solid organ transplants, it seems intuitive that it plays a role after lung transplantation, where CLAD predominantly manifests as BOS. In 1998 two retrospective analyses demonstrated that the development of antibodies to HLA after transplantation correlated significantly with BOS ($p=0.02$) [28]. Work by Sundaresan et al. determined that HLA antibodies were a significant predictor of BOS by both univariate and multivariate analysis [29].

While this fits with the putative process of AMR in other SOT, at present there are only limited data on the effects of DSA as a marker of AMR after lung transplantation. No studies have a follow-up of greater than 2 years. To our knowledge, no studies in LTX have yet examined the impact of pre-sensitization with DSA. In 2010 Hachem et al. [30] reported on a protocol change at their institute in which they preemptively treated patients who developed de novo DSA with intravenous gammaglobulin and rituximab (the specifics of therapy for AMR will be discussed later in the chapter). Given the nature of the therapy, it is not surprising that they did not detect an association between the treatment arm and BOS ($n=116$), although there was an association between those who had persistent DSA after treatment and the development of BOS ($p=0.03$). Four patients who did not receive treatment due to concurrent critical illness all died within 30 days.

The strong evidence from other SOT [17, 19, 21] makes it unlikely that a prospective, placebo-controlled study will provide a direct comparison between

patients with untreated DSA and DSA negative patients due to ethical concerns. However, it is possible that studies with longer follow-up periods may elicit further information. A study by Worthington et al. in renal transplant recipients found that the mean time from antibody production to graft failure was 996.9 days [21], while the longest follow-up period in a study directly investigating DSA in LTX was 2 years by Hachem et al. [30]. The LTX literature may simply not yet have reports for which the duration of follow-up time required to demonstrate the full effects *of* de novo DSA is adequate.

One of the most salient points of evidence from other SOT is that the detection of anti-HLA DSA represents a more sensitive predictor of chronic graft dysfunction when it is used in conjunction with C4d staining of the graft [25, 26]. In 2003 Magro et al. [31] reported that C4d deposition in septal capillaries corresponded to morphological evidence of AMR as defined by septal capillary necrosis in 30 of 33 cases, with higher deposition patterns corresponding to more marked capillary necrosis and absent or limited deposition demonstrating minimal or no necrosis. Additionally, all patients with symptomatic acute rejection showed histopathological evidence compatible with AMR, and patients with BOS were found to have deposits of C4d and other immunoreactants in the bronchial wall. However, the only statistically significant finding for BOS was the deposition of C1q within the bronchial wall. They did not find any association with HLA antibodies as detected by panel-reactive antibodies (PRA), which led them to conclude that AMR after LTX was not HLA mediated. With the benefit of hindsight, it is arguable that more sensitive screens for HLA antibodies now available may have detected DSA in these cases. Alternatively, these cases may indeed have represented true non-HLA-mediated AMR.

Concurrent work by the same group [32] directly explored the involvement of humoral immunity as a potential cause of BOS. Fresh frozen tissue from 13 single-lung transplant recipients was analyzed for deposition of C1q, C4d, C5b-9, and IgG, IgM, and IgA. An indirect immunofluorescent assay was also conducted with patient serum against cytospins of the pulmonary endothelium. In each case, the tissue samples showed a microvascular injury syndrome involving the bronchial wall that was characterized by one or more of hemorrhage, fibrin deposition, and endothelial cell necrosis. Other features included bronchial epithelial and chondrocyte necrosis. The end-stage lesion was a thinned bronchial epithelial lining with mural fibrosis. Immunofluorescent analysis showed deposition of C1q, C3, C4d, C5b-9, and Ig in the bronchial epithelium, chondrocytes, basement membrane zone of the bronchial epithelium, and bronchial wall microvasculature. The indirect anti-endothelial cell antibody assay result was positive in all instances where it was tested. It was concluded that AMR may be involved in the pathogenesis of BOS and that the antigenic targets included the bronchial wall microvasculature, the bronchial epithelium, and chondrocytes.

