American Journal of Pathology, Vol. 150, No. 2, February 1997

Analysis of Chronic Rejection and Obliterative Arteriopathy

Possible Contributions of Donor Antigen-Presenting Cells and Lymphatic Disruption

A. J. Demetris,* N. Murase,† Q. Ye,†
F. H. F. Galvao,† C. Richert,* R. Saad,*
S. Pham,† R. J. Duquesnoy,* A. Zeevi,*
J. J. Fung,† and Thomas E. Starzl†

From the Transplant Institute, Departments of Pathology and Surgery,† Divisions of Transplantation, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania*

Sequential analysis of changes that lead to chronic rejection was undertaken in an animal model of chronic rejection and obliterative arteriopathy. Brown Norway rats are pretreated with a Lewis bone marrow infusion or a Lewis orthotopic liver allograft and a short course of immunosuppression. They are challenged 100 days later with a Lewis heterotopic heart graft without immunosuppression. The heart grafts in both groups undergo a transient acute rejection, but all rats are operationally tolerant; the heart grafts are accepted and remain beating for more than 100 days. Early arterial remodeling, marked by arterial bromodeoxyuridine incorporation, occurred in both groups between 5 and 30 days during the transient acute rejection. It coincided with the presence of interstitial (but not arterial intimal) inflammation and lymphatic disruption and resulted in mild intimal thickening. Significant arterial narrowing occurred only in the bone-marrow-pretreated rats between 60 and 100 days. It was associated with T lymphocyte and macrophage inflammation of the heart graft that accumulated in the endocardium and arterial intima and adventitia near draining lymphatics. There also was loss of passenger leukocytes from the heart graft, up-regulation of cytokine mRNA and