# Human Leukocyte Antigen Antibodies and Chronic Rejection: From Association to Causation

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Considerable research has established an association between human leukocyte antigen antibodies and chronic rejection. Two new major developments now provide evidence that this relationship is in fact causative. First, recent studies of *serial* serum samples of 346 kidney transplant patients from four transplant centers show that de novo antibodies, can be detected *before* rejection. Moreover, serial testing revealed that when antibodies were *not* present, 528 patient years of good function was demonstrable in 149 patients. Second, among 90 patients whose grafts chronically failed, 86% developed antibodies *before* failure. To assess the likelihood of a causal link, we applied the nine widely accepted Bradford Hill criteria and conclude that the evidence supports a causal connection between human leukocyte antigen antibodies and chronic rejection. The clinical implication is significant because we hope this review will stimulate centers to begin the one remaining task of showing that antibody *removal* will indeed *prevent* chronic failure.

**Keywords:** Chronic rejection, Kidney transplant, HLA antibody.

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Although studies have shown an association between human leukocyte antigen (HLA) and major histocompatibility complex class I chain-related gene A (MICA) antibodies and chronic rejection of transplants (*1–3*), the question arises whether antibodies *cause* chronic rejection. To argue that these antibodies cause chronic rejection, in the same sense that smoking causes lung cancer, we can examine the evidence for this relationship using the same criteria applied to support a causal link between smoking and lung cancer. The two classical descriptions of Doll and Hill (*4*, *5*) were critical in establishing the link. Nine criteria for moving from a claim of an association to one of causation were proposed by Sir Austin Bradford Hill, coauthor of the seminal papers on smoking and lung cancer (*6*): strength; consistency; specificity; temporality; biological gradient; plausibility; coherence; experiment; and analogy. We demonstrate here that most of these criteria can now be fulfilled and that HLA antibodies can operationally be considered a primary cause of chronic rejection.

## Strength of Association

Preliminary data suggesting that posttransplant antibodies are associated with poor survival first appeared in 1968 (*7*, *8*), followed by other studies in the 1970s (*9*). Eighteen such articles were reviewed in 2000 (*1*), 14 additional ones in 2003 (*2*) and a further three in 2005 (*3*). Since the 2005 review, 26 publications on posttransplant antibodies and chronic failure have emerged (*10–36*). We discuss here several striking published findings of association.

### Human Leukocyte Antigen Antibodies Are Detected After Chronic Failure

It is commonly believed today that chronic renal allograft failure results from the cumulative effects of multiple injurious factors (*37*). However, if HLA antibodies, cause most chronic failures, then, in most instances, HLA antibodies should be found at the time of failure. Indeed, this has been demonstrated by many publications. For instance, of 826 instances of failure in five centers, 96% of the patients had formed HLA antibodies (*38*). Almost all patients had antibodies, despite the fact that no effort was made to isolate different "causes" of failure among these patients waiting for a second transplant.

### Most Chronic Failures Are Preceded by Human Leukocyte Antigen Antibody Development

Assuming antibodies are present when a graft fails, if they cause failure, they should be evident, well *before* failure. Fortunately, such evidence became recently available. Four transplant centers had meticulously stored serial samples of sera from their transplant patients taken at several month intervals for as long as 12 years. The first study of this sera "gold mine" was from Dr. Miller of Miami, who analyzed 679 serial samples from 65 patients. Of 39 patients whose grafts failed, 95% had antibodies to HLA or MICA, compared with 58% of 26 patients with functioning transplants ($P<0.01$) (*39*). The more recent serial sample studies from Miami (*40*), Nagoya (*41*), Greenville (*42*), and Maastricht (*43*), are sum-

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**TABLE 1.** HLA/MICA antibodies found in serial serum samples of patients before chronic failure

| Center | Total fail | With antibody | % | Reference |
|---|---|---|---|---|
| Miami | 25 | 23 | 92 | Mizutani et al. (*40*) |
| Nagoya | 30 | 26 | 87 | Kinukawa et al. (*41*) |
| Maastricht | 12 | 7 | 58 | Van den Berg-Loonen et al. (*43*) |
| Greenville | 26 | 24 | 92 | Ozawa et al. (*42*) |
| Total | 93 | 80 | 86 | |

marized in Table 1. Each patient had a failed graft, the causes of which in a few instances were attributed to either recurrence or other nonimmunologic causes. Surprisingly, of these 93 patients 86%, had formed antibodies.