While this intriguing body of work stands as direct evidence for an antibody-mediated process as a cause of BOS, at present there is a lack of consensus in the literature, with conflicting reports on the utility of C4d and other immunohistochemical markers in AMR. Wallace et al. [33] retrospectively stained transbronchial

biopsies ($n=68$) from recipients with acute cellular rejection, obliterative bronchiolitis, or diffuse alveolar damage for C4d and found a variable, focal, nonspecific staining pattern of C4d that was not consistent across the different diagnostic groups. Another study by the Pittsburgh group [34] reported that specific subendothelial C4d deposition was seen in 5 of 16 (31 %) patients with anti-HLA-Ab and was absent in 16 patients without anti-HLA-Ab ($p<0.05$). Because only 4 of 15 of those who developed BOS demonstrated positive C4d staining, they concluded that C4d was not a sensitive marker for BOS. With no large studies on the utility of C4d to date, this clinical question remains unanswered. Perhaps the devil is in the detail, and the variability of conclusions reflects a lack of consensus criteria for C4d staining positivity. The Pathology Council Working Group of the International Society for Heart and Lung Transplantation (ISHLT) has just released their initial consensus statement on the pathological criteria of pulmonary AMR, which should address exactly this source of confounding and allow a greater uniformity of definition [35].

There is also an increasing body of evidence to support the relevance of non-HLA antibody targets for AMR as a potential cause of BOS. A study of LTX recipients with BOS who had no detectable anti-HLA antibodies by low-PRA, cytotoxicity, or ELISA used flow cytometry to test for the presence of non-HLA antibodies directed against AEC [9]. Twelve of 36 patients with BOS had antibodies that bound to AEC, while none of the controls did. They also noted acceleration of the fibroproliferation cascade when AEC were incubated with the patient sera, which is one of the major recognized pathways that leads to chronic allograft dysfunction. The target antigen was found to be K-α(alpha)1 tubulin on Western blot analysis.

Tiriveedhi et al. [11] examined a case series of 12 LTX recipients with collagen V (Col-V) antibodies who developed BOS and reported that, antibodies to the α(alpha)1 chain of the Col-V antibody were present at the time of BOS onset in the sera of all 12 patients, while antibodies to the α(alpha)2 chain were only present in two patients. They suggested that antibodies to the α1 chain were immunodominant and could potentially represent a cause of BOS. This was further supported by the detection by immunohistochemistry of Col α1 (V) antibodies on frozen sections of biopsies taken 6 months after the onset of BOS [11]. Col-V antibodies have also been implicated as a potential cause of primary graft dysfunction [36], a known risk factor for the subsequent development of BOS [37]. The role of autoantibodies against Col-V and K-α1 tubulin as risk factors for BOS certainly requires further investigation; however, there can be little doubt that anti-HLA antibodies alone represent only a portion of the spectrum of AMR. Further support for the concept has been provided by Hagedorn et al., who found that BOS grades could be differentiated by a profile of autoantibodies binding to 28 proteins or their peptides [38]. Fukami et al. reported that animals receiving anti-MHC class I, but not control antibodies, developed marked cellular infiltration around vessels and bronchiole of lung by day 15 followed by epithelial hyperplasia, fibrosis, and occlusion of the distal airways similar to chronic rejection following human lung transplantation. Lungs of mice receiving anti-MHC class I showed increased expression of chemokines, their receptors, and growth factors and induced IL-17 as well as de novo antibodies to self-antigens, K-α1 tubulin, and collagen V [39].

Taken together, these pieces of evidence provide strong support for the notion that there is an antibody-mediated process contributing to BOS. Whether that process is driven primarily by anti-HLA antibodies or a spectrum of antigenic targets in addition to HLA molecules remains to be determined, as does the total contribution of AMR to BOS. It is likely that different individuals will have different profiles that are dependent on factors such as HLA match, history of cellular rejection, graft infection, and gastric aspiration with the response modulated by genetic polymorphisms.

Screening for DSA

There is no consensus on the frequency of screening for anti-HLA antibodies before and after LTX despite the potential risk of graft dysfunction secondary to AMR [40]. Of course, the detection of anti-HLA antibodies alone is not synonymous with the presence of DSA, which represent the centerpiece of the immunological diagnosis of AMR. Prior to the development of the new technologies such as single-antigen bead assays (Luminex testing), screening for individual DSA was impractical, and tests for the presence of HLA antibodies could only play a surrogate role. However, it is now possible to test specifically for individual HLA antibodies and thereby detect the presence of true DSA.

