American Journal of Pathology, Vol. 145, No. 5, November 1994


# Patterns of Myocardial Cell Adhesion Molecule Expression In Human Endomyocardial Biopsies After Cardiac Transplantation

## *Induced ICAM-1 and VCAM-1 Related to Implantation and Rejection*

Ahvie Herskowitz,*† Ann E. Mayne,‡ Sharon B. Willoughby,† Kirk Kanter,§ and Aftab A. Ansari‡

*From the Departments of Medicine* and Molecular Microbiology and Infectious Diseases,† The Johns Hopkins Medical Institutions, Baltimore, Maryland, and the Department of Pathology and Winship Cancer Center‡ and the Division of Cardiothoracic Surgery, Department of Surgery,§ Emory University School of Medicine, Atlanta, Georgia*

***Conflicting patterns of myocardial cell adhesion molecule expression associated with cardiac rejection have emerged from numerous studies of randomly selected cardiac biopsies. We designed a prospective, longitudinal study which reports both qualitative and quantitative levels of myocardial ICAM-1, VCAM-1, E-selectin, and P-selectin expression in sequential human cardiac allograft biopsies. Intense ICAM-1 and VCAM-1 staining was found in all biopsies during the first three weeks after transplant and coincided with elevated serum levels of troponin T, a sensitive marker of ischemic myocyte injury. Baseline ICAM-1 and VCAM-1 expression returned within three to four weeks, as did serum troponin T levels in all patients who did not develop rejection. All 29 rejection episodes encountered were associated with intense ICAM-1 staining, while 24 of the 29 (83%) had intense VCAM-1 staining. Increased ELAM-1 and CD62 staining was only rarely observed. Persistence of increased ICAM-1 and VCAM-1 staining after treated rejection episodes predicted a recurrent rejection episode within two months (75% positive and 100% negative predictive value). Objective quantitative measurements by radioimmunoassay (RIA) confirmed these patterns of induced ICAM-1 and VCAM-1 expression. Thus, longitudinal monitoring of serial biopsies for myocardial ICAM-1 and VCAM-1 expression could be useful in the early detection of rejection episodes and monitoring the efficacy of immunosuppressive therapy. (Am J Pathol 1994, 145:1082–1094)***

The histological examination of endomyocardial biopsies (EMBs) performed at routine intervals after cardiac transplantation is the most reliable means to assess the patients for allograft rejection. The histological criteria used to classify mild, moderate and severe rejection has been standardized by Billingham et al.[1] which provides a diagnostic uniformity to biopsy interpretation. Clinical experience, however, has shown that whereas biopsies containing multiple foci of interstitial inflammation with myocyte necrosis represent ongoing rejection, histology alone cannot readily distinguish nonspecific myocardial inflammation from the early stages of rejection.

Recently, a growing body of evidence has shown the importance of the expression of cell adhesion molecules (CAMs) on endothelial cells and their respective ligands on subpopulations of mononuclear cells in regulating leukocyte-endothelial cell interactions.[2] Effective interaction between CAMs and their ligands facilitate interactions between T cells and antigen-presenting cells and help direct adhesion, activation and trafficking of lymphocytes. Numerous studies

Supported by NIH grants 1R01-HL-47272-04 and P50-HL17655-16.

Accepted for publication August 3, 1994.

Address reprint requests to Ahvie Herskowitz, MD, The Johns Hopkins Medical Institutions, 615 North Wolfe Street, Room 5017, Baltimore, MD, 21205.

have shown induction of CAMs on endothelial cells in human and experimental liver,[3,4] kidney,[5,6] pancreas[4] and heart grafts,[7–15] as well as reduced severity of allograft rejection when adhesion molecule expression is effectively blocked.[16–18] Immunohistology studies examining the role of CAM expression in human cardiac allograft rejection performed to date[7–15] have analyzed relatively small numbers of selected biopsies and have described variable expression of ICAM-1, VCAM-1 and ELAM-1. In a recent review of these studies,[7] no consensus pattern of CAM expression associated with rejection was evident. Potential explanations for such conflicting results include: 1) variability of inducible CAM expression in individual patients; 2) varied temporal relationships between different CAMs and histological rejection; and 3) a lack of objective measurements of CAM expression. Therefore, to more clearly define the temporal sequence of induced myocardial CAM expression in patients after cardiac transplantation, we designed a prospective, longitudinal study to qualitatively and quantitatively measure changes in relative levels of ICAM-1, VCAM-1, E-selectin, and P-selectin in sequential human cardiac allograft biopsies from the time of transplantation. The temporal relationships between myocardial CAM expression and the development of rejection constitute the basis for this report.

## Materials and Methods

### Patients

A total of 20 consecutive adult patients undergoing cardiac transplantation at Emory University Hospital were prospectively studied. Patient demographics, donor ischemia times, and donor/recipient MHC class I and class II typing results were available for each patient. All transplant patients were routinely treated with immunosuppressive therapy including cyclosporine A, azathioprine, and prednisone. Percutaneous transvenous endomyocardial biopsies were performed on cardiac transplant recipients within the first week and then weekly for the first eight weeks and every other week for the next eight weeks, or as clinically indicated. The biopsies were collected in sterile medium and transported to the laboratory for all studies listed below. Serum samples were obtained immediately before EMB sampling during each biopsy-related visit. Informed consent for all EMB studies described below were obtained from each patient and all studies were approved by the Human Investigations Committee of the Emory University Medical Center.

### Histological Rejection Grades

Aliquots of the biopsies were fixed in 10% neutral buffered formalin, processed in a rapid tissue processor in a 4-hour cycle, and embedded in paraffin as a single block. The biopsies were serially sectioned, which provides slides at 16 levels. Every other slide was stained with hematoxylin and eosin and microscopic evaluation performed. Intervening slides were used for special stains if deemed necessary. The grading of the biopsies was performed using the standardized histological grading system devised by Billingham et al:[1] negative, grade 0; minimal/mild lymphocytic infiltrates, grades 1A and 1B; focal moderate rejection, grade 2; and moderate/severe rejection, grades 3A, 3B, and 4. Episodes of significant rejection (biopsies demonstrating focal moderate, moderate, or severe rejection) were routinely treated with additional immunosuppression (see below).

### Immunohistology

Aliquots of EMB were snap-frozen in OCT compound, and serial sections were utilized to semiquantitatively assess the expression of relative levels of ICAM-1 (CD54), VCAM-1, ELAM-1, and P-selectin (CD62) on microvascular endothelial cells and cardiac myocytes, essentially as previously described.[19–21] The monoclonal antisera specific for ICAM-1, ELAM-1, and P-selectin were purchased from Becton-Dickinson (Mountain View, CA), and the anti-VCAM-1 was a generous gift from Dr. J. Harlan (University of Washington, Seattle, WA). Each of these antisera was titrated to define optimum concentrations to be utilized, using the human umbilical vein endothelial cell line (HUVEC, ATCC, CR1730, Rockville, MD) pretreated with tumor necrosis factor-$\alpha$ (TNF-$\alpha$), an EBV-transformed human lymphoblastoid cell line (prepared in our laboratory), and thrombin-activated platelets. The optimum concentrations were determined to be a 1:50 dilution of the anti-ICAM-1 and VCAM-1 antibodies, a 1:100 dilution for the anti-ELAM-1, and a 1:25 dilution for the anti-P-selectin antibody. The developing antibody was a peroxidase-labeled goat anti-mouse IgG antisera (Sigma Chemical Co., St. Louis, MO) diluted 1:500 before use. The staining procedure is described in detail elsewhere.[16,17] The semi-quantitative scoring system was defined as follows: 0 to 1+, slight patchy staining

of endothelial cells, myocytes negative; 2+, moderate, diffuse endothelial staining, myocytes negative; 3+, intense, diffuse endothelial staining, myocytes negative; and 4+, intense, diffuse endothelial staining, myocytes low level positive.

