Circulation
Volume 91, Issue 6, 15 March 1995, Pages 1647-1654
https://doi.org/10.1161/01.CIR.91.6.1647

ARTICLE

Cardiac Allograft Vascular Disease

Relationship to Microvascular Cell Surface Markers and Inflammatory Cell Phenotypes on Endomyocardial Biopsy

Mario C. Deng, Steven Bell, Philip Huie, Fausto Pinto, Sharon A. Hunt, Edward B. Stinson, Richard Sibley, Bruce M. Hall, and Hannah A. Valantine

ABSTRACT: *Background* Cardiac allograft vascular disease is characterized by accelerated and diffuse intimal proliferation involving both the microvasculature and epicardial vessels. Because in vivo documentation of this complication is now possible with intracoronary ultrasound imaging, we can examine the relationship of intimal proliferation to markers of immunity and endothelial activation. We hypothesize that alterations of microvascular cell surface markers likely mirror changes in the epicardial vessels that may be important in the pathophysiology of intimal proliferation. *Methods and Results* Forty-three heart transplant patients were examined by intracoronary ultrasound more than 1 year after transplantation, and these images were analyzed to obtain mean intimal thickness and intimal thickness class (I through IV), calculated from the mean thickness and circumferential involvement. Right ventricular endomyocardial biopsies obtained at the time of intracoronary ultrasound were examined by immunohistochemistry to

detect microvascular expression of histocompatibility leukocyte antigen (HLA) classes I and II (HLA ABC, DR, DP, and DQ); endothelial-specific antigen detected by the monoclonal antibody E 1.5; intercellular adhesion molecules (ICAM-1); $CD4^+$ and $CD8^+$ lymphocytes and macrophages ($CD\ 14^+$). Microvascular antigen expression was graded 1 through 5 on the basis of the diffuseness of positive staining. The number of each inflammatory cell phenotype present per high-power field was counted. By ANOVA, scores for HLA DR, HLA DQ, and E1.5 expression were lower in intimal thickness classes II, III, and IV compared with class I. This inverse relationship was significant by linear regression analysis of mean intimal thickness. Inflammatory cells were not significantly correlated with intimal thickening. Rejection incidence was higher, and time since transplantation longer, in intimal thickness classes II, III, and IV compared with class I. *Conclusion* Transplant coronary artery intimal proliferation is associated with alteration of microvascular endothelial cell surface markers. These changes in cell surface antigen expression could provide the substrate for coronary artery intimal proliferation and narrowing.

Key Words: endothelium ■ antigens ■ transplantation

Cardiac allograft vascular disease (CAVD), the major limitation to long-term survival after cardiac transplantation, is characterized by diffuse intimal proliferation during the early phase of the disease and ultimately manifests as luminal stenosis of epicardial branches, occlusion of smaller vessels, and myocardial infarction.[1] Although the angiographic and histopathological

characteristics of CAVD, as opposed to naturally occurring coronary disease, have been well described,[2] [3] [4] [5] its pathophysiology is poorly understood. The introduction of intracoronary ultrasound (ICUS) imaging to study CAVD in vivo supports earlier pathological data indicating that coronary angiography underestimates the severity of the disease.[6] [7] ICUS can therefore be used in vivo to characterize early changes in the vessel wall morphology and applied to the study of pathophysiological mechanisms during the development of CAVD. We hypothesized that the donor blood vessels lying at the interface between the donor and recipient tissues bear the brunt of immune attack, and that such immune injury to the microvasculature is likely paralleled by injury to the epicardial vessels, ultimately leading to intimal proliferation.

The specific aim of the study was to determine whether epicardial coronary artery intimal hyperplasia assessed by ICUS in vivo was significantly correlated with the expression of histocompatibility leukocyte antigen (HLA) ABC, DR, DP, DQ; E 1.5–associated antigens; and intercellular adhesion molecules (ICAM-1) on arterioles, venules, and capillaries. A secondary objective was to determine whether the inflammatory cell phenotype (lymphocytes, $CD4^+$ and $CD8^+$; macrophages, $CD14^+$) was significantly correlated with intimal proliferation. The rationales for targeting these particular cell surface markers were the following: (1) Increased expression of HLA class I and class II antigens, particularly HLA DR, in the renal allograft is associated with the triggering of acute rejection[8] [9] [10] ; (2) upregulation of HLA DR expression in the coronary arteries of patients with CAVD has been reported[11] ; (3) expression of adhesion molecules is crucial to the effector arm of cell-mediated immunity; (4) recipient T lymphocytes normally express high levels of LFA-1 and LFA-2, the adhesion molecules that bind to ICAM-1 and LFA-3,

respectively, on donor endothelial target cells[12] ; (5) because cardiac vascular endothelial cells express HLA class I and class II antigens, ICAM-1, and LFA-3,[13] [14] they are potential targets as well as stimulators of rejection; (6) expression of the vascular endothelial cell–specific antigen identified by the antibody E 1.5 has been shown to decrease during rejection, possibly because of destruction of the microvasculature[15] ; and (7) $CD4^+$ and $CD8^+$ T lymphocytes and $CD14^+$ macrophages are attracted to the allograft upon cytokine activation as part of the delayed-type hypersensitivity reaction.[16]

METHODS

Patients

The study population included 43 consecutive heart transplant recipients from a pool of 168 patients surviving for more than 1 year who consented to routine annual coronary arteriography, ICUS examination, and endomyocardial biopsy. Patients who had moderate to acute rejection on the surveillance endomyocardial biopsy performed at the time of this study were excluded. Immunosuppression in all patients included prophylactic anti–T cell antibody therapy, either OKT3 or rabbit antithymocyte globulin, administered during the initial 2 weeks after transplantation. Maintenance immunosuppression was a cyclosporine-based, triple-drug regimen that included azathioprine and prednisone in all patients. Acute rejection episodes, defined as the presence of a lymphocytic infiltrate with myocyte necrosis on endomyocardial biopsy (International Society for Heart and Lung Transplantation [ISHLT] grade 2, 3a, 3b, or 4),[17] were treated with high-dose steroids, either intravenous methylprednisolone (3 g) or oral prednisone (100 mg daily for 3 days followed by gradual tapering to

baseline dose during the ensuing 30 days). The number of rejection episodes, total pulsed steroid dose, average maintenance dose of each immunosuppressive agent, and time since transplantation were calculated for each patient.