Complement-dependent cytotoxicity (CDC) cross-matching was one of the initial techniques used to detect clinically relevant antibodies before transplantation in order to determine the viability of the graft for a specific donor-recipient match [41]. In this technique, separated donor B- and T-cell lymphocytes are incubated with the potential recipient's serum in the presence of complement. If death of the donor cells above control levels is detected, cytotoxic antibodies are considered to be present, and the presence of these antibodies is considered a contraindication to transplant [36]. This technique is time consuming, and can only be performed in LTX when donor cells are available before retrieval, due to the importance of minimizing the ischemic time. It also has a low sensitivity compared to newer techniques, and it is unable to detect low-level antibody titers, which can contribute to graft failure [42].

One of the limitations of the CDC cross-match is that anti-HLA antibodies may be present that adsorb to the target lymphocytes but do not activate complement and cause cell lysis. These monovalent antibodies are unable to affect the high-affinity, bivalent interactions with C1q required to activate the complement cascade and cause cell lysis. The addition of goat antihuman kappa light chain immunoglobulin (IgL) reagent (AHG) to the incubating serum allows these antibodies to cause direct cell lysis [43]. Therefore, the AHG-CDC has largely replaced the classical CDC cross-match [43].

Flow cytometry cross-matching was developed as a more sensitive screen for donor-reactive antibodies. This technique involves incubation of patient serum with donor lymphocytes that are then stained with fluorochrome-conjugated secondary

antibodies that are typically anti-IgG. The presence of antibody can then be detected by the surface fluorescence of the antibodies. This allows for the detection of donor-reactive antibodies independent of complement fixation. Depending on the sample, flow cytometry can be 1–3 logs more sensitive than AHG-CDC cross-matching. Though these antibodies are present in a far lower titer, they are clinically significant. In a study of flow cytometry cross-match-positive CDC cross-match-negative kidney transplants, Piatosa et al. [44] found an absolute reduction in 5-year graft survival of 11.5 % vs. negative controls.

The panel-reactive antibody (PRA) allows for a surrogate measure of donor-reactive antibodies as part of the workup for transplant. By performing the tests outlined in our discussion of cross-matching on lymphocyte cell lines of people with known HLA types (see above), we are able to approximate the percentage of the population against whom the potential recipient has antibodies. This allows detection of people who have been hypersensitized to HLA antibodies, as may occur with pregnancy or multiple blood transfusions. The level of sensitivity depends on the number of patients whose lymphocytes are included in the panel, which varies from center to center. Shah investigated the clinical implications of pre-transplant PRA and found that graft loss was increased in the PRA-positive patients vs. PRA-negative ones with a hazard ratio of 1.01 ($p < 0.01$) [40].

Solid-phase antibody techniques are the newest development in the detection of anti-HLA antibodies. In this technique, purified HLA antibodies bound to a solid matrix (e.g., beads) are used as the substrate to which the antibodies from the patient's serum can bind. These antibodies can then be detected either through ELISA or via flow cytometry [45]. A study of PRA in kidney transplants comparing AHG-CDC with solid-phase assays by ELISA and flow cytometry found concordance of the results in 83 % of samples ($n = 264$). In the remaining 32 samples, 0 of 32 were positive by AHG-CDC, 20 of 32 were positive by ELISA, and 32 of 32 were positive by flow cytometry [46]. They concluded that flow cytometry was the most sensitive technique available, and subsequent studies have supported this finding.

The development of increasingly sensitive techniques for the detection of anti-HLA antibodies is driven, in part, by the understanding that the antibody levels detected do not necessarily correspond to their clinical effects. A study of flow PRA in kidney transplant recipients with negative AHG-CDC PRA found that those with a positive flow PRA were more likely to suffer an episode of rejection (36 %, 4/11) than those without (8 %, 3/36, $p < 0.02$) [47]. This is not to suggest that noncomplement fixing antibodies detected by flow represent an absolute contraindication to transplant. Shah's retrospective review of 10,000 LTX from 1987 to 2005 found that though a positive PRA was associated with an increased 30-day (HR, 2.6) and overall mortality (HR 1.3) on multivariate analysis, when the cohort from 1998 to 2005 was analyzed alone, the effect was not seen. They concluded that the development of more sensitive screening techniques in this era has allowed for better management of the sensitized patient [40]. The presence of positive flow PRA indicates that the patient is at an increased risk of graft dysfunction and acute AMR, and, therefore, requires closer monitoring than those with negative flow PRA to achieve the best outcomes.