All samples were read by one trained observer who was blinded to patient identity and history. For the purposes of this study, the term intense staining is limited to 3+ to 4+ staining patterns. Because arterioles are present in a minority of biopsies, arteriolar staining patterns were not analyzed. In order to assess different patterns of CAM induction in biopsy samples following a treated rejection episode, the following definitions were used: non-repeaters, those patients whose histologic rejection episodes were not followed by another rejection episode within 2 months following additional immunosuppression; repeaters, those patients whose histological rejection episodes were followed by another rejection episode within 2 months after additional immunosuppression.

Frozen heart samples from an unused potential transplant donor without any history of heart disease were used as negative controls. Positive controls consisted of TNF-$\alpha$ pretreated HUVECs and biopsy samples from 4+ rejection episodes for ICAM-1, ELAM-1, and VCAM-1 and thrombin-activated platelets for P-selectin (CD62).

## *RIA for ICAM-1 and VCAM-1*

To derive a more objective evaluation, a quantitative RIA was set up as previously described[19,20] to measure myocardial levels of ICAM-1 and VCAM-1. Adequate tissue was available for all serial biopsies from 18 patients. Briefly, three to five sections of each biopsy (4 μ each) were transferred into siliconized 12 × 75 mm glass tubes which had been previously coated overnight with a cocktail of 1% bovine serum albumin, 10% normal goat serum, and 1% gelatin in PBS pH 7.4 containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiodipyridine, 1 mM benzamidine, 2.5 μg/ml each of chymostatin, leupeptin, elastinal, and antipain, and 5 μg/ml of aprotinin. To triplicate samples was added 1 ml of PBS containing the above listed enzyme inhibitors and 25 μl of either normal mouse serum, anti-ICAM-1 antisera, or anti-VCAM-1 monomorphic antisera, and the tubes incubated for 1 hour at 4 C. To control for variations in biopsy sizes from one sample to another, all samples were similarly analyzed using 100 μl of anti-myosin, which we have previously shown provides a representative measure of the myocardial sample size.[20] Monoclonal anti-myosin reagent (EU-1H1) was prepared in our laboratory and has been characterized to react with cardiac but not skeletal myosin.[20,22,23] After incubation, the samples were washed four times by centrifugation with PBS containing 10% normal goat serum and inhibitors with 10 minutes incubation between washes. After the final wash, to each sample was added 10 μl of 1:20 dilution of $^{125}$I-labeled goat anti-mouse Ig (NEN, Boston, MA). The radiolabelled goat anti-mouse Ig was absorbed with human thymocytes and cardiac myocytes and then affinity purified on a mouse Ig column (mouse Ig conjugated to CNBr-Sepharose-4B; Pharmacia, Piscataway, NJ) before use. The dilution of antibody used per sample contained approximately 50,000 cpm. After the addition of the $^{125}$I-labeled goat anti-mouse Ig, the samples were again washed four times in PBS pH 7.4 by centrifugation and then counted for radioactivity using an LKB gamma counter. The mean cpm of the triplicate sample was calculated. With each RIA, a positive and negative control was used. Positive control consisted of similarly prepared tissue from a patient who had died of acute viral myocarditis obtained at autopsy. Negative control consisted of normal heart tissue obtained from an unused potential transplant donor without any history of heart disease. Aliquots of tissue for both positive and negative control were frozen in mini-vials and used after thawing with each RIA. The cpm between negative and positive control had to show a 10-fold difference for the test to be valid. The following formula was used to calculate the RIA index for ICAM-1 and VCAM-1: (mean cpm of EMB + anti-ICAM-1 or VCAM-1 – mean cpm of EMB + normal mouse serum)/(mean cpm of EMB + anti-cardiac myosin – mean cpm of EMB + normal mouse serum).

## *Controls for Quantitative RIA*

Standard curves for quantitative ICAM-1 and VCAM-1 levels were generated using increasing concentrations of TNF-$\alpha$-treated HUVECs (Figure 1). HUVECs were added to individual wells of a 96-well microtiter plate in triplicate. The plate was centrifuged at 150 × *g* for 5 minutes at 4 C and 0.1 ml of 1:100 dilution of ICAM-1 or VCAM-1 antibody was added. After incubation for 1 hour, wells were washed with PBS and 0.1 ml of 1:1000 dilution of rabbit anti-mouse Ig was added to each well, followed by 0.1 ml of an alkaline phosphatase conjugated goat anti-rabbit Ig. Mean optical density was determined using the MULTISKAN plate reader. Repeated measures of single biopsy samples revealed <10% variability with either ICAM-1 or VCAM-1 expression.

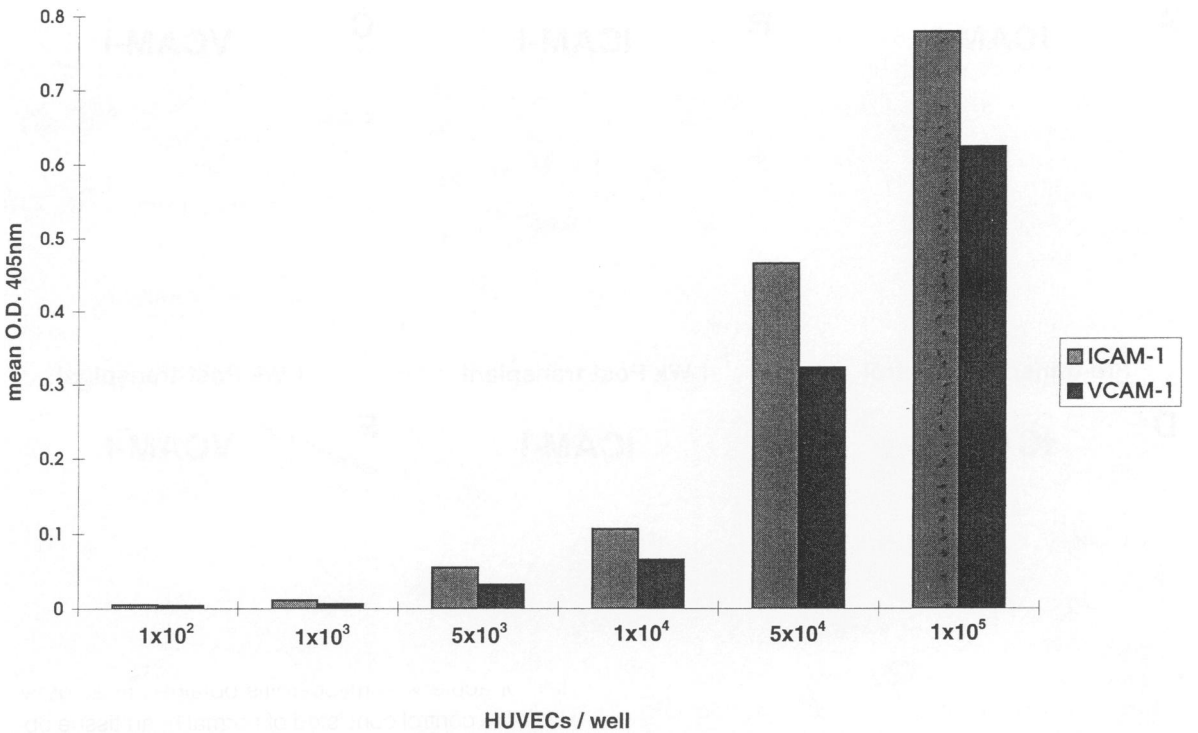


Figure 1. *Standardization curve for the ICAM-1 and VCAM-1 radioimmunoassay. Mean optical density (O.D.) readings were obtained using increasing numbers of TNF-α-pretreated HUVECs.*