### Serial Testing of Patients Over Time Shows Good Function is Associated With no Antibodies

If antibodies cause chronic failure, we should be able to show that patients who do *not* develop antibodies, have good functional transplants. Before the serial studies, no other studies reported that long surviving patients were *consistently shown to not* have antibodies. The patients from the serial studies had been tested at frequent intervals, and at each time, neither class I nor class II HLA antibodies were found. All patients had excellent graft function. In one study, 143 patient years of good function was found (*43*), and in the other, 385 patient years of good function (*42*): a total of 528 patient years of good function in a total of 149 patients. As with findings that associate antibodies with failure, the finding that no antibodies are associated with good function strongly suggests a causal link between antibodies and graft rejection.

## Consistency of Observed Association

The above studies reported a significant association in patients receiving different organ transplants in different parts of the world. To our knowledge, only two studies found no association. One analyzed sera samples collected during acute rejection (*44*). However, a later study by the same authors using more sensitive flow and luminex methods revealed that 20 of 55 patients with donor specific antibodies (DSA) had higher rates of primary nonfunction, delayed graft function, and lower graft survival rates at 6 months than patients without antibodies (*45*). In another study of posttransplant antibodies, although no association with failures was initially observed (*46*), later tests with single antigen luminex beads showed a closer association ($P$=0.07–0.003) (*43*).

## Specificity of the Association

Although many factors are associated with chronic rejection (*37*), the association with antibodies is stronger than any other single factor. More significantly, *specific* antibodies, directed at the incompatibilities present in the donor have been shown to be associated with chronic rejection.

Antibody response can now be identified more precisely by using luminex beads with absorbed single HLA antigens (*47*). These beads can precisely identify the site on the HLA molecule (epitope) to which the antibody reacts (*48*). Early more theoretical attempts identified many epitopes (*49*), but the experimental basis for the epitopes was possible only with single antigen beads. Currently, 103 class I ABC locus (*50*) 61 class II DR (*51*), and 16 class II DQ (*52*) epitopes have been identified.

Piazza et al.'s (*53*) study of 55 patients who had rejected kidney transplants, attributed many antibody reactions after graft rejection to these epitopes. Specifically, 91% of the "multispecific" antibodies produced could be explained by a single amino acid position. This finding that antibodies are directed at an epitope explained the common earlier observation that many antibodies are produced after transplant rejection, far in excess of the mismatched "antigens" (*54*). Similar findings were described in 27 patients who developed DSA and eight who did not after rejection of kidney allografts (*55*). Among patients with DSA, 68% also had antibodies to non-DSA which could be attributed to 66 epitopes on the molecule. De novo antibodies produced after transplants can also now be shown to be directed at these epitopes (*56*). In a study of antibodies to HLA-DR, among 19 patients who developed non-DSA, 77.3% of the antibodies could be shown to react with targets sharing an amino acid sequence with the mismatched donor DR antigens (*57*). In a study of 138 patients with functioning grafts 5.1% had anti-DP antibodies, whereas 19.5% of 185 patients with rejected grafts had anti-DP antibodies ($P$<0.001) (*58*).

## Temporal Relationship of the Association: Which is the Cart and Which is the Horse?

### Serial Testing

In Dr. Rebellato's study of 493 serial serum samples from 54 kidney transplant patients, graft failure occurred in 21 of 32 patients with antibodies and 4 of 22 patients without antibodies ($P$=0.0006) (*42,56*). Among 15 patients with DSA 13 patients failed ($P$=0.000004). Subsequent to these studies, a total of 4033 serum samples from 400 patients were investigated (*40–43*). An example of serial testing on two patients are shown in Figures 1 and 2, which are representative of similar figures for 90 different patients in (*40–43*). Figure 1 shows that one patient's

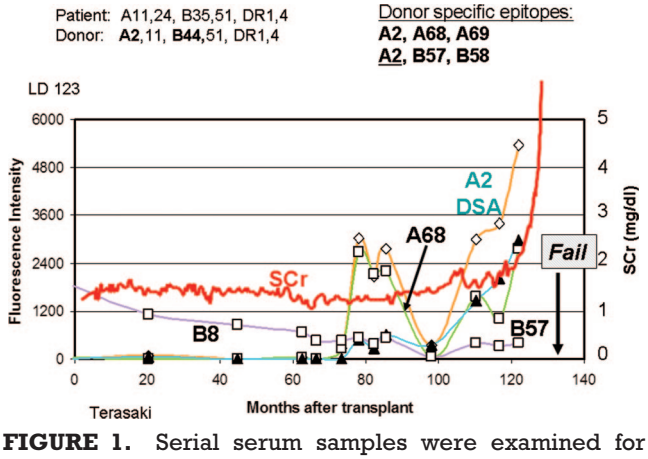


**FIGURE 1.** Serial serum samples were examined for HLA antibodies over a 10-year period after transplantation. Note that antibodies became detectable 6.5 years after transplantation, before the failure of the graft after 11 years. The antibodies were directed against the two donor specific epitopes.