The study protocol was approved by the Human Subjects Committee of Stanford University Medical Center. Informed patient consent to routine annual catheterization including ICUS was obtained before each patient's inclusion in the study. Patient demographics are summarized in Table 1.

ICUS Examination

The ICUS protocol routinely used in our institution in this patient population has been described in detail.[6] A 4.5F intracoronary ultrasound catheter (CVIS Cardiovascular Imaging Systems) housing a 30-MHz transducer at the tip is introduced through an 8F guiding catheter after full anticoagulation with heparin. It is advanced into the left anterior descending artery to a position at which the vessel is at least 50% larger than the catheter diameter. The imaging catheter is then withdrawn, scanning the vessel and pausing at regions of interest from the midportion of the vessel to the ostium. Two sites are selected for ultrasound quantification, and their precise positions are documented by angiography. The ICUS parameters used in this study were (1) intimal thickness, deterimined by using a planimeter to measure the leading edge of the luminal echodense band and the leading edge of the media band, calculating the difference between the two measurements, and averaging the results from the two sites; and (2) the "intimal thickness class," using a semiquantitative grading scale that combines the severity of intimal thickness and the extent of circumferential involvement of the vessel, as we have previously described.[6] A modification of the intimal thickness classification was used to determine whether patients with various

severities of CAVD differed in the extent to which microvascular antigens or inflammatory cell infiltrates were detectable on endomyocardial biopsy. For the purpose of this analysis, data from patients with no, minimal, and mild intimal hyperplasia (classes I, II, and III) were combined. Fig 1A shows examples of mild intimal thickening (class I) and Fig 1B shows severe thickening (class IV).

Immunohistochemical Examination of Endomyocardial Biopsy

Endomyocardial biopsy was performed within 24 hours of ICUS. Four specimens were obtained for routine hematoxylin and eosin staining for histological grading of acute rejection.[17] Biopsies were graded as showing no rejection; mild rejection, indicating the presence of a lymphocytic infiltrate but no myocyte necrosis (ISHLT grades 1a and 1b); or moderate rejection, indicating the presence of cellular infiltration with myocyte necrosis (ISHLT grades 2, 3a, 3b, and 4). A fifth specimen for immunohistochemical studies was immediately immersed in OCT compound and snap-frozen in liquid nitrogen. Subsequently, 6-mm sections were mounted on gelatinized slides, fixed in acetone, and incubated with the first-step monoclonal antibody. First-step monoclonal antibodies to microvascular antigens included E 1.5 (source, Alan Krensky); HLA ABC (Sera Labs); HLA DR, HLA DQ, and HLA DP (Becton Dickinson); and ICAM-1 (Tim Springer). Monoclonal antibodies to inflammatory cells were RPA $T4^+$ ($CD4^+$), RPA $T8^+$ ($CD8^+$), and RPA $M1^+$ ($CD14^+$) (Bruce Hall). A four-step technique was then used for detection of specific antigen binding. This involved (1) incubating with a negative control antibody consisting of a mixture of equal parts of mouse IgG1, IgG2a, and IgG2b to a final concentration of 5 mg/mL; (2) incubating with rabbit antiserum to mouse immunoglobulin (Dako) at 1:200 dilution; (3)

incubating with swine antiserum to rabbit immunoglobulin (Dako) at 1:25 dilution; and (4) incubating with rabbit peroxidase antiperoxidase complex (Dako) at 1:50 dilution. These steps were followed by rinsing, washing in PBS, applying substrate solution (Sigma), and rinsing. Finally, sections were counterstained with hematoxylin.

Using this immunoperoxidase method, a positive signal is identified by a dark brown color, as shown in Fig 2A and 2B. Sections were graded semiquantitatively for the fraction of the microvasculature staining positively for microvascular antigens by reference to a negative control section that was from the same biopsy tissue but from which the primary antibody had been omitted during preparation of the sections (Fig 2C). Grading of the microvasculature was based on a scale of 0 through 5, assessing the fraction of vasculature staining on the section using a grid (0, <5%; 1, 5% to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, 75% to 95%; and 5, >95% of positive staining on the section). In 50% of sections, larger arteries and veins, distinct from arterioles, venules, and capillaries, were identified. In these cases, vessels were graded individually, on the basis of the extent of circumferential staining (0, <5%; 1, 5% to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, 75% to 95%; and 5, >95% of the vessel). Because the staining pattern in these larger vessels was always identical to that in the arterioles, venules, and capillaries, as demonstrated in Fig 2, the scores were combined to obtain a mean score for the expression of each antigen on a given section. Inflammatory cells were counted using a grid counter and expressed as the number per high-power field. Reproducibility of microvascular antigen grading and cell counts were assessed by comparison of results from two independent observers, both of whom were blinded to all clinical data on the patients.

Statistical Analysis

ANOVA was used to determine whether patients with various classes of intimal hyperplasia differed significantly with respect to the immunohistochemical markers studied. The difference in antigen expression in patients with no, minimal, or mild average intimal thickness compared with that in patients with moderate or severe average intimal thickening (>0.35 mm) was analyzed for statistical significance using ANOVA. The relationship between average intimal thickness and antigen expression was also examined using a linear regression model. Because we have previously reported a significant association of CAVD with time since transplantation, rejection incidence, and pulsed and maintenance steroid dose,[18] we performed an analysis to confirm a similar association in the current cohort of patients and to examine the relationship of these clinical factors with microvascular antigen expression and inflammatory cell counts. For each of these statistical methods, $P < .05$ was considered to be significant. Interobserver and intraobserver variability for ICUS measurements, microvascular antigen grading, and inflammatory cell counts were determined from the percent standard error and correlation coefficients between two measurements.

RESULTS

Intimal Proliferation by ICUS

The distribution of intimal thickness measured in 43 heart transplant patients is displayed in Table 2. Of 43 patients studied, 33 had either no, minimal or mild intimal thickening, while 10 had moderate or severe thickening. Time since transplantation within these groups is also shown for comparison in Table 2. As previously reported, there was a trend towards increasing intimal proliferation with time. Time since transplant was significantly longer in patients with the most

severe intimal proliferation (>0.45 mm) compared with patients with no, minimal, or mild intimal hyperplasia (<0.26 mm). However, the differences between the other groups in terms of time since transplantation were not statistically significant. Fig 1A is an example of an ICUS image of minimal intimal thickness (class I); Fig 1B shows severe intimal thickening (class IV).