## *Serum Assays for Troponin T and Creatine Kinase Isoenzyme MB (CK-MB)*

To determine whether serological evidence of myocardial cell damage was evident after cardiac transplantation, serial serum samples were evaluated for troponin T and CK-MB levels. Cardiac troponin T is a regulatory protein not normally found in blood. Its detection in the circulation has been shown to be a sensitive and specific marker of myocardial cell damage.[24,25] For the quantitative determination of serum troponin T, an enzyme immunological assay (Troponin-T kit; Boehringer Mannheim, Indianapolis, IN) was used to determine whether its presence in the serum of patients after cardiac transplantation could be demonstrated. Values ≥0.1 ng/ml were considered positive for troponin T in this study. CK-MB was assessed by a microparticle enzyme immunoassay (STAT CK-MB kit, Abbott Laboratories, Abbott Park, IL; values > 7.0 ng/ml were considered positive for this study). All assays were performed by technicians unaware of the patients' histories.

## *Statistics*

All results are expressed as means ± SD unless otherwise noted. Categorical data were analyzed with standard two-tailed $\chi^2$ or Fisher's exact test. Continuous data were summarized with standard measures of central tendency and dispersion using Student's *T*-test or, for skewed distributions, the Mann-Whitney *U* test to guide interpretation, using conventional levels of significance. Correlation coefficients between immunohistology and quantitative RIA results were determined by simple linear regression. Comparisons among multiple groups were performed using an ANOVA followed by a Fisher's PLSD. A *P* value of 0.05 was considered statistically significant. Positive predictive value was calculated as the number of true positive test results among all positive test results observed and negative predictive value as the number of true negative test results among all negative test results observed.

## *Results*

A total of 267 serial EMBs were analyzed by immunohistology from 20 patients (mean, 13.4 per patient; range, 8 to 18 biopsies per patient). Mean follow-up time was 137 days (range, 72 to 302 days). The mean age of the transplant patients was 44.8 years (range, 20 to 63 years). Median donor ischemia time was 93 minutes (range, 42 to 202 minutes). Of the 20 patients,

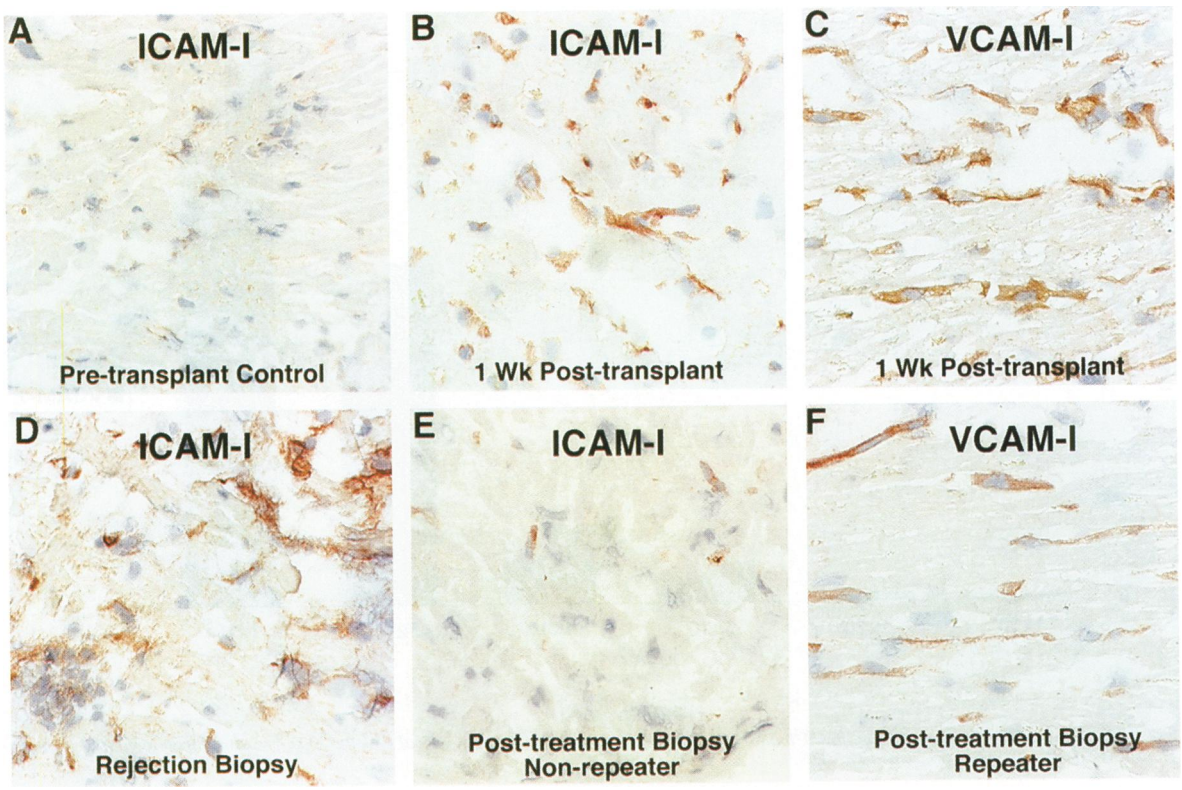


Figure 2. *Immunoperoxidase staining patterns for ICAM-1 and VCAM-1 in frozen sections of EMB samples after transplantation.* A: *ICAM-1 staining in a pre-transplant ventricular biopsy showing minimal reactivity within scattered microvessels. Diffuse increased staining for ICAM-1* (B) *and VCAM-1* (C) *in EMB samples without histologic rejection 1 week post-transplantation.* D: *Intense ICAM-1 staining within the venous and capillary microvasculature as well as focal myocyte surface staining (upper right corner) in a biopsy with grade 3A histological rejection. Similar staining patterns were found in biopsies preceding, as well as during, histologic rejection episodes.* E: *After immunosuppressive therapy for grade 3A rejection was instituted, the repeat biopsy revealed resolved rejection, and immunoperoxidase staining for ICAM-1 (and VCAM-1, not shown) showed a return to control levels. This patient did not experience a repeat rejection episode within 2 months following the treated rejection episode (non-repeater). In contrast, in another patient, a repeat biopsy 2 weeks after a treated rejection episode showed continued increased staining for VCAM-1* (F) *within the microvasculature, despite a significant histological improvement (hematoxylin and eosin stain, not shown). A similar persistent increase in ICAM-1 staining was also noted (not shown). This patient experienced a repeat rejection episode 4 weeks after his first rejection episode was treated and was therefore labeled a repeater.* (A–F, *magnification* ×400).

5 patients did not experience rejection episodes. Of the remaining 15 patients, a total of 29 rejection episodes were encountered. Eighty-six per cent were treated with a 3-day course of high dose intravenous methylprednisolone, 10% with increased oral prednisone, and 4% with other regimens. None of the patients in this study had detectable infection or malignancy.