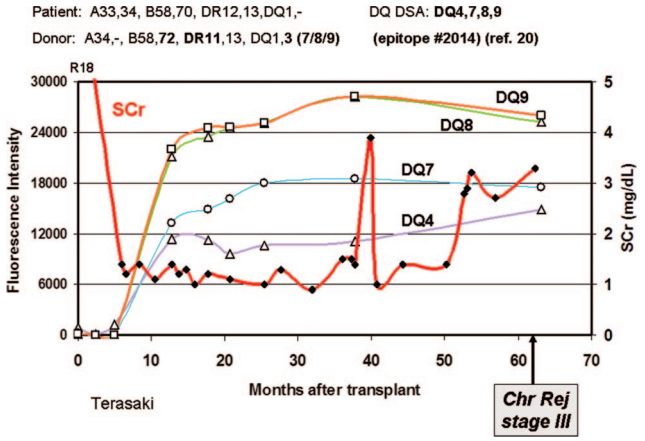


**FIGURE 2.** This patient produced antibodies against DQ4, 7, 8, and 9 after receiving a mismatched kidney for DQ3 (7, 8, 9). The epitope that the antibody had been made against was #2014 (*20*), which includes DQ4.

course of chronic rejection was clearly preceded by development of HLA antibodies (*41*). The B8 antibody was a preformed antibody, which decreased in activity with time. However, donor specific, de novo A2 antibody, increased with time, and presumably reacted against the graft, causing failure. In this instance, although the mismatch was against the A2 "antigen," the antibody produced was against the two A2 epitopes: epitope 142T/145H: A2, A68, A69 and epitope 62G: A2, B57, B58 (*48*). Thus, while A69, B57, and B58 might be considered non-DSA, clearly, they are antibodies against specificities that are in common with the A2 donor specific mismatch, and are donor specific epitope antibodies.

In a patient who developed antibodies approximately 1 year after transplantation, along with an increase in serum creatinine and subsequent failure at 5 years (*42*), the antibodies produced reacted with DQ4, 7, 8, and 9 single antigen beads (Fig. 2). Although we may have earlier thought this antibody was multispecific, the antibody reacts to a single amino acid substitution, epitope #2014 at the four possible locations indicated on the molecule (*52*).

### Presence of Antibodies With Good Function

It is a common observation and "complaint" that some patients with HLA antibodies have excellent kidney graft function. The exact frequency of this occurrence has been documented to be about 20% in studies of 2658 patients with functioning grafts (*59*) Thus, at any transplant center roughly 20% of patients would likely have antibodies and good function. According to prospective studies, when 158 patients with antibodies were followed for as long as 4 years, their graft survival was 58% as compared with 81% for 806 patients without antibodies (*59*).

Significantly, the presence of antibodies did not foretell immediate or certain graft failure. Studies by Worthington et al. (*60*) have shown that the mean time from antibody development to failure for class I antibodies was 2.7 years and 3.9 years for class II antibodies. Additionally, antibodies causing humoral rejection may not appear until as many as 13 years (*61*), or even after 26 years (*62*) posttransplant.

The reason for this long interval between antibody appearance and graft failure is the time needed for the endothelial walls of arteries to hypertrophy and close the lumen, or for the tubules to disappear because of peritubular capillary damage produced by antibodies (*63*). In both instances, defense mechanisms could be triggered as the endothelium is damaged and repair mechanisms are triggered (*64*). For instance, in a recent extensive analysis, transplant glomerulopathy was characterized by double contours of glomerular basement membranes and was a characteristic of late antibody-mediated kidney allograft rejection (*65–67*). Of 41 patients with transplant glomerulopathy HLA antibodies were seen in 73% of biopsies.

In protocol biopsies the incidence of C4d staining has been shown to be about 2% and in indication biopsies about 10% in a large multicenter trial of 551 protocol and 377 indication biopsies (*68*). One interpretation is that C4d in biopsies is transient, and at any moment, may be undetectable, because complement can be further degraded, past the C4d stage.