Immunohistochemistry

Expression of HLA AntigensMean scores (±SEM) for HLA antigens were as follows: ABC, 3.7±1.00; DR, 2.3±0.84; DP, 2.4±0.82; and DQ, 1.4±0.15 (Table 2). The distribution of HLA ABC was generally greater than those of the class II antigens HLA DR, HLA DP, and HLA DQ. HLA ABC was present in all biopsies and was graded >3 (distribution of >75% on the sections) in 80% of the biopsies examined. The scores for all three HLA class II antigens were significantly less than that of HLA class I, and within HLA class II, the scores for the three phenotypes were in the order DR>DP>DQ. Using linear regression analysis to compare HLA expression with intimal proliferation (Table 3), there was an inverse relationship between intimal thickness and expression of HLA DR and HLA DQ; a similar, though nonsignificant, relationship with HLA DP was also demonstrated. No correlation was demonstrated with HLA ABC expression. Examples of HLA DR in patients with mild intimal hyperplasia are shown in Fig 2A and Fig 2B.

Because of the relationship between time since transplantation and CAVD, we examined the correlation of HLA antigens with time since transplantation. Results indicate that there was no correlation between HLA antigen expression and time since transplantation (Table 3). We also examined the relationship between HLA antigen expression and incidence of rejection; HLA DP was inversely

correlated with rejection incidence, but other class II and class I antigens showed no correlation.

Expression of Endothelial-Specific Antigen E 1.5 The endothelial-specific antigen E 1.5 was less diffusely expressed on endomyocardial biopsies than were the HLA antigens (Table 2). The mean grade for the group as a whole was 1.07±0.90, indicating that on average E 1.5 was expressed in 5% to 25% of each biopsy section examined. The range of grades, however, was 0 to 3, indicating that in some instances the expression was distributed in up to 75% of the section under examination. E 1.5 grading in biopsies from patients with intimal thickening class II, III, or IV was significantly less than that in biopsies from patients with intimal thickening class I. By linear regression analysis this inverse relationship was significant (r=−.59, P<.001 [Table 3]). Because of the relationship between time since transplantation and CAVD, we examined the correlation of E 1.5 with time since transplantation. Results showed no significant correlation (Table 3). However, E 1.5 expression was inversely correlated with rejection incidence (r=−.4, P<.01).

Expression of ICAM-1 The mean score for ICAM-1 in the group as a whole was 2.30±0.81, with a range of 1 through 3 (Table 2). This indicates that ICAM-1 expression was detectable in all biopsies, and was distributed in up to 75% of the sections from some patients. However, ICAM-1 expression was not significantly different in patients with intimal thickening class II, III, or IV compared with those with intimal thickening class I. Regression analysis likewise revealed no correlation of intimal thickening with ICAM-1 expression. No significant correlation with time since transplantation was discernible

(Table 3). However, ICAM-1 was inversely correlated with rejection incidence (r=−.4, P<.02).

Expression of Inflammatory Cell Phenotypes Although a primary exclusion criterion for the study was evidence of acute rejection (ie, a cellular infiltrate and myocyte necrosis on hematoxylin and eosin–stained sections) on concurrent surveillance endomyocardial biopsy, examination of the specimens obtained for immunohistochemistry revealed the presence of T lymphocytes and macrophages in some instances. However, these counts were generally low: RPA $T4^+$ and RPA $T8^+$ lymphocytes, 2.1±1.0 and 1.3±1.2 per high-power field, respectively; RPA $M1^+$ positive cells, 2.7±1.5 per high-power field.

There was no correlation between inflammatory cell counts and intimal thickness (Table 3); however, RPA $T4^+$ cell counts were inversely correlated with rejection incidence but not time since transplantation (Table 3).

Correlation of Immunohistochemical Markers With Immunosuppression RPA $T4^+$ and RPA $T8^+$ cell counts were inversely correlated with pulsed steroid dose but positively correlated with average daily maintenance prednisone dose (Table 4). HLA DQ and ICAM-1 were inversely correlated with pulsed steroid dose but positively correlated with average daily maintenance prednisone dose. No correlation of immunosuppression with HLA ABC, HLA DR, HLA DP, or E 1.5 was demonstrated. Average daily cyclosporine dose was not significantly correlated with any of the immunohistochemical markers.

Relationship of Inflammatory Cells to Microvascular Antigens

RPA $T4^+$ and RPA $T8^+$ counts were positively correlated with HLA ABC (respectively, r=.4, P<.02 and r=.3, P<.03) and HLA DP (r=.4, P=.01 and r=.4, P<.01); RPA $T4^+$ was also positively correlated with HLA DQ, but RPA $T8^+$ was not (r=.3, P<.05 and r=.2, P=NS). No correlation with HLA DR, ICAM-1, or E 1.5 was demonstrated.

Relationship of Rejection Incidence to Intimal Thickness, Immunohistochemical Markers, Time Since Transplantation, and Immunosuppression

As previously reported, there was a significant correlation between intimal thickness and number of moderate acute rejection episodes (r=.53, P=.0003). By ANOVA, the incidence of rejection was significantly higher in patients with intimal thickness classes II, III, and IV than in those with intimal thickness class I (Table 2). Rejection incidence was inversely correlated with RPA $T4^+$ cells and with HLA DP, E 1.5, and ICAM-1 expression (Table 3). There was no significant correlation between rejection incidence and time since transplantation (r=.27, P=.08). Rejection incidence was positively correlated with total pulsed steroid dose (r=.6, P<.001) and average maintenance prednisone dose (r=.4, P≤.05) but not with cyclosporine dose.

Relationship of Immunohistochemical Markers to Patient Demographics

None of the microvascular or inflammatory cell surface markers were correlated with gender, donor or recipient age, or number of HLA mismatches at the A, B, or C loci.

Reproducibility of ICUS and Immunohistochemistry Measurements

The interobserver variability for intimal thickness measurements was 5.6%, and for grading of immunohistochemical markers it ranged from 4.5% to 7.2%. For immunohistochemistry the interobserver variability was between 4.5% and 7.2%.