## *Induction of ICAM-1 and VCAM-1 Staining during the Immediate Post-Transplant Period*

Control EMBs taken at the time of transplant revealed intense (3+ to 4+) staining for ICAM-1 and VCAM-1 in 10% and 15% of the samples, respectively, whereas none had intense staining for ELAM-1 and CD62. The majority of the control biopsies showed VCAM-1 and ICAM-1 to be minimally present on scattered microvessels (Figure 2). There was no relationship between ICAM-1 and VCAM-1 staining and ischemia times or HLA mismatches. In contrast, EMBs within the first week of transplant (median, 6 days post-transplantation) revealed intense ICAM-1 staining (involving both the microvasculature and myocytes) in all patients, whereas 60% had intense staining with VCAM-1 (limited to the microvasculature). Significant staining for ELAM-1 and CD62 were randomly observed in only 3% and 1% of all EMBs and were unrelated to rejection episodes. In five patients who did not experience any episodes of rejection, increased ICAM-1 and VCAM-1 staining returned to baseline levels within 28 days after transplantation (mean, 17 days; range, 10 to 28 days). Quantitative levels of myocardial ICAM-1 and VCAM-1 levels determined by RIA also returned to control levels during this time period (see below).

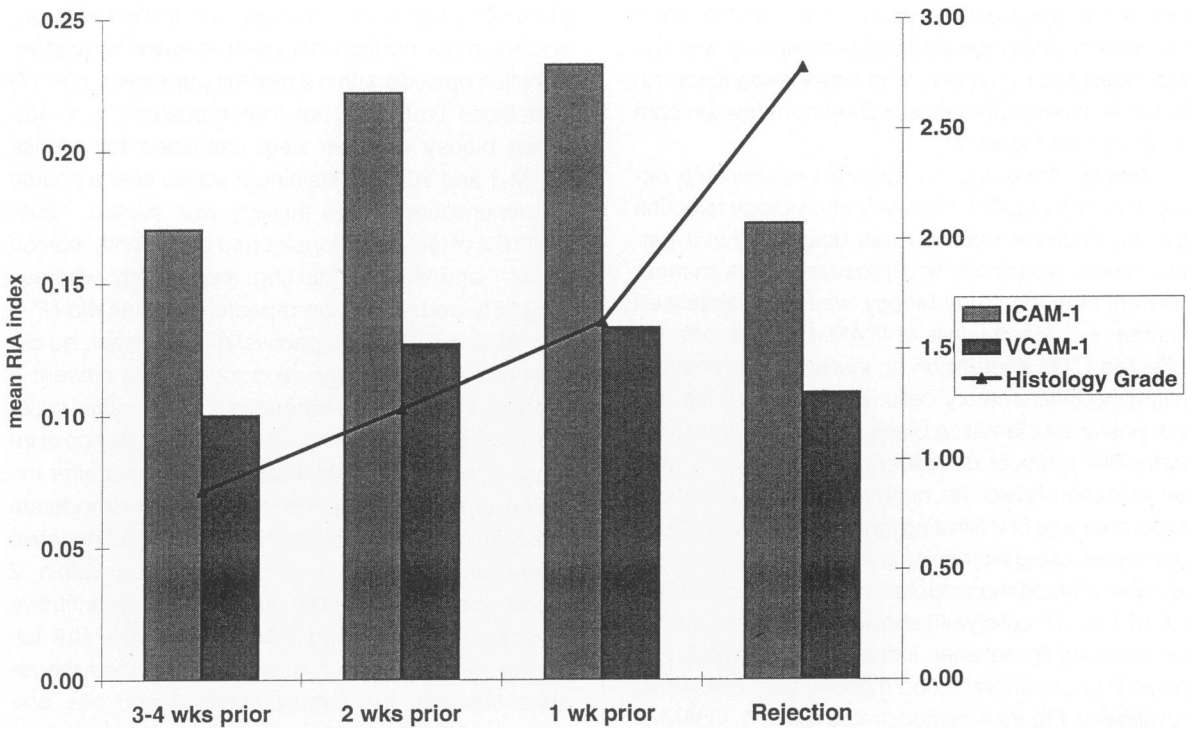


**Figure 3.** *Mean histological rejection grade and quantitative myocardial ICAM-1 and VCAM-1 levels determined by RIA prior to and at the time of rejection.*

## *Serum Troponin T Levels*

Mildly elevated serum troponin T was present in one patient at the time of transplant. In contrast, during the first biopsy after transplantation, serum troponin T levels peaked and were elevated above control values in all patients (mean, 1.7 ng/ml; range, 0.5 to 2.7 ng/ml), whereas CK-MB levels remained normal in all patients. Increased serum levels of troponin T persisted in all patients at biopsy number 2 (mean, 0.5 ng/ml) and in 90% of patients at biopsy number 3 (mean, 0.3 ng/ml). Thereafter, serum troponin T levels returned to baseline levels and were not found to be elevated during any rejection episode.

## *Induction of ICAM-1 and VCAM-1 Staining Associated with Histological Rejection*

Fifteen patients experienced a total of 29 rejection episodes. All episodes were associated with intense ICAM-1 staining whereas 24 of 29 (83%) had intense VCAM-1 staining. Eleven patients experienced early rejection episodes within 2 months after transplantation (median, 22 days). All such episodes were uniformly associated with persistence of intense ICAM-1 and VCAM-1 staining from the first biopsy after transplant. ICAM-1 staining was generally diffuse in distribution and was most intense on venous and capillary microvessels although focal myocyte staining was also seen. VCAM-1 staining was limited to the microvasculature and was generally more focal in distribution, usually associated with the presence of mononuclear cell infiltration in close proximity of VCAM-1-positive microvessels (Figure 2). EMBs from four patients who developed transplant rejection episodes more than 2 months after transplantation (median, 94 days), ICAM-1 and VCAM-1 staining initially returned to baseline levels and then subsequently increased, preceding histological rejection by up to 22 days. Of these four late rejection episodes, three were predicted by a significant rise in ICAM-1 (14, 14 and 22 days before histological rejection) and two were predicted by a significant rise in VCAM-1 staining (both 14 days before histological rejection). In the one case of late rejection that was not predicted with either intense ICAM-1 or VCAM-1 staining, the only EMB before histological rejection was 29 days before the diagnostic biopsy. Quantitative measurements of serial myocardial ICAM-1 and VCAM-1 levels (by RIA) before histological rejection are presented in Figure 3. Myocardial ICAM-1 and VCAM-1 levels rise slowly before rejection and peak levels are frequently found 1 to 2 weeks before histological rejection. Even at 3 to

4 weeks before rejection, mean ICAM-1 and VCAM-1 levels are significantly elevated as compared with values measured in patients who never experience rejection (typically with ratios of CAM cpm/myosin cpm of <0.05; see Figure 7).

Although there was no apparent relationship between ICAM-1/VCAM-1 levels and histology rejection grades, because rejection was diagnosed in a parallel biopsy specimen and processed in a manner different from the study biopsy, we further assessed whether increased levels of ICAM-1/VCAM-1 simply reflected CAM expression on increased numbers of infiltrating inflammatory cells. We subjected the first four post-transplantation biopsies in all patients to the same RIA protocol described above using 25 μl of anti-common leukocyte antigen (CLA) to provide a direct measure of infiltrating leukocytes. Standardization curves using increasing concentrations of human peripheral blood mononuclear cells (ranging from $1 \times 10^2$ to $1 \times 10^6$ cells/well) showed an appropriate linear relationship between increasing CLA levels and increasing peripheral blood mononuclear cells (data not shown). Figure 4 demonstrates ICAM-1, VCAM-1, and CLA levels determined by RIA in post-transplant biopsies 1 through 4. No apparent relationship between the extent of infiltrating leukocytes and CAM induction could be identified in these biopsies.