### Biological Gradient or Dose Response

The strength of antibodies in 39 patients whose grafts subsequently failed was markedly higher than that in the sera of 26 patients who continued to have good graft function ($P<0.0084$). In the failure group, there were nine patients with DSA with MESF (molecules of equivalent soluble fluorochrome) values which were very significantly different from the functioning transplants ($P=0.00000027$) (*69*). This highlights the importance of examining and reporting antibody strength. To express the "titer" of antibodies converting the fluorescence intensity to MESF values taken at only one dilution was shown to be adequate.

### Plausibility

Transplants are routinely performed despite HLA incompatibilities between the donor and recipient. Because HLA antigens are the main difference between donors and recipients, it is logical that the immunologic response is directed against the mismatched HLA antigens. There exist many organ specific antigens such as those specific for the heart, kidney, and liver, but these are shared among all humans. The only unique aspect of an allograft is that the donor and recipient HLA and other histocompatibility antigens are different. HLA is therefore the naturally expected target. Many would argue that *cellular* rather than *humoral* immunity against these differences causes rejection. Despite the existence of many in vitro tests, such as mixed lymphocyte culture, cell mediated lympholysis, and cytotoxic precursor assays measuring cellular immunity, using these tests, few studies of actual patients demonstrate that a cellular reaction against donor specific mismatches is associated with graft failure (*70*).

### Coherence With Laboratory Data

Many publications show that in experimental animals antibodies cause graft rejection. For example, among mice with aortic grafts, those deficient in helper T cells and humoral response did not develop neointimal concentric proliferation (*71*). In another study, intimal proliferation was induced in aortic grafts in rats by humoral antibody transfer (*72*). One very convincing finding from animal studies is that

passive transfer of antibodies causes rejection of heart grafts (*73*). From the very early experiments with skin grafts (*74*), a separate review will be required to cover the animal experiments in the intervening 40 years.

## Experimental Proof of the Thesis

### Prospective Studies of Patients With Functioning Transplants

During the 12th International Histocompatibility workshop, a multicenter prospective study was initiated to test patients with functioning kidney transplants *once* for HLA antibodies posttransplantation. The 806 patients without HLA antibodies, had a subsequent 4 year graft survival of 81%, compared with 58% for 158 patients with HLA antibodies (*59*). These results were obtained from 21 centers worldwide with many variables, but recently three large individual centers independently reported their own survival statistics. Among 512 patients followed for 1 year posttesting in Sao Paulo, 12% of antibody positive patients lost their grafts, whereas graft failure occurred in only 5.5% of those without HLA antibodies ($P=0.03$) (*75*). These results have been updated, demonstrating that at 3 years posttransplantation, patients without HLA antibodies had a 94% survival rate compared with 79% for those with HLA class II antibodies (*76*). Figure 3 provides results from independent studies from Perth (*77*), and from 1043 patients transplanted in Berlin (*78*). In these three studies the half lives of patients without HLA antibodies were similar to that of HLA identical siblings (Fig. 3), and may be considered to be relatively "safe" from rejection.

Results of frequent intervals of monitoring over a 16-year period, were quite similar (*79*). The 16-year graft survival of 375 patients without DSA was 80% compared with 14% in 73 patients who developed DSA.

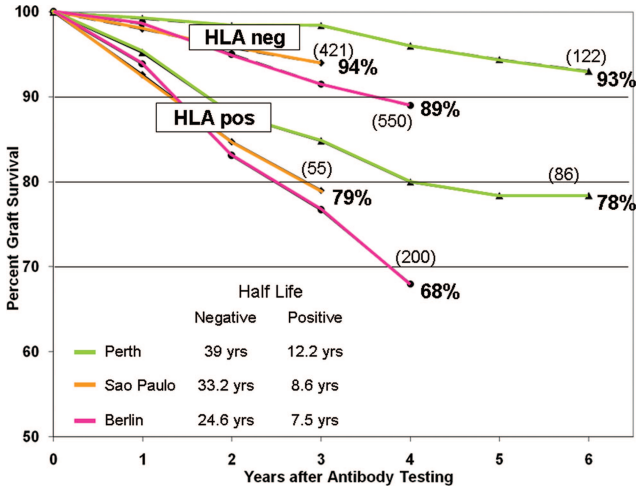


**FIGURE 3.** In three different large centers, patients with functioning kidneys for more than 6 months were tested for HLA antibodies on one occasion and then followed for up to 6 years. Patients who did not have HLA antibodies have survived at a much higher rate than those who had HLA antibodies.