Single-antigen bead flow cytometry provides the ability to detect specific anti-HLA antibodies, which, when combined with donor HLA typing, directly informs us of the presence and level of DSA. The most well-known of these is the LUMINEX single-antigen bead assay, which operates by using beads coated with known individual HLA antigens such that flow cytometry can determine the individual HLA antigens to which the recipient's antibodies are binding. Currently, the mean fluorescence intensity (MFI) and standard deviation (SD) of the cutoff between positive and negative are set at 1,000 ± 500 [48, 49]. Though the single-antigen bead LUMINEX provides a quantitative measure, the MFIs do not have a clinical impact based on their level. Seemingly low MFIs may translate into AMR. Equally important, the majority of patients pre-sensitized with DSA detected by LUMINEX do not go on to have episodes of AMR [27, 48, 49]. The findings in those who develop de novo DSA are similar [19, 21, 22].

While it is evident that screening for DSA by LUMINEX prior to transplantation is worthwhile, at present there is no consensus on appropriate post-transplant screening intervals, which is not surprising given the valid questions regarding their clinical significance. The Pathology Council of the ISHLT encourages the development of site protocols for regular DSA surveillance and biopsy [50]. At our center, we screen potential recipients as part of the transplant workup, on the night of transplant, at regular intervals after transplant, and when clinically mandated by a drop in lung function.

Diagnosis

Though the Banff reports [2, 51] have provided diagnostic criteria for AMR in kidney transplantation since 2003, it is only recently that a consensus agreement has been reached by the Pathology Council of the ISHLT with the caveat that pulmonary AMR remains an area of investigation in which there are no large unifying studies [50]. Pragmatically, it has been agreed that the diagnosis of AMR requires the "triple-test" of clinical allograft dysfunction, circulating DSA, and pathological findings.

The classical histopathological findings of AMR comprise capillary injury with neutrophilic margination, defined by the Council as neutrophilic infiltrates within the interstitial capillaries and septae in the absence of karyorrhectic changes and fibrin accumulation. The histopathological findings in general represent nonspecific patterns of inflammation and injury, which can also be produced by a broad spectrum of disorders. Histopathologically, AMR should be considered a diagnosis of exclusion, and current recommendations state that reporting should use the terms "No evidence of AMR" or "Findings suggestive of AMR," thereby informing the treating physician of the need for serological studies, if such had not been conducted prior to the biopsy.

The list of histopathological indications for performing immunostaining is diverse (Table 7.2). C4d staining is reported as strong or weak. *Strong* C4d staining

Table 7.2 Histopathological indications for immunopathological evaluation

1. Neutrophilic capillaritis
2. Neutrophilic septal margination
3. High-grade acute cellular rejection (≥A3)
4. Persistent/recurrent acute cellular rejection (any A grade)
5. Acute lung injury pattern/diffuse alveolar damage
6. High-grade lymphocytic bronchiolitis (grade B2R)
7. Persistent low-grade lymphocytic bronchiolitis (grade B1R)
8. Obliterative bronchiolitis (grade C1)
9. Arteritis in the absence of infection or cellular rejection
10. Graft dysfunction without morphological explanation
11. Any histological findings in setting of de novo DSA

demonstrates continuous linear endothelial deposition that outlines the capillary vasculature in longitudinal sectioning and creates ringed or "doughnut" shapes in cross section. *Weak* staining has a fainter pattern that appears patchy or granular.

In light of the limited published data, the ISHLT has defined C4d positivity in lung allografts as being immunoreactivity in >50 % of the interstitial capillaries, including multifocal and diffuse staining. Focal staining (<50 %) is classified as negative, but should be included in reporting, as serological studies may be indicated. Recommended follow-up for positive C4d staining is 1 month after treatment has been completed, with continued staining until there is complete resolution with negative C4d staining follow-up biopsy specimens.

C4d positivity is required to achieve a clinical diagnosis of renal AMR [51], but there is a subset of LTX recipients who have been clinically diagnosed with AMR in the past despite being C4d-negative, in light of consistent histopathological findings and in the absence of an alternative diagnosis [34]. The ISHLT Pathology Council Working Group affirmed that the definitive diagnosis of pulmonary AMR requires the combination of clinical dysfunction, circulating DSA, and C4d immunoreactivity. Certainly AMR may present as an acute illness or simply with an otherwise unexplained drop in lung function that is potentially the harbinger of BOS. Pulmonary AMR can occur at any time and should always be considered as a potential cause in the differential diagnosis of allograft dysfunction [52]. DSA can be detected using the methods discussed earlier in the chapter, but single-antigen flow cytometry (LUMINEX) is the most sensitive technology utilized for this purpose and is becoming a widely used method for DSA detection [19, 30].