## *Induction of ICAM-1 and VCAM-1 Staining as a Predictor of Recurrent Histologic Rejection Episodes (Figure 5)*

To determine whether persistently increased ICAM-1 and VCAM-1 staining could help monitor the efficacy of immunosuppressive therapy, we divided all rejection episodes into those that were followed by another rejection episode within 2 months (repeaters, n = 17) and those that were not (non-repeaters, n = 12). When biopsy samples were examined for intense ICAM-1 and VCAM-1 staining 2 weeks after a course of immunosuppressive therapy was started, 100% and 76% of repeater biopsies had persistently intense ICAM-1 and VCAM-1 staining, respectively, whereas only 25% and 9% of non-repeater biopsies did ($P$ = 0.0001 and 0.0008, respectively). In contrast, no differences in histology rejection scores were present in repeaters *versus* non-repeaters 2 weeks after initiation of immunosuppressive therapy. Persistence of intense ICAM-1 or VCAM-1 staining 2 weeks after initiation of immunosuppressive therapy for moderate rejection was associated with a positive and negative predictive value for recurrent rejection within 2 months of 75% and 100%, respectively. Quantitative myocardial ICAM-1 and VCAM-1 levels by RIA following treated rejection episodes confirmed the results obtained by immunohistology and are presented in Figure 6. Those patients who develop a recurrent rejection episode within 2 months of treatment (repeater) clearly have sustained elevations in quantitative myocardial ICAM-1 and VCAM-1 levels as compared to patients who do not develop recurrent rejection (non-repeaters).

To determine whether there were significant differences in the quantity of myocardial ICAM-1 and VCAM-1 expression among individual patients, we measured myocardial ICAM-1 and VCAM-1 levels determined by RIA in patients with no rejection (n = 4), early rejection (n = 8) and late rejection (n = 6) (Figure

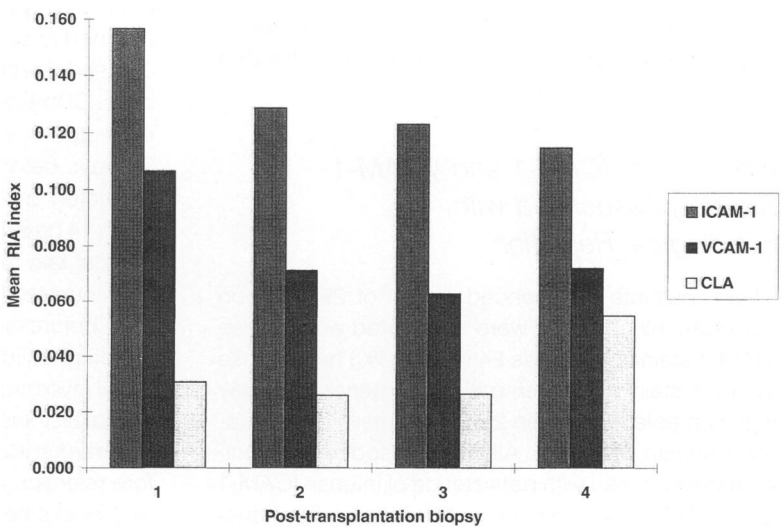


**Figure 4.** *Mean quantitative myocardial ICAM-1, VCAM-1, and CLA levels determined by RIA in post-transplantation biopsies 1 through 4.*

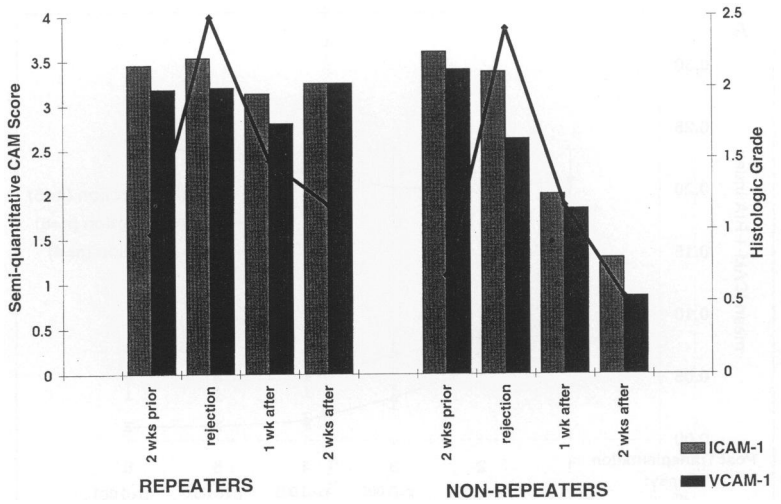


Figure 5. *Mean histological grade and semi-quantitative grades for ICAM-1 and VCAM-1 immunostaining surrounding rejection episodes in repeater and non-repeater biopsies. Repeater biopsies are defined as those patients whose histologic rejection episodes were followed by another rejection episode within 2 months following additional immunosuppression. Non-repeater biopsies are defined as those patients whose histological rejection episodes were not followed by another rejection episode within 2 months following additional immunosuppression.*

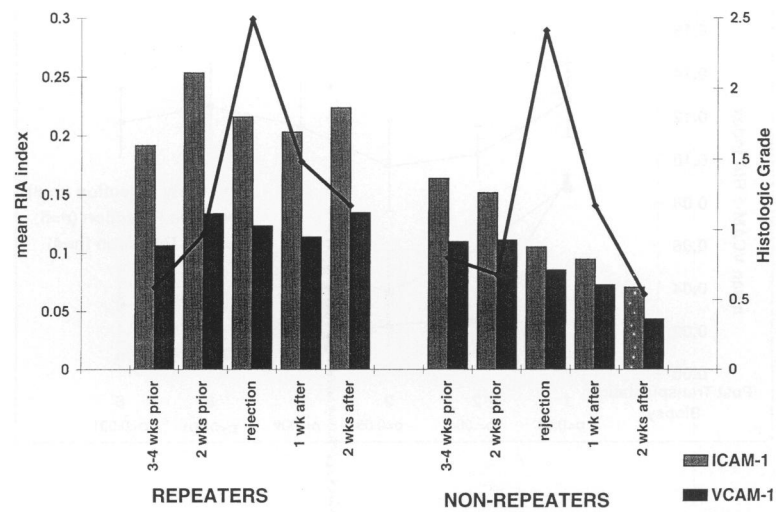


Figure 6. *Mean histologic grade and quantitative myocardial ICAM-1 and VCAM-1 levels determined by RIA surrounding rejection episodes in repeater and non-repeater biopsies. Repeater biopsies are defined as those patients whose histological rejection episodes were followed by another rejection episode within 2 months following additional immunosuppression. Non-repeater biopsies are defined as those patients whose histological rejection episodes were not followed by another rejection episode within 2 months following additional immunosuppression.*

7A, B). In patients who experience early rejection episodes, increased myocardial ICAM-1 levels and, to a lesser extent, VCAM-1 levels are more pronounced at post-transplantation biopsy 1 and remain persistently elevated through biopsy 6.

Immunohistology results for ICAM-1 and VCAM-1 were strongly correlated, with an $r$ value of 0.9 ($P < 0.0001$). RIA values for ICAM-1 and VCAM-1 were likewise significantly correlated ($r = 0.89$, $P < 0.0001$). Correlation coefficients for immunohistology and RIA results were as follows: ICAM-1, $r = 0.72$ ($P < 0.0001$) and VCAM-1, $r = 0.71$ ($P < 0.0001$).