DISCUSSION

We have previously described the spectrum of coronary artery vessel wall morphology seen by ICUS examination in heart transplant recipients.[6 7] The current study was undertaken to further our understanding of the effect of the immune response on the pathophysiology of coronary artery intimal proliferation. Specifically, we aimed to determine whether alterations in vascular endothelial cell surface markers on endomyocardial biopsy, were correlated with intimal hyperplasia of epicardial arteries. Because coronary arteries cannot be readily biopsied, we hypothesized that examination of vessels seen on endomyocardial biopsies might reveal changes in conduit vessels, because there are many antigens in common. To the best of our knowledge, this is the first study examining the correlation of coronary artery intimal hyperplasia in vivo with microvascular cell surface markers and inflammatory cell phenotypes. Laboratory data support our hypothesis that immune injury directed at vascular endothelial cell surface antigens is an important initiating event for CAVD. This response leads to a cascade of events involving activated lymphocytes and the release of several cytokines, which are potent initiators of vascular smooth muscle cell and extracellular matrix proliferation.[19 20 21 22 23 24 25 26 27]

In this study we observed a significant inverse correlation between the expression of some microvascular cell surface markers and intimal hyperplasia. No significant correlation of intimal

proliferation with inflammatory cells or ICAM-1 was demonstrated. One possible explanation is that cell surface antigens expressed by the macrovasculature do not reflect those expressed by the conduit vessels. This might be particularly true for HLA class II antigens, which are thought to have low levels of constitutive expression that nonetheless are upregulated after transplantation. Our preliminary unpublished data examining HLA expression in coronary arteries in parallel with the microvasculature from autopsy specimens would suggest that in the case of the transplanted heart, HLA class II expression in the microvasculature is paralleled by expression in the conduit vessels. Furthermore, Billingham and coworkers (Russell et al[28]) have reported the concomitant involvement of microvasculature with CAVD in parallel with the epicardial vessels. Whether these structural changes are mirrored by similar changes in cell surface antigen expression requires further investigation.

In the present study, HLA DR and HLA DQ were inversely correlated with intimal hyperplasia, ie, patients with the most severe intimal hyperplasia had the least expression of HLA DR and HLA DQ on endomyocardial biopsy. There has been considerable controversy about the expression of HLA class II antigens as markers of acute rejection.[29 30] Although it is well accepted that HLA class I antigens are constitutively expressed in human vascular endothelial cells, class II antigens have a low level of expression that appears to be upregulated after transplantation. Indeed, some results suggest that HLA DR expression may be increased during acute rejection episodes. Furthermore, Salomon et al[11] have documented upregulation of HLA DR in the coronary arteries of patients with CAVD that may be explained by a chronic immune response. Despite acute rejection being an exclusion criterion in the current study, HLA class II antigens were widely expressed, but the fraction

of microvasculature expressing HLA class II antigens was inversely correlated with intimal hyperplasia. This observation would suggest that microvascular expression of HLA class II antigens by vascular endothelial cells may differ during acute rejection compared with their expression chronically or when there is CAVD. In the latter instance, their decreased expression is consistent with downregulation of the specific cell surface antigen, which may be mediated through functional rather than structural changes of the vascular endothelial cell. Downregulation of microvascular endothelial cell surface markers was further reflected by the decreased expression of the endothelial-specific antigen E 1.5 in patients with the most severe intimal hyperplasia. A similar observation has also been reported in patients with transplanted kidneys that were removed because of chronic rejection.[15] As in the current study, the investigators reported diminished HLA class II antigens as well as diminished E 1.5 antigens in peritubular capillaries. The relationship of microvascular changes to those occurring in the epicardial vessels cannot be ascertained from the current study, and warrants further investigation.

Another possible explanation for the inverse correlation of HLA class II antigens and E 1.5 with intimal proliferation is that this is a circumstantial association dependent on time since transplantation, because the alloimmune response diminishes with time. The evidence against this explanation is that no significant correlation of HLA antigens with time since transplantation was discernible. Furthermore, expression of E 1.5, an endothelial-specific antigen, also showed an inverse correlation with intimal hyperplasia. Because downregulation of this endothelial antigen is a marker associated with allograft dysfunction even in the absence of an inflammatory cell infiltration,[31] its decreased expression in association with intimal

hyperplasia in the present study very likely reflects microvascular injury.

In the present study there was no association of ICAM-1 expression with intimal hyperplasia. The expression of this particular antigen has been associated with acute rejection, and in animal models graft survival has been prolonged by the administration of antibodies to ICAM-1.[32 33] The lack of a demonstrable association of this marker of acute rejection with intimal hyperplasia in the present study is consistent with our exclusion of patients with acute rejection. This negative observation further supports the hypothesis that intimal hyperplasia observed in these patients was not an acute event, but was rather a manifestation of a chronic immune response. The persistent expression of ICAM-1 in this study likely reflects the fact that it is a constitutively expressed antigen modulated during acute rejection.

Relationship of Rejection Incidence to Intimal Thickness, Immunohistochemical Markers, and Time Since Transplantation

In the present study, intimal thickening was significantly correlated with the number of treated acute rejection episodes. This observation, which we have previously reported, is consistent with the hypothesis that immunologic injury sustained during acute rejection is an important factor in the development of coronary artery disease. Because we do not routinely characterize the inflammatory cell phenotypes in surveillance biopsies, it is not possible to comment on which cell types are important in the pathophysiology of this disease. Observation of an inverse correlation between the RPA $T4^+$ cells with rejection incidence in this study is most readily explained by the fact that patients who demonstrate acute rejection

in their biopsies are treated with augmented immunosuppression, with the result that these inflammatory cells are chronically decreased within the graft. Thus, as might be anticipated, patients who had more frequent augmentation of their immunosuppression were indeed found to have fewer RPA $T4^+$ cells on endomyocardial biopsy.

Correlation of Immunohistochemical Markers With Immunosuppression

The observed inverse correlation of markers of acute rejection such as RPA $T4^+$, RPA $T8^+$, HLA DQ, and ICAM-1 with total pulsed steroid dose is consistent with rejection therapy. All rejection episodes are treated with 3 g methylprednisolone or an augmented course of 100 mg prednisone daily, with a gradual taper over the ensuing 30 days. The results of this study indicate that patients with rejection received high doses of pulsed steroids, and as a consequence had chronic diminution of conventional markers of acute rejection on endomyocardial biopsy. Despite the effectiveness of steroids in decreasing markers of acute rejection, this did not translate to a protective effect against CAVD. Rather, the data indicate that, despite treatment, intimal hyperplasia occurs, suggesting that once injury to the vasculature is sustained during acute rejection, the environment for intimal proliferation is initiated. This implicates an important role for endogenous cells of the graft in propagating the proliferative response following their activation, as has been proposed by Libby et al.[27]

The positive correlation of average daily maintenance prednisone dose with RPA $T4^+$, RPA $T8^+$, and HLA DQ is consistent with conventional management of rejection. In these patients, the recurrent episodes of rejection are usually accompanied by a

response from physicians to keep maintenance prednisone doses high rather than rapidly tapering, and these results would be consistent with such a management approach. Whether these high doses of steroids play a direct role in the development of intimal hyperplasia by modulating endothelial function is unclear. It is possible that the steroids could decrease microvascular antigen expression, rendering the graft less susceptible to acute rejection, but altering normal vessel wall homeostasis in favor of vascular smooth muscle proliferation. In vitro data suggest that such an effect would be counterbalanced by cyclosporine, which inhibits smooth muscle cell proliferation in the vascular response to injury.[34] However, the lack of correlation of cyclosporine dose with intimal hyperplasia in the current study does not support this hypothesis.