Management

The basic tenet of therapy for AMR is to remove circulating DSA from the patient's serum and prevent further production of DSA, which perhaps is a lofty goal. DSA are central to the pathogenesis of AMR, and it is likely that their removal prevents

further damage to the graft. However, it should be emphasized that it is not the circulating antibody that does the damage; it is the antibody bound to the graft (a simple concept, but one best remembered). Nevertheless, the three treatment modalities in common usage in the treatment of AMR are therapeutic plasma exchange (plasmapheresis), intravenous immunoglobulin (IVIG), and the anti-CD20 monoclonal antibody, rituximab, and these are usually used in combination with each other. The ultimate therapeutic goal is maintenance of graft function, but measurements of circulating antibody are often used as a surrogate goal.

Plasmapheresis

Plasmapheresis is an extracorporeal treatment involving the removal of blood from the patient followed by separation of the plasma from the other blood products, after which the plasma is either filtered or replaced before the blood is returned to the patient's circulation. There are several forms of plasmapheresis. *Plasma exchange*, in which the plasma is discarded and substituted (usually with albumin); *double filtration plasmapheresis*, in which the separated plasma is filtered again into large and small molecular weight components with the large molecular weight component discarded and the low molecular weight component, which contains albumin but not the IgG, is returned to the circulation; and *immunoadsorption plasmapheresis*, in which the plasma is passed through an adsorption column such that antibodies are adsorbed depending on affinity for the membrane that it contains. Plasmapheresis can remove DSA from the patient's circulation more rapidly than other interventions, and it has the benefit of reducing complement levels in the blood for up to 48 h after it has been performed. One disadvantage of plasmapheresis is that it adds a level of immunosuppression that may not be desirable due to risk of infections. Also, antibody levels quickly rebound if it is used as monotherapy. Hence, it is commonly used with adjunctive therapies, particularly IVIG, which can be used in a lower dose when combined with plasmapheresis [1, 38].

Intravenous Immunoglobulin

Although high-dose IVIG (2 mg/kg IV that is often given in three divided doses on alternate days) has been recognized as an effective treatment for AMR, the mechanisms by which it induces desensitization remain unknown. There are several theories that have been proposed to explain the effect of IVIG. The benefits of IVIG were originally thought to be due to the neutralizing effects on circulating antibodies, but the benefit extends well beyond the half-life, suggesting that regulation of adaptive cellular immunity occurs. The likely mechanism for this is through the saturation of Fc receptors on the surface of a number of immune cell subsets. Some Fc receptors are known to have immunosuppressant effects, particularly via

expression of FcgRIIb, which is induced in response to IVIG and can induce B-cell apoptosis. There is also some evidence that IVIG can inhibit T-cell activation as well as the actions of monocytes and macrophages. Although immunoglobulin is known to be a potent activator of the complement cascade, new data have shown that immunoglobulins can also act as inhibitor of this cascade via binding to complement and scavenging activated complement, thereby suppressing AMR. It is likely that the mechanisms by which IVIG lowers DSA and prevents recurrence are multiple [53].

Rituximab is a monoclonal antibody to CD20; a receptor expressed on the surface of immature B lymphocytes and B memory lymphocytes. Rituximab does not work by reducing circulating DSA but by reducing long-term production of DSA. As the antibody-secreting plasma cells do not express CD20 on their surface, rituximab has an indirect influence on the production of DSA by causing apoptosis of the immature B cells, which prevents clonal expansion of the DSA-producing cell line that is causing AMR. It is also possible that additional therapeutic benefit may come from modifications of cellular immunity as well as the effect on DSA production.

Therapy can be given either as desensitization therapy prior to transplantation to prevent AMR or when an episode of AMR is detected following the development of de novo DSA post-transplant. No evidence-based, standardized protocols for the treatment of AMR currently exist for any SOT. However, there is a clear need to establish best practice, which will likely constitute IVIG or IVIG/plasmapheresis as the standard of care. The efficacy of novel therapeutics also needs to be assessed following standardization of an accepted treatment regime [54].