## Discussion

A large body of evidence has shown that the expression of CAMs on endothelial cells and their respective ligands on leukocytes are responsible for adhesion, activation, and trafficking of lymphocytes. Essentially, three molecular families of adhesion molecules and their ligands have been identified: integrins (LFA-1$\alpha$/LFA-1$\beta$ and VLA-4), the immunoglobulin supergene family (CD4, CD8, CD58, CD54, BB1/B7, VCAM-1, major histocompatibility complex Class I and Class II antigens, CD2, and CD28), and the selectins (ELAM-1, LECAM, and CD62).[21,26] It is clear that leukocytes that normally remain in the circulation only cross the endothelium and enter the extravascular parenchyma following adhesion to activated endothelial cells. Inflammatory cytokines such as IL-1$\beta$ and TNF-$\alpha$ lead to the expression of CAMs on endothelial cells[27–30] and myocytes[21] and thus may facilitate adhesion and extravasation of circulating leukocytes into the myocardial interstitial space. In efforts to gain insight into the possible utility of monitoring

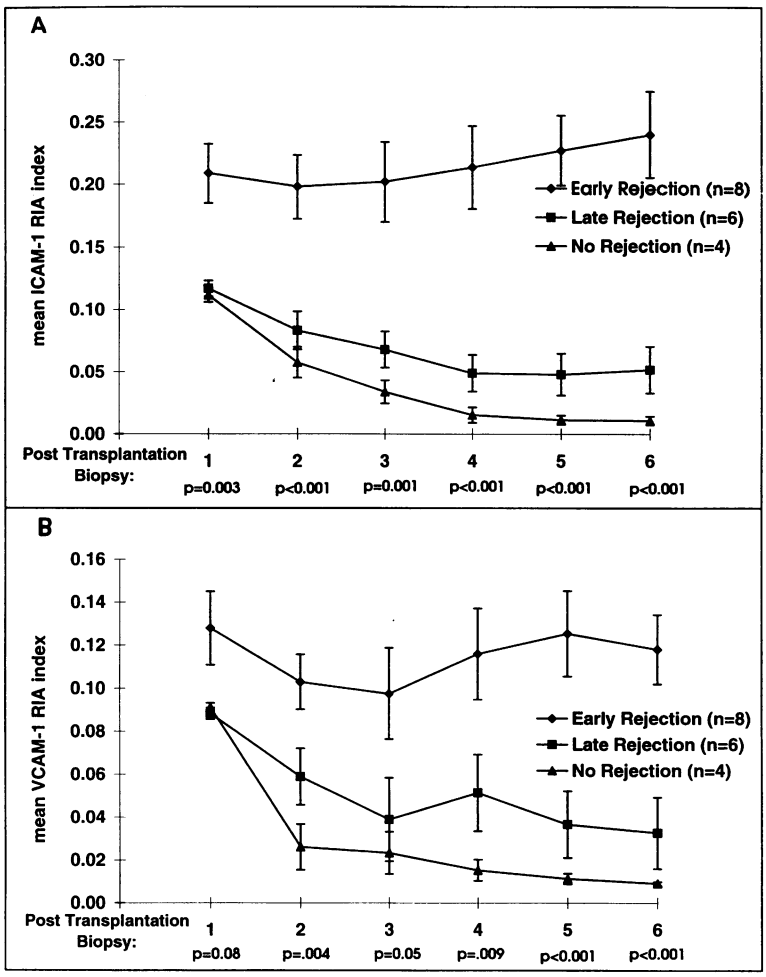


Figure 7. *Quantitative myocardial ICAM-1 levels* (A) *and VCAM-1 levels* (B) *as determined by RIA in select patients with no rejection* ($n = 4$), *early rejection* ($n = 8$), *and late rejection* ($n = 6$), *after cardiac transplantation. Results are expressed as means and standard errors. Comparisons of RIA values at each post-transplant biopsy were made between the three groups using an ANOVA.*

myocardial CAM induction as a marker of rejection, qualitative levels of ICAM-1, VCAM-1, ELAM-1, and P-selectin were measured prospectively in sequential human cardiac allograft biopsies. In addition, quantitative measurements for the two induced CAMs, ICAM-1, and VCAM-1 were also performed by RIA.

Our results demonstrate three main findings. First, the initial myocardial induction of ICAM-1 and VCAM-1 after transplantation is seen in the majority of, if not all, patients and may represent a manifestation of either an early immune response that gets attenuated with further immunosuppression or to post-implantation reperfusion injury. The expression of ICAM-1 and VCAM-1 protein within the myocardium then returns to baseline levels typically within 14 to 28 days unless a histological rejection episode develops. Second, induction of ICAM-1 and VCAM-1 within the myocardium is typically associated with histological rejection episodes and may precede diagnostic histological findings by 2 to 3 weeks. Third, persistence of CAM induction in follow-up biopsies 1 to 2 weeks after a treated rejection episode is associated with recurrent rejection episodes within a 2-month period.

Immunohistology revealed intense induction of ICAM-1 and VCAM-1 in virtually every biopsy specimen immediately after transplantation. This pattern of induction persisted for up to 4 weeks after transplantation before returning to baseline levels in those patients who did not develop a superimposed rejection episode. Increased ICAM-1 and VCAM-1 levels were not apparently related to the extent of leukocyte infiltration as determined either by histology grade or by quantitative CLA levels. In support of the hypothesis that myocardial induction of ICAM-1 and VCAM-1 after transplantation is related to post-implantation reperfusion injury is the uniform post-transplant rise in serum levels of troponin T noted in all patients. Troponin T is a cardiac-specific marker of ischemic myocyte damage, which is significantly more sensitive than routine CK-MB measurements.[24,25] Although a relationship between increased serum troponin T lev-

els and operative trauma cannot be ruled out, the persistence of serum troponin T elevations were more consistent with ongoing ischemic myocyte injury rather than transient operative trauma. In addition, the time courses of both elevated serum troponin T levels and myocardial ICAM-1 and VCAM-1 induction are coincident with the time frame of histological evidence of subendocardial ischemia, which also persists in EMBs up to 3 to 4 weeks after transplant.[31] Induced CAM expression during the immediate posttransplantation period is consistent with other studies that have shown induction of CAMs on microvascular endothelium with ischemia/reperfusion.[32] The importance of CAM induction is also supported by studies that have demonstrated that therapy with antibodies to ICAM-1 lead to reduction in reperfusion injury.[33]

Quantitative measurements by RIA suggest significant differences in the quantity of myocardial ICAM-1 and VCAM-1 expression among individual patients. Patients who experienced early rejection episodes appeared to have significantly higher myocardial ICAM-1 and VCAM-1 levels in the immediate 3 to 4 weeks after transplantation. These findings suggest that variations in the levels of induction of CAMs in response to either reperfusion or operative trauma or as a manifestation of the earliest phases of alloactivation before steady state immunosuppression is reached may play a role in the development of early rejection episodes.