### Elimination of Antibodies and Achievement of Long-term Graft Survival

The final evidence of the humoral theory would be to demonstrate that eliminating de novo antibodies resulted in long-term graft survival. This will require some time.

Studies have repeatedly shown that about 20% of patients have antibodies, despite current immunosuppressive therapy (*59*). This means that the drugs effectively suppress antibodies in 80% of patients (*80*). When de novo antibodies are first detected, the immunosuppressants could be increased or changed to other common immunosuppressants. Switching from azathioprine to mycophenolate mofetil has been shown to reduce antibody levels, and is associated with longer graft survival (*81*).

Many studies have investigated removal of presensitized antibody existing before transplantation, or antibody during acute rejection. In an extensive study of 67 patients with DSA present before transplantation and treated with plasmapheresis and intravenous immunoglobulin (IVIGG), antibodies could be removed in 53% of patients (*82*). The most important factors affecting removal were antibody strength and specificity. Among 32 patients treated with IVIGG, plasmapheresis, and CD20 antibodies, successful desensitization was directly correlated with the titer of the preformed antibody, as measured by antiglobulin crossmatch (*83*), indicating that desensitization depends entirely on treating patients under a certain threshold of antibody strength. This may also be true for the use of IVIGG (*84*).

If detected early, antibodies will likely be of lower strength and be more readily removed than if detected after many antibody producing clones have become active and high titers of antibodies are present. Relatively frequent monitoring, for example, every 4 months, may be required for timely detection of de novo antibody formation. Monitoring for antibody formation might also be the ideal means of checking for noncompliance to medication, estimated as high as 22% in adult renal patients (*85*). If no antibodies have formed, the patient may not need immunosuppression; if antibodies are present, obviously compliance should be enforced.

One important unknown is whether once removed, antibodies will again return. There is no evidence in the literature demonstrating that antibodies can be removed for long sustained periods. Possibly clones are continually regenerated, and cannot be removed permanently. If the half life of grafts is 10 years, one might estimate that clones are regenerated with a half life of 10 years.

## Analogy

HLA antibodies are now universally accepted as the *cause* of hyperacute rejection. As confirmed by many publications (*86–89*), if HLA antibodies against the donor are present, the graft will be hyperacutely rejected and destroyed within minutes. There is also a wealth of publications showing that pretransplantation HLA antibodies markedly influence graft failure in the first 3 months, from the first article in 1971 (*90*) to the most recent in 2007, which adds the closely linked MICA antibodies (*91*). Introduction of more sensitive methods of antibody detection were also shown to identify patients at risk for acute early rejections posttransplantation (*92–96*). Thus, antibodies present before transplantation

clearly affect the transplant. We believe these same antibodies are involved in chronic rejections. Unlike hyperacute rejections, however, antibodies formed posttransplantation do not immediately reject the graft, but, as noted above, damages it over time.

## Conclusion

Conventional wisdom is that cumulative damage from numerous possible factors such as ischemic injury damages the kidney leading to interstitial fibrosis and tubular atrophy (*37*). Reviews such as this by Nankivell and Chapman, can be cited as a "balanced" view of chronic rejection, emphasizing that there are many causes of chronic rejection. However, we conclude that ample evidence points to antibodies as the main cause of chronic rejection and that the criteria needed to argue for causation are essentially fulfilled. According to the humoral theory, this one *main* factor—antibody—triggers an attack on blood vessel endothelium, causing a loss of blood supply, ultimately leading to transplant glomerulopathy and tubular atrophy. But are antibodies *the* cause? Clearly, there exist other causes of failure such as drug toxicity to the kidney, recurrence of disease, senescence, so forth. However, based on the arguments above, HLA antibodies are one major cause (86%, Table 1) of chronic rejection.

If antibody removal eliminates chronic failures, antibodies can be said to conclusively cause rejection. Just as many years were needed to demonstrate that the reduction in smoking led to a lower lung cancer incidence, some time will be needed to obtain this information. In the meantime, there is now sufficient evidence to stop smoking . . . or stop antibodies produced de novo.

Stopping chronic rejection is clearly a life or death matter for heart and lung transplant patients. For kidney transplant patients, loss of a kidney results in the patient becoming highly sensitized and enduring a lengthy wait for another graft. For society, the loss of a functioning graft costs $53,757 more per year than if the graft is retained (*97*). Thus, transplant centers may wish to implement appropriate antibody monitoring procedures and institute measures to reduce antibodies when detected.

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