The 2010 retrospective trial by Hachem et al. [30] determined that there was no increased risk of BOS with preemptive treatment of de novo DSA-positive patients using a single dose of rituximab and a monthly regime of IVIG (0.5 g/kg) for at least 6 months if follow-up DSA screens were negative. Monthly treatment continued if DSA screens remained positive.

It is important to recognize that these extremely potent therapies for AMR are capable of treating for all circulating antibodies that may be causing damage to the graft. However, our impression that the treatment of declining graft function in lung transplant recipients with anti-HLA DSA leads to remission of AMR may be naive. Certainly, there are other antibodies for which we do not routinely screen that may contribute to allograft dysfunction, and perhaps it is the reduction of these antibodies that actually leads to recovery of allograft function. Autoantibodies of note include Col-V and k-α1-tubulin, and multiple antibodies directed against currently unknown antigens may prove to be extremely important mediators of AMR-associated graft dysfunction but have yet to be discovered.

Future Directions

As a lung transplant community, it appears we may finally be nearing the threshold of answering the enigma of why the transplanted lung fails even in the absence of cellular rejection or infection. Allograft rejection is, perhaps not surprisingly, due to

lack of tolerance to the graft and its components, as the lung allograft is variably challenged by the vicissitudes of constant exposure to the external environment but ultimately at the mercy of the immune system, which has had millennia to develop sophisticated responses to nonself-antigens, however presented to immune surveillance.

With this understanding, combined with an expanding technology platform, we can now look forward to offering our patients the hope of better survival and quality of life, although the cynics amongst us might reply that all we can hope to know is why the graft failed. That at least is a beginning to solving the problem of lung allograft rejection.

References

1. Takemoto SK, Zeevi A, Feng S, Colvin RB, Jordan S, Kobashigawa J, et al. National conference to assess antibody-mediated rejection in solid organ transplantation. Am J Transplant. 2004;4(7):1033–41.
2. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, et al. Banff '05 meeting report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 2007;7(3):518–26.
3. Cooper NR. The classical complement pathway: activation and regulation of the first complement component. Adv Immunol. 1985;37:151–216.
4. Lanier LL, Phillips JH. Evidence for three types of human cytotoxic lymphocyte. Immunol Today. 1986;7(5):132–4.
5. Piertney SB, Oliver MK. The evolutionary ecology of the major histocompatibility complex. Heredity. 2006;96(1):7–21.
6. Glanville AR, Tazelaar HD, Theodore J, Imoto E, Rouse RV, Baldwin JC, et al. The distribution of MHC class I and II antigens on bronchial epithelium. Am Rev Respir Dis. 1989;139(2):330–4.
7. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. Science. 1994;265(5168):106–9.
8. Montgomery RA, Cozzi E, West LJ, Warren DS. Humoral immunity and antibody-mediated rejection in solid organ transplantation. Semin Immunol. 2011;23(4):224–34.
9. Goers TA, Ramachandran S, Aloush A, Trulock E, Patterson GA, Mohanakumar T. De novo production of K-alpha1 tubulin-specific antibodies: role in chronic lung allograft rejection. J Immunol. 2008;180(7):4487–94.
10. Burlingham WJ, Love RB, Jankowska-Gan E, Haynes LD, Xu Q, Bobadilla JL, et al. IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants. J Clin Invest. 2007;117(11):3498–506.
11. Tiriveedhi V, Angaswamy N, Brand D, Weber J, Gelman AG, Hachem R, et al. A shift in the collagen V antigenic epitope leads to T helper phenotype switch and immune response to self-antigen leading to chronic lung allograft rejection. Clin Exp Immunol. 2012;167(1):158–68.
12. Williams GM, Hume DM, Hudson Jr RP, Morris PJ, Kano K, Milgrom F. "Hyperacute" renal-homograft rejection in man. N Engl J Med. 1968;279(12):611–8.
13. Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC, et al. Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. Am J Transplant. 2010;10(3):464–71.
14. (SRTR). OPaTNOaSRoTR. OPTN/SRTR 2010 Annual Data Report. Rockville, MD: Department of Health and Human Services HRaSA, Healthcare Systems Bureau, Division of Transplantation; 2011.