A growing number of studies have shown increased expression of CAMs during rejection episodes in solid tissue allografts.[34–36] It has also been well demonstrated that proinflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$, which are known to induce CAM expression, play an important local role in the early phases of acute rejection, presumably by activating endothelial cells to upregulate the expression of adhesion molecules.[35,37–40] In our current study, the localization of maximum induction of ICAM-1 and VCAM-1 protein expression on microvascular endothelium was frequently within areas of mononuclear cell infiltration. Although we did not determine whether these mononuclear cells secreted cytokines, it is likely that cytokine-induced CAM expression plays an important role in allograft rejection. Data from our current study suggests that 1) myocardial induction of intense ICAM-1 and VCAM-1 expression by immunohistochemistry may precede histologic rejection by 2 to 3 weeks and 2) quantitatively, ICAM-1 and VCAM-1 levels appear to peak one week before histologic rejection. Induction of CAM expression in the myocardium may therefore precede the influx of large numbers of mononuclear cells typical of histologic rejection, a pattern that has been similarly described with liver allograft rejection.[41]

It is important to note that, although induced VCAM-1 expression was limited to the microvasculature, induced expression of ICAM-1 on myocytes was also demonstrated in intensely staining biopsy samples. This pattern of ICAM-1 expression is likely not an artifact. Our previous studies[21] and others[42] have demonstrated that increased ICAM-1 expression can be induced on cultured human cardiac myocytes upon exposure to proinflammatory cytokines. Seko et al[42] similarly found cytokine-mediated induction of myocyte expression of ICAM-1 in mouse cardiac myocytes *in vitro*. Induced ICAM-1 expression on myocytes has also been described in the setting of ischemia/reperfusion injury[43] and myocarditis.[44] In any case, selective induction of ICAM-1 on cardiac myocytes may represent a mechanism by which enhanced mononuclear cell-myocyte adherence is accomplished.

The failure to detect increased levels of ELAM-1 and CD62 on cardiac biopsy samples in our study was somewhat surprising. Several reasons could account for this finding. First of all, both these CAMs are only transiently induced following activation of endothelial cells.[45,46] Thus, it is possible that the sequential biopsies studied may have been obtained from patients at time intervals that did not allow for the assessment of such increases. Second, while other CAMs such as ICAM-1 and VCAM-1 are either constitutively expressed and/or induced to express by several cell lineages, the expression of ELAM-1 and CD62 may be more restricted, which would contribute to the sensitivity of either the immunohistological assays or the RIA that we utilized. Finally, it should be noted that several other studies have similarly shown a failure in the detection of ELAM-1[8,9,14] during cardiac rejection episodes. In addition, our finding of a lack of ELAM-1 and CD62 inducibility on myocardial endothelium and a distinct distribution of ICAM-1 and VCAM-1 is similar to that described in liver transplant rejection.[3] These results may be due to the problems discussed above or may suggest that posttransplantation inflammation *in vivo* may not mirror *in vitro* findings for the expression of certain CAMs.

Histological examination of post-rejection follow-up biopsies usually demonstrates marked improvement in interstitial inflammation and myocyte injury. Repeat or recurrent rejection episodes within short periods of follow-up, however, are common and, in our current study, 59% of the rejection episodes encountered by our study population were followed by recurrent rejection episodes within a 2-month period. Our data suggest that monitoring myocardial

ICAM-1 and VCAM-1 expression in the first two post-rejection biopsies is a sensitive and specific method to distinguish which patients are at high risk for recurrent rejection episodes. Our data are consistent with the hypothesis that, in the subgroup of patients who experience recurrent rejection, clones of alloactivated lymphoid cells may persist and cause persistent induction of ICAM-1 and VCAM-1. We have recently also found a similar pattern of persistent induction of major histocompatibility complex class I and class II antigens in those post-rejection biopsies that are followed by subsequent episodes of recurrent rejection (unpublished obervations).

A number of recent studies have described different patterns of expression of CAMs, largely in randomly selected biopsy samples in cardiac transplant recipients.[7–12,14,15] Carlos et al[11] showed a clear correlation between VCAM-1 and rejection but not with ICAM-1. Tanio et al[7] described a strong correlation between rejection and ICAM-1 expression but not VCAM-1 or ELAM-1. Ferran et al[12] described increased ELAM-1 and VCAM-1 staining in serial biopsies from four of six patients who developed rejection during the course of study. Our current study results suggests three important factors that underscore the need to longitudinally study serial biopsy samples from individual patients starting at the time of cardiac transplantation in order to determine the precise role of CAM induction in human cardiac transplant rejection: 1) the significant rise in myocardial ICAM-1 and VCAM-1 levels in all patients immediately after transplantation, apparently unrelated to rejection; 2) the induction of ICAM-1 and VCAM-1 expression preceding histologic rejection by as much as 3 weeks; and 3) persistence of ICAM-1 and VCAM-1 expression in a subgroup of post-rejection follow-up biopsies in patients subject to recurrent rejection. These factors may help explain apparent discrepancies relating the temporal association between CAM induction and rejection.

In conclusion, our study suggests that longitudinal monitoring of myocardial ICAM-1 and VCAM-1 expression could be useful both in monitoring the development of initial rejection episodes and in determining the efficacy of immunosuppressive therapy more sensitively than histology. In addition, it demonstrates that CAM induction may precede histological evidence of rejection by up to several weeks. The concept that rejection may take weeks or longer to develop is consistent with the clinical observation that rejection episodes are slower to progress since the advent of the immunosuppressive drug cyclosporine. The potential role of individual patient variations in the quantity of constitutive and induced myocardial ICAM-1 and VCAM-1 levels after transplantation and the development of initial and subsequent rejection episodes requires further study.

## Acknowledgments

We wish to acknowledge the assistance of Dr. Daniel Chan in performing the serum troponin T and CK-MB assays.