Limitations

One limitation to this study is that there was no stratification for time since transplantation. Although some of the observations described here reflect factors primarily related to time since transplantation, it is unlikely that this is the full explanation, as already discussed. However, a larger study in which patients are stratified by time since transplantation would be most helpful to further clarify these potentially confounding factors.

The second limitation is due to the fact that there has been little characterization of the antigen to which the antibody E 1.5 binds, thus complicating the interpretation of any results regarding detection of antigen binding to E 1.5. In two previous studies, this antibody was used as a marker of microvascular endothelium in human renal transplantation.[9 15] In both, E 1.5 bound to peritubular capillary and endothelial cells, and there was diminished binding in chronically rejected organs. Data from our laboratory demonstrated

E 1.5 binding to microvascular endothelial cells and downregulation of binding during episodes of acute allograft dysfunction.[31]

The third limitation is that the grading of the vascular endothelial antigen expressed is semiquantitative. Morphometric quantitation was not used because it is also semiquantitative. Furthermore, because we examined only a single endomyocardial biopsy from each subject, there is considerable potential for variations that would relate to sampling error.[35] Because multiple biopsies cannot be obtained for frozen section from a single patient, these limitations are difficult to overcome. In the future, the focus of these investigations would be on developing immunohistochemistry methods to be applied to formalin-fixed tissue, to enable examination of all four specimens obtained for clinical surveillance.

Finally, more precise identification of the cell types expressing the antigens on endomyocardial biopsy would be enhanced by use of the double-staining immunohistochemical method.

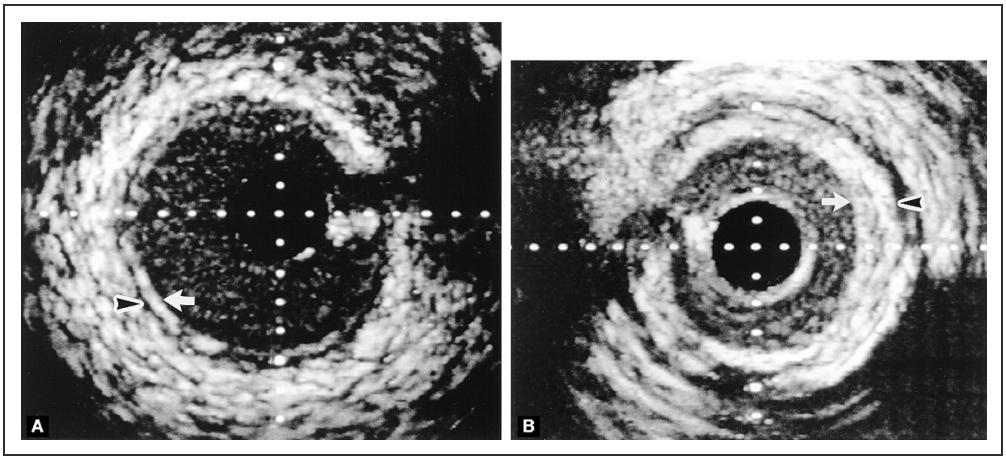

Figure 1. Intracoronary ultrasound images from heart transplant patients. A, Minimal intimal proliferation, indicated by the black and white arrows. B, Marked intimal proliferation, identified by the area between the lumen-intima interface (white arrow) and the media-intima interface (black arrow). The lumina-intima interface and the intima-media interface were measured by planimetry to obtain the intimal thickness.

Figure 2. Sections of endomyocardial biopsy from a patient with minimal intimal thickening, showing expression of HLA DR. All sections have been processed by a four-step peroxidase-antiperoxidase method, using the primary monoclonal antibody for HLA DR. Note expression of HLA DR by microvascular endothelium, including capillaries, venules, and arterioles denoted by the brown staining. A and B, Original magnification ×20 and ×40. C, Primary antibody has been omitted: negative control (original magnification ×40).

Table 1. Patient Demographics (Table view)

	n	45	
	Sex, F/M	10/35	
	Age, y	36-61 (47)	
	Age at transplantation, y	31-46 (44)	
	Donor age, y	31-46 (38)	
	Time since transplantation, mo	12-46 (31±35)	
	Ischemic time, min	0-278 (143±57)	
	HLA A,B,C mismatch, n	1-5 (3.2±3.5)	

HLA indicates histocompatibility lymphocyte antigen.
Values in parentheses are means (±SEM).

Table 2. Severity of Intimal Thickness as It Relates to Time Since Transplantation; Expression of HLA Classes I and II, ICAM-1, and E 1.5 Antigens; and Rejection Incidence (Table view)

IT Class	n	Range, mm	IT, mm	Years Since Tx	HLA ABC	HLA DR	HLA DP	HLA DQ	E 1.
I	28	0.00-0.25	0.13 ±0.08	1.9±2.8	3.8±0.9[1]	2.6±0.8	2.5±0.8	1.6 ±1.1	1.4±
II	5	0.26-0.35	0.29±0.03[2]	3.3±2.8	3.0 ±1.6[1]	2.0±0.7[3]	2.2±0.8	1.2±1.0[3]	0.7 ±0.8
III	7	0.36-0.45	0.40 ±0.03[2]	3.3±2.0	3.5±0.9[1]	2.4±0.5[3]	2.4±0.6	1.0 ±1.3[3]	0.4±
IV	3	>0.45	0.51±0.07[2]	5.5±3.7[6]	4.3 ±1.0[1]	2.0±1.0[3]	2.0±0.0	1.0±1.0[3]	0.3 ±0.6

IT indicates intimal thickness; Tx, transplantation; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules. Values are given as mean±SD.