15. Morris PJ, Mickey MR, Singal DP, Terasaki PI. Serotyping for homotransplantation. XXII. Specificity of cytotoxic antibodies developing after renal transplantation. BMJ. 1969; 1(5646):758–9.
16. Lee P-C, Terasaki PI, Takemoto SK, Lee PH, Hung CJ, Chen YL, et al. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. Transplantation. 2002;74(8):1192–4.
17. Mauiyyedi S, Pelle PD, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin NE, et al. Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. J Am Soc Nephrol. 2001;12(3):574–82.
18. Worthington JE, Martin S, Dyer PA, Johnson RW. An association between posttransplant antibody production and renal transplant rejection. Transplant Proc. 2001;33(1–2):475–6.
19. Einecke G, Sis B, Reeve J, Mengel M, Campbell PM, Hidalgo LG, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant. 2009;9(11):2520–31.
20. Tambur AR, Bray RA, Takemoto SK, Mancini M, Costanzo MR, Kobashigawa JA, et al. Flow cytometric detection of HLA-specific antibodies as a predictor of heart allograft rejection. Transplantation. 2000;70(7):1055–9.
21. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RWG. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. Transplantation. 2003;75(7):1034–40.
22. Mao Q, Terasaki PI, Cai J, Briley K, Catrou P, Haisch C, et al. Extremely high association between appearance of HLA antibodies and failure of kidney grafts in a five-year longitudinal study. Am J Transplant. 2007;7(4):864–71.
23. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. J Am Soc Nephrol. 2002;13(1):234–41.
24. Nickeleit V, Zeiler M, Gudat F, Thiel G, Mihatsch MJ. Detection of the complement degradation product C4d in renal allografts: diagnostic and therapeutic implications. J Am Soc Nephrol. 2002;13(1):242–51.
25. Kieran N, Wang X, Perkins J, Davis C, Kendrick E, Bakthavatsalam R, et al. Combination of peritubular c4d and transplant glomerulopathy predicts late renal allograft failure. J Am Soc Nephrol. 2009;20(10):2260–8.
26. Rodriguez ER, Skojec DV, Tan CD, Zachary AA, Kasper EK, Conte JV, et al. Antibody-mediated rejection in human cardiac allografts: evaluation of immunoglobulins and complement activation products C4d and C3d as markers. Am J Transplant. 2005;5(11):2778–85.
27. Lefaucheur C, Suberbielle-Boissel C, Hill GS, Nochy D, Andrade J, Antoine C, et al. Clinical relevance of preformed HLA donor-specific antibodies in kidney transplantation. Am J Transplant. 2008;8(2):324–31.
28. Smith MA, Sundaresan S, Mohanakumar T, Trulock EP, Lynch JP, Phelan DL, et al. Effect of development of antibodies to HLA and cytomegalovirus mismatch on lung transplantation survival and development of bronchiolitis obliterans syndrome. J Thorac Cardiovasc Surg. 1998;116(5):812–20.
29. Sundaresan S, Mohanakumar T, Smith MA, Trulock EP, Lynch J, Phelan D, et al. HLA-A locus mismatches and development of antibodies to HLA after lung transplantation correlate with the development of bronchiolitis obliterans syndrome. Transplantation. 1998;65(5):648–53.
30. Hachem RR, Yusen RD, Meyers BF, Aloush AA, Mohanakumar T, Patterson GA, et al. Anti-human leukocyte antigen antibodies and preemptive antibody-directed therapy after lung transplantation. J Heart Lung Transplant. 2010;29(9):973–80.
31. Magro CM, Pope Harman A, Klinger D, Orosz C, Adams P, Waldman J, et al. Use of C4d as a diagnostic adjunct in lung allograft biopsies. Am J Transplant. 2003;3(9):1143–54.
32. Magro CM, Ross Jr P, Kelsey M, Waldman WJ, Pope-Harman A. Association of humoral immunity and bronchiolitis obliterans syndrome. Am J Transplant. 2003;3(9):1155–66.
33. Wallace WD, Reed EF, Ross D, Lassman CR, Fishbein MC. C4d staining of pulmonary allograft biopsies: an immunoperoxidase study. J Heart Lung Transplant. 2005;24(10): 1565–70.