## References

1. 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 Transplant 1990, 9:587–592
2. Shimizu Y, Newman W, Gopal TV, Horgan KJ, Graber N, Beall LD, van Seventer G, Shaw S: Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. J Cell Biol 1992, 113:1203–1212
3. Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K: Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia: Lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2, and LFA-3. Am J Pathol 1993, 142:481–488
4. Bacchi CE, Marsh CL, Perkins JD, Carithers RL Jr, McVicar JP, Hudkins KL, Benjamin CD, Harlan JM, Lobb R, Alpers CE: Expression of vascular cell adhesion molecule (VCAM-1) in liver and pancreas allograft rejection. Am J Pathol 1993, 142:579–591
5. Hancock WH, Whitley D, Tullius SG, Heemann UW, Wasowska B, Baldwin WM, Tilney NL: Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. Transplantation 1993, 56: 643–650
6. Brockmeyer C, Ulbrecht M, Schendel DJ, Weiss EH, Hillebrand G, Burkhardt K, Land W, Gokel MJ, Riethmuller G, Feucht HE: Distribution of cell adhesion molecules (ICAM-1, VCAM-1, ELAM-1) in renal tissue during allograft rejection. Transplantation 1993, 55: 610–615
7. Tanio JW, Basu CB, Albelda SM, Eisen HJ: Differential expression of the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin in normal and posttransplantation myocardium cell adhesion molecule expression in human cardiac allografts. Circulation 1994, 89: 1760–1768
8. Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober JS: Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. Transplantation 1991, 51:537–539
9. 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
10. Rose M, Page C, Hengstenberg C, Yacoub M: Immunocytochemical markers of activation in cardiac transplant rejection. Eur Heart J 1991, 12:147–150
11. Carlos T, Gordon D, Fishbein D, Himes VE, Coday A, Ross R, Allen MD: Vascular cell adhesion molecule-1 is induced on endothelium during acute rejection in human cardiac allografts. J Heart Lung Transplantation 1992, 11:1103–1109
12. Ferran C, Peuchmaur M, Desruennes M, Ghoussoub JJ, Cabrol A, Brousse N, Cabrol C, Bach JF, Chatenoud L: Implications of de novo ELAM-1 and VCAM-1 expression in human cardiac allograft rejection. Transplantation 1993, 55:605–609
13. Pelletier RP, Morgan CJ, Sedmak DD, Miyake K, Kincade PW, Ferguson RM, Orosz CG: Analysis of inflammatory endothelial changes, including VCAM-1 expression in murine cardiac grafts. Transplantation 1993, 55:315–320
14. Taylor PM, Rose ML, Yacoub MH, Pigott R: Induction of vascular adhesion molecules during rejection of human cardiac allografts. Transplantation 1992, 54:451–457
15. Qiao JH, Ruan XM, Trento A, Czer LSC, Blanche C, Fishbein MC: Expression of cell adhesion molecules in human cardiac allograft rejection. J Heart Lung Transplant 1992, 11:920–925
16. Kameoka H, Ishibashi M, Tamatani T, Takano Y, Moutabarrik A, Jiang H, Kokado Y, Takahara S, Okuyama A, Kinoshita T, Miyasaka M: The immunosuppressive action of anti-CD18 monoclonal antibody in rat heterotopic heart allotransplantation. Transplantation 1993, 55:665–667
17. Sadahiro M, McDonald TO, Allen MD: Reduction in cellular and vascular rejection by blocking leukocyte adhesion molecule receptors. Am J Pathol 1993, 142: 675–683
18. Isobe M, Yagita H, Okumura K, Ihara A: Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science 1992, 255: 1125–1127
19. Ansari AA, 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 posttransplantation. Transplantation 1988, 45:972–978
20. Herskowitz A, Ansari AA, Neumann DA, Beschorner WE, Rose NR, Soule LM, Burek CL, Sell KW, Baughman KL: Induction of major histocompatibility complex (MHC) antigens within the myocardium of patients with active myocarditis: a nonhistologic marker of myocarditis. J Am Coll Cardiol 1990, 15:624–632
21. Ansari AA, Sundstrom B, Runnels H, Jensen P, Kanter K, Mayne A, Herskowitz A: The absence of constitutive and induced expression of critical cell-adhesion molecules on human cardiac myocytes. Transplantation 1994, 57:942–949
22. Ansari AA, Wang Y-C, Danner DJ, Gravanis MB, Mayne A, Neckelmann N, Sell KW, Herskowitz A: Abnormal expression of histocompatibility and mitochondrial antigens by cardiac tissue from patients with myocarditis and dilated cardiomyopathy. Am J Pathol 1991, 139:337–354
23. Wang YC, Neckelmann N, Mayne A, Herskowitz A, Srinivasan A, Sell KW, Ansari AA: Establishment of a human fetal cardiac myocyte cell line. In Vitro Cell Dev Biol 1991, 27A:63–74
24. Hamm CW, Ravkilde J, Gerhardt W, Jorgensen P, Peheim E, Ljungdahl L, Goldmann B, Katus HA: The prognostic value of serum Troponin T in unstable angina. N Engl J Med 1992, 327:146–150
25. Zimmerman R, Baki S, Dengler TJ, Ring GH, Remppis A, Hagi S, Kubler W, Katus HA: Troponin T release after heart transplantation. Br Heart J 1993, 69:395–398
26. 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
27. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA, Jr: Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J Clin Invest 1985, 76:2003–2011
28. Pober JS, Lapierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA, Jr: Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. J Immunol 1987, 138:3319–3324
29. Bevilacqua MP: Endothelial-leukocyte adhesion molecules. Annu Rev Immunol 1993, 11:767–804
30. Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R: Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 1989, 59:1203–1211
31. Hossein-Nia M, Brown P, Jennison SH, McKenna WJ, Holt DW: The use of CK-MB isoforms as a noninvasive marker of rejection following heart transplantation. Clin Chem 1992, 38:976
32. Kukielka GL, Hawkins HK, Michael L, Manning AM, Youker K, Lane C, Entman ML, Smith CW, Anderson DC: Regulation of intercellular adhesion molecule-1 (ICAM-1) in ischemic and reperfused canine myocardium. J Clin Invest 1993, 92:1504–1516
33. Byrne JG, Smith WJ, Murphy MP, Couper GS, Appleyard RF, Cohn LH: Complete prevention of myocardial stunning, contracture, low-reflow, and edema after heart transplantation by blocking neutrophil adhesion molecules during reperfusion. J Thorac Cardiovasc Surg 1992, 104:1589–1596
34. Lukacs NW, Kunkel SL, Burdick MD, Strieter RM: The

production of chemotactic cytokines in an allogeneic response: the role of intercellular adhesion molecule-1 and lymphocyte function-associated antigen-3. Am J Pathol 1993, 143:1179–1188

35. Hoffmann MW, Wonigeit K, Steinhoff G, Herzbeck H, Flad H-D, Pichlmayr R: Production of cytokines (TNF-alpha, IL-1-beta) and endothelial cell activation in human liver allograft rejection. Transplantation 1993, 55: 329–335
36. Adams DH, Shaw J, Hubschler SG, Rothlein R: Intercellular adhesion molecule 1 on liver allografts during rejection. Lancet 1989, 1122–1124
37. Wu CJ, Lovett M, Wong Lee J, Moeller F, Kitamura M, Goralski TJ, Billingham ME, Starnes VA, Clayberger C: Cytokine gene expression in rejecting cardiac allografts. Transplantation 1992, 54:326–332
38. Krams SM, Falco DA, Villaneuva JC, Rabkin J, Tomlanovich SJ, Vincenti F, Amend WJC, Melzer J, Garovoy MR, Roberts JP, Ascher NL, Martinez OM: Cytokine and T cell receptor gene expression at the site of allograft rejection. Transplantation 1992, 53:151–156
39. Vandenbroecke C, Caillat-Zucman S, Legendre C, Noel L-H, Kreis H, Woodrow D, Bach J-F, Tovey MG: Differential *in situ* expression of cytokines in renal allograft rejection. Transplantation 1991, 51:602–609
40. Ruan XM, Qiao JH, Trento A, Czer LSC, Blanche C, Fishbein MC: Cytokine expression and endothelial cell and lymphocyte activation in human cardiac allograft rejection: an immunohistochemical study of endomyocardial biopsy samples. J Heart Lung Transplant 1992, 11:1110–1115
41. Lautenschlager I, Höckerstedt K: Induction of ICAM-1 on hepatocytes precedes the lymphoid activation of acute liver allograft rejection and cytomegalovirus infection. Transplant Proc 1993, 25:1429–1430
42. Seko Y, Matsuda H, Kato K, Hashimoto Y, Yagita H, Okumura K, Yazaki Y: Expression of intercellular adhesion molecule-1 in murine hearts with acute myocarditis caused by coxsackievirus B3. J Clin Invest 1993, 91:1327–1336
43. Youker K, Smith CW, Anderson DC, Miller D, Michael LH, Rossen RD, Entman ML: Neutrophil adherence to isolated adult cardiac myocytes. J Clin Invest 1992, 89:602–609
44. Toyozaki T, Saito T, Takano H, Yorimitsu K, Kobayashi S, Ichikawa H, Takeda K, Inagaki Y: Expression of intercellular adhesion molecule-1 on cardiac myocytes for myocarditis before and during immunosuppressive therapy. Am J Cardiol 1993, 72:441–444
45. Sugama Y, Tiruppathi C, Janakidevi K, Andersen TT, Fenton II JW, Malik AB: Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. J Cell Biol 1992, 119:935–944
46. McEver RP: Selectins. Curr Opin Immunol 1994, 6:75–84.