1 *P*≤.05 vs HLA DR, HLA DP, and HLA DQ.

2 *P*≤.05 for IT class II, III, and IV vs class I (by definition).

3 *P*≤.05 for IT class II vs class I; class III vs class I; and class IV vs class I.

4 *P*≤.01 vs IT class I.

5 *P*≤.05 for IT classes II and III vs classes I and IV.

6 *P*≤.05 for IT class IV vs class I. Time since transplantation was significantly longer in patients with IT >0.45 mm (class IV) compared with patients with IT <0.26 mm (class I). The difference between the other groups was not statistically significant.

7 *P*≤.05 for IT class IV vs class I, II, or III.

Table 3. Correlation of Antigen Expression With Intimal Thickness, Time Since Transplantation, and Number of Rejection Episodes (Table view)

MAB/Antigen	n	Intimal Thickness		Time Since Transplantation		Number of Rejection Episodes	
		r	*P*	*r*	*P*	*r*	*P*
RPA T4^{+}	41	−.29	.060	−.30	.060	−.34	.02
RPA T8^{+}	43	−.21	.170	−.35	.200	−.28	.07
RPA M1^{+}	35	.35	.080	−.07	.680	−.03	.84
HLA DQ	43	−.36	.010	−.01	.990	−.13	.40
HLA DP	43	−.14	.360	−.03	.200	−.31	.04
HLA DR	41	−.56	.001	−.16	.290	−.12	.44
HLA ABC	42	−.02	.886	−.25	.100	−.14	.34
E 1.5	43	−.59	.001	−.12	.450	−.38	.01
ICAM-1	40	−.21	.176	−.23	.130	−.36	.02

MAB indicates monoclonal antibody; RPA, Royal Prince Albert; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules.

Table 4. Correlation of Microvascular Antigens and Inflammatory Cell Phenotypes With Immunosuppression (Table view)

Antigen	Total Pulsed Steroid Dose		Average Daily Prednisone Dose		Average Daily Cyclosporine Dose	
	r	*P*	*r*	*P*	*r*	*P*
RPA $T4^+$	−.34	.037	.42	.008	−.10	.500
RPA $T8^+$	−.32	.040	.52	.001	.10	.490
RPA $M1^+$	−.12	.500	.10	.570	.34	.060
HLA ABC	−.22	.150	.24	.130	−.22	.160
HLA DR	−.05	.170	.17	.490	−.28	.250
HLA DP	−.14	.430	.05	.740	−.19	.260
HLA DQ	−.33	.030	.43	.005	.20	.190
E 1.5	−.23	.200	.12	.510	.10	.580
ICAM-1	−.52	.008	.34	.050	.23	.270

RPA indicates Royal Prince Albert; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules.

ARTICLE INFORMATION

accepted 28 September 1994; Received 1 August 1994.

Correspondence

Correspondence to Hannah A. Valantine, MD, Falk Cardiovascular Research Center, Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305-5246.

Affiliations

From the Division of Cardiovascular Medicine (M.C.D., F.P., S.A.H., H.A.V.), the Department of Pathology (S.B., P.H., R.S., B.M.H.), and the Department of Cardiovascular Surgery (E.B.S.), Stanford University School of Medicine, Stanford, Calif.

Acknowledgments

This work was supported in part by NIH grants 5K081-IL024 41-03 and HL4 3312 to Drs Valantine and Hall.

REFERENCES

1. Miller LW. Transplant coronary artery disease: editorial. *J Heart Lung Transplant.* 1992;*11*:S1-S4. PubMed.
2. Billingham ME. Cardiac transplant atherosclerosis. *Transplant Proc*. 1987;19(suppl 5):19-25.
3. Billingham ME. Histopathology of graft coronary disease. *J Heart Lung Transplant.* 1992;*11*:S38-S44. PubMed.
4. Johnson DE, Alderman ED, Schroeder JS, Gao S-Z, Hunt S, DeCampli WM, Stinson E, Billingham M. Transplant coronary artery disease: histopathologic correlations with angiographic morphology. *J Am Coll Cardiol.* 1991;*17*:449-457. Crossref. PubMed.
5. Gao SZ, Alderman EL, Schroeder JS, Silverman JF, Hunt SA. Accelerated coronary vascular disease in the heart transplant patient: coronary arteriographic findings. *J Am Coll Cardiol.* 1988;*12*:334-340. Crossref. PubMed.
6. St Goar FG, Pinto FJ, Alderman EL, Valantine HA, Schroeder JS, Gao S-Z, Stinson EB, Popp RL. Intracoronary ultrasound in cardiac transplant recipients: in vivo evidence of 'angiographically silent' intimal thickening. *Circulation.* 1992;*85*:979-987. Crossref. PubMed.
7. Valantine HA, Pinto FJ, St Goar FG, Alderman EL, Popp RL. Intracoronary ultrasound imaging in heart transplant recipients: the Stanford experience. *J Heart Lung Transplant.* 1992;*11*:S60-S64. PubMed.
8. Bishop GA, Hall BM, Suranyi MG, Tiller DJ, Horvath JS, Duggin GG. Expression of HLA antigens on renal tubular cells in culture, I: evidence that mixed lymphocyte culture supernatants and gamma interferon increase both class I and class II HLA antigens. *Transplantation.* 1986;*42*:671-679. Crossref. PubMed.
9. Bishop GA, Waugh JA, Hall BM. Expression of HLA antigens on renal tubular cells: effect of increased HLA antigen expression on tubular cell stimulation of lymphocytes and their vulnerability to cell-mediated lysis. *Transplantation.* 1988;*46*:303-310. Crossref. PubMed.
10. Halloran PF, Wadgymar A, Autenreid P. The regulation of the expression of major histocompatibility antigens (overview). *Transplantation.* 1986;*41*:413-420. Crossref. PubMed.
11. Salomon RN, Hughes CCW, Schoen FJ, Payne DD, Pober JS, Libby P. Human coronary transplantation-associated arteriosclerosis: evidence of a chronic immune reaction to activated graft endothelial cells. *Am J Pathol.* 1991;*138*:791-798. PubMed.

12. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immunol.* 1987;*5*:223-252. Crossref. PubMed.
13. Libby P, Swanson SJ, Tanaka H, Murray A, Schoen FJ, Pober JS. Immunopathology of coronary arteriosclerosis in transplanted hearts. *J Heart Lung Transplant.* 1992;*11*:S5-S6. PubMed.
14. Allen MD, McDonald TO, Carlos T, Himes V, Fishbein D, Aziz S, Gordon D. Endothelial adhesion molecules in heart transplantation. *J Heart Lung Transplant.* 1992;*11*:S8-S13. PubMed.
15. Bishop GA, Waugh JA, Landers DV, Krensky AM, Hall BM. Microvascular destruction in renal transplant rejection. *Transplantation.* 1989;*48*:408-414. Crossref. PubMed.
16. Schoen FJ, Libby P. Cardiac transplant graft arteriosclerosis. *Trends Cardiovasc Med.* 1991;*1*:216-223. Crossref. PubMed.
17. Billingham ME, Cary NRB, Hammond ME, Kemnitz J, Marboe C, McCallister HA, Snovar DC, Winters GL, Zerbe A. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. *J Heart Lung Transplant.* 1990;*9*:587-593.
18. Pinto FJ, St Goar FG, Chiang M, Popp RL, Valantine HA. Intracoronary ultrasound evaluation of intimal thickening in cardiac transplant recipients: correlation with clinical characteristics. *J Am Coll Cardiol.* 1991;*17*:103A. Abstract. Crossref.
19. Ross R, Glomset JA. The pathogenesis of atherosclerosis. *N Engl J Med.* 1976;*295*:369-420. Crossref. PubMed.
20. Ross R. The pathogenesis of atherosclerosis: an update. *N Engl J Med.* 1986;*314*:488-520. Crossref. PubMed.
21. Laden AMK. The effects of treatment on the arterial lesions of rat and rabbit cardiac allografts. *Transplantation.* 1972;*13*:281-290. Crossref. PubMed.
22. Minick CR, Murphy GE. Experimental induction of atheroarteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet. *Am J Pathol.* 1973;*73*:265-292. PubMed.
23. Alonso DR, Starek PK, Minick CR. Studies on the pathogenesis of atheroarteriosclerosis induced in rabbit cardiac allografts by the synergy of graft rejection and hypercholesterolemia. *Am J Pathol.* 1977;*87*:415-435. PubMed.
24. Cramer DV, Qian S, Harnaha J, Chapman FA, Starzl TE, Makowka L. Accelerated graft arteriosclerosis is enhanced by sensitization of the recipient to donor lymphocytes. *Transplant Proc.* 1989;*21*:3714-3715. PubMed.
25. Cramer DV, Chapman FA, Wu GD, Harnaha JB, Qian S, Makowka L. Cardiac transplantation in the rat, II: alteration of the severity of donor graft arteriosclerosis by

modulation of the host immune response. *Transplantation.* 1990;*50*:554-558. Crossref. PubMed.

26. Davis MM, Björkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature.* 1988;*334*:395-402. Crossref. PubMed.
27. Libby P, Salomon RN, Payne DD, Schoen FJ, Pober JS. Functions of vascular wall cells related to development of transplantation-associated coronary arteriosclerosis. *Transplant Proc*. 1989;21: 3677-3684.
28. Russell ME, Fujita M, Billingham M. Cardiac graft vascular disease: nonselective involvement of large and small vessels. *Transplantation.* 1993;*56*:762-764. Crossref.
29. Ahmed-Ansari A, Tadros TS, Knopf WD, Murphy DA, Hertzler G, Feighan J, Leatherbury A, Sell KW. Major histocompatibility complex class I and class II expression by myocytes in cardiac biopsies post-transplantation. *Transplantation.* 1988;*45*:972-978. Crossref. PubMed.
30. Wood KJ, Hopley A, Dallman MJ, Morris PJ. Lack of correlation between the induction of donor class I and class II major histocompatibility complex antigens and graft rejection. *Transplantation.* 1988;*45*:759-767. Crossref. PubMed.
31. Valantine H, Keogh A, Waugh JA, Starkey T, Anderson W, Bell S, Hall B, Sibley R. Acute cardiac allograft dysfunction: correlation with microvascular injury. *Circulation*. 1991;84(suppl II):II-353. Abstract.
32. Flavin R, Ivens K, Rothlein R, Faanes R, Clayberger C, Billingham M, Starnes VA. Monoclonal antibodies against intercellular adhesion molecule 1 prolong cardiac allograft survival in cynomolgus monkeys. *Transplant Proc.* 1991;*23*:533-534. PubMed.
33. Kobayashi H, Miyano T, Yamataka T. Prolongation of rat cardiac allograft survival by a monoclonal antibody: anti-rat intercellular adhesion molecule-1. *Cardiovasc Surg.* 1993;*1*:577-580. PubMed.
34. Jonasson L, Holm J, Hansson GH. Cyclosporin A inhibits smooth muscle proliferation in the vascular response to injury. *Proc Natl Acad Sci U S A.* 1988;*85*:2303-2306. Crossref. PubMed.
35. Nakhleh RE, Jones J, Goswitz JJ, Anderson EA, Titus J. Correlation of endomyocardial biopsy findings with autopsy findings in human cardiac allografts. *J Heart Lung Transplant.* 1992;*11*:479-485. PubMed.

Sections

LIST OF ILLUSTRATIONS

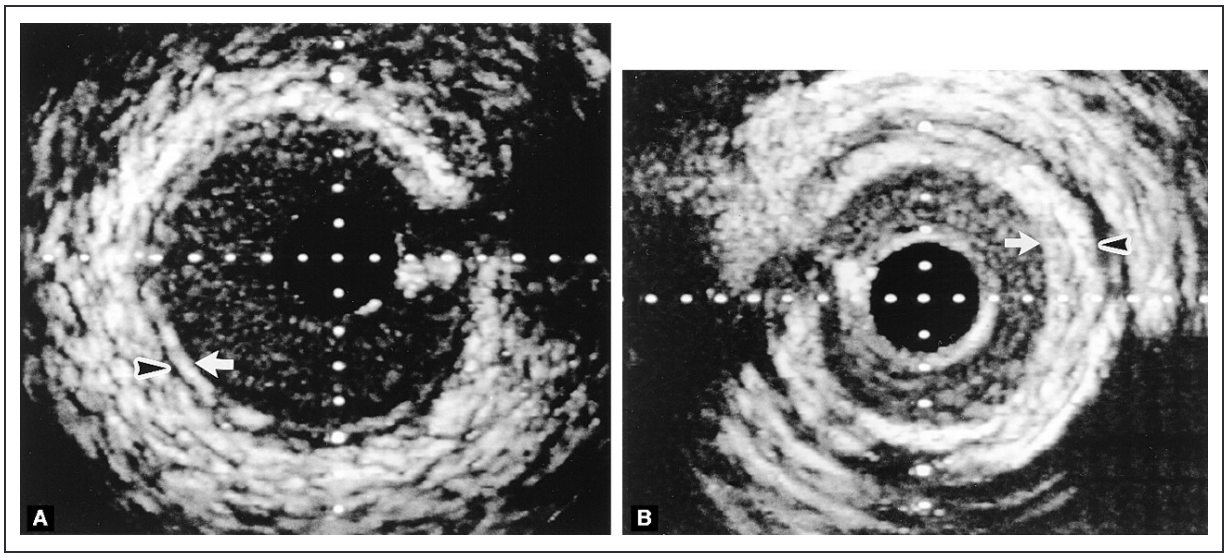

Figure 1. Intracoronary ultrasound images from heart transplant patients. A, Minimal intimal proliferation, indicated by the black and white arrows. B, Marked intimal proliferation, identified by the area between the lumen-intima interface (white arrow) and the media-intima interface (black arrow). The lumina-intima interface and the intima-media interface were measured by planimetry to obtain the intimal thickness.


THIS FIGURE IS
CURRENTLY UNAVAILABLE
We apologize for
any inconvenience.

Figure 2. Sections of endomyocardial biopsy from a patient with minimal intimal thickening, showing expression of HLA DR. All sections have been processed by a four-step peroxidase-antiperoxidase method, using the primary monoclonal antibody for HLA DR. Note expression of HLA DR by microvascular endothelium, including capillaries, venules, and arterioles denoted by the brown staining. A and B, Original magnification ×20 and ×40. C, Primary antibody has been omitted: negative control (original magnification ×40).

Table 1. Patient Demographics

	n	45	
	Sex, F/M	10/35	
	Age, y	36-61 (47)	
	Age at transplantation, y	31-46 (44)	
	Donor age, y	31-46 (38)	
	Time since transplantation, mo	12-46 (31±35)	
	Ischemic time, min	0-278 (143±57)	
	HLA A,B,C mismatch, n	1-5 (3.2±3.5)	

HLA indicates histocompatibility lymphocyte antigen.
Values in parentheses are means (±SEM).

Table 2. Severity of Intimal Thickness as It Relates to Time Since Transplantation; Expression of HLA Classes I and II, ICAM-1, and E 1.5 Antigens; and Rejection Incidence

IT Class	n	Range, mm	IT, mm	Years Since Tx	HLA ABC	HLA DR	HLA DP	HLA DQ	E 1.
I	28	0.00-0.25	0.13 ±0.08	1.9±2.8	3.8±0.9[1]	2.6±0.8	2.5±0.8	1.6 ±1.1	1.4±
II	5	0.26-0.35	0.29±0.03[2]	3.3±2.8	3.0 ±1.6[1]	2.0±0.7[3]	2.2±0.8	1.2±1.0[3]	0.7 ±0.8
III	7	0.36-0.45	0.40 ±0.03[2]	3.3±2.0	3.5±0.9[1]	2.4±0.5[3]	2.4±0.6	1.0 ±1.3[3]	0.4±
IV	3	>0.45	0.51±0.07[2]	5.5±3.7[6]	4.3 ±1.0[1]	2.0±1.0[3]	2.0±0.0	1.0±1.0[3]	0.3 ±0.6

IT indicates intimal thickness; Tx, transplantation; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules. Values are given as mean±SD.

1 *P*≤.05 vs HLA DR, HLA DP, and HLA DQ.

2 *P*≤.05 for IT class II, III, and IV vs class I (by definition).

3 *P*≤.05 for IT class II vs class I; class III vs class I; and class IV vs class I.

4 *P*≤.01 vs IT class I.

5 *P*≤.05 for IT classes II and III vs classes I and IV.

6 *P*≤.05 for IT class IV vs class I. Time since transplantation was significantly longer in patients with IT >0.45 mm (class IV) compared with patients with IT <0.26 mm (class I). The difference between the other groups was not statistically significant.

7 *P*≤.05 for IT class IV vs class I, II, or III.

Table 3. Correlation of Antigen Expression With Intimal Thickness, Time Since Transplantation, and Number of Rejection Episodes

MAB/Antigen	n	Intimal Thickness		Time Since Transplantation		Number of Rejection Episodes	
		r	*P*	*r*	*P*	*r*	*P*
RPA $T4^+$	41	−.29	.060	−.30	.060	−.34	.02
RPA $T8^+$	43	−.21	.170	−.35	.200	−.28	.07
RPA $M1^+$	35	.35	.080	−.07	.680	−.03	.84
HLA DQ	43	−.36	.010	−.01	.990	−.13	.40
HLA DP	43	−.14	.360	−.03	.200	−.31	.04
HLA DR	41	−.56	.001	−.16	.290	−.12	.44
HLA ABC	42	−.02	.886	−.25	.100	−.14	.34
E 1.5	43	−.59	.001	−.12	.450	−.38	.01
ICAM-1	40	−.21	.176	−.23	.130	−.36	.02

MAB indicates monoclonal antibody; RPA, Royal Prince Albert; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules.

Table 4. Correlation of Microvascular Antigens and Inflammatory Cell Phenotypes With Immunosuppression

Antigen	Total Pulsed Steroid Dose		Average Daily Prednisone Dose		Average Daily Cyclosporine Dose	
	r	*P*	*r*	*P*	*r*	*P*
RPA $T4^+$	−.34	.037	.42	.008	−.10	.500
RPA $T8^+$	−.32	.040	.52	.001	.10	.490
RPA $M1^+$	−.12	.500	.10	.570	.34	.060
HLA ABC	−.22	.150	.24	.130	−.22	.160
HLA DR	−.05	.170	.17	.490	−.28	.250
HLA DP	−.14	.430	.05	.740	−.19	.260
HLA DQ	−.33	.030	.43	.005	.20	.190
E 1.5	−.23	.200	.12	.510	.10	.580
ICAM-1	−.52	.008	.34	.050	.23	.270

RPA indicates Royal Prince Albert; HLA, histocompatibility lymphocyte antigen; and ICAM-1, intercellular adhesion molecules.