34. Ionescu DN, Girnita AL, Zeevi A, Duquesnoy R, Pilewski J, Johnson B, et al. C4d deposition in lung allografts is associated with circulating anti-HLA alloantibody. Transpl Immunol. 2005;15(1):63–8.
35. Berry G, Burke M, Andersen C, Angelini A, Bruneval P, Calbrese F, et al. Pathology of pulmonary antibody-mediated rejection: 2012 update from the Pathology Council of the ISHLT. J Heart Lung Transplant. 2013;32(1):14–21.
36. Talbot D, Givan AL, Shenton BK, Stratton A, Proud G, Taylor RM. The relevance of a more sensitive crossmatch assay to renal transplantation. Transplantation. 1989;47(3):552–5.
37. Daud SA, Yusen RD, Meyers BF, Chakinala MM, Walter MJ, Aloush AA, et al. Impact of immediate primary lung allograft dysfunction on bronchiolitis obliterans syndrome. Am J Respir Crit Care Med. 2007;175(5):507–13.
38. Hagedorn PH, Burton CM, Carlsen J, Steinbrüchel D, Andersen CB, Sahar E, et al. Chronic rejection of a lung transplant is characterized by a profile of specific autoantibodies. Immunology. 2010;130(3):427–35.
39. Fukami N, Ramachandran S, Saini D, Walter M, Chapman W, Patterson GA, et al. Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection. J Immunol. 2009;182(1):309–18.
40. Shah AS, Nwakanma L, Simpkins C, Williams J, Chang DC, Conte JV. Pretransplant panel reactive antibodies in human lung transplantation: an analysis of over 10,000 patients. Ann Thorac Surg. 2008;85(6):1919–24.
41. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med. 1969;280(14):735–9.
42. Reinsmoen NL, Nelson K, Zeevi A. Anti-HLA antibody analysis and crossmatching in heart and lung transplantation. Transpl Immunol. 2004;13(1):63–71.
43. Fuller TC, Fuller AA, Golden M, Rodey GE. HLA alloantibodies and the mechanism of the antiglobulin-augmented lymphocytotoxicity procedure. Hum Immunol. 1997;56(1–2):94–105.
44. Piatosa B, Rubik J, Grenda R. Is positive flow cytometric cross-match a risk factor for early cadaveric kidney graft dysfunction? Transplant Proc. 2006;38(1):53–5.
45. Pajaro OE, George JF. On solid-phase antibody assays. J Heart Lung Transplant. 2010;29(11):1207–9.
46. Gebel HM, Bray RA. Sensitization and sensitivity: defining the unsensitized patient. Transplantation. 2000;69(7):1370–4.
47. Gebel HM, Bray RA, Ruth JA, Zibari GB, McDonald JC, Kahan BD, et al. Flow PRA to detect clinically relevant HLA antibodies. Transplant Proc. 2001;33(1–2):477.
48. Vlad G, Ho EK, Vasilescu ER, Colovai AI, Stokes MB, Markowitz GS, et al. Relevance of different antibody detection methods for the prediction of antibody-mediated rejection and deceased-donor kidney allograft survival. Hum Immunol. 2009;70(8):589–94.
49. Ho EK, Vasilescu ER, Colovai AI, Stokes MB, Hallar M, Markowitz GS, et al. Sensitivity, specificity and clinical relevance of different cross-matching assays in deceased-donor renal transplantation. Transpl Immunol. 2008;20(1–2):61–7.
50. Berry GJ, Burke M, Anderson C, Angelini A, Bruneval P, Calbrese F, et al. The pathology of pulmonary antibody mediated rejection: 2012 update from the Pathology Council of ISHLT. J Heart Lung Transplant. 2012;32(1):14–21.
51. Mengel M, Sis B, Haas M, Colvin RB, Halloran PF, Racusen LC, et al. Banff 2011 meeting report: new concepts in antibody-mediated rejection. Am J Transplant. 2012;12(3):563–70.
52. Glanville AR. Antibody-mediated rejection in lung transplantation: myth or reality? J Heart Lung Transplant. 2010;29(4):395–400.
53. Jordan SC, Toyoda M, Vo AA. Intravenous immunoglobulin a natural regulator of immunity and inflammation. Transplantation. 2009;88(1):1–6.
54. Archdeacon P, Chan M, Neuland C, Velidedeoglu E, Meyer J, Tracy L, et al. Summary of FDA antibody-mediated rejection workshop. Am J Transplant. 2011;11(5):896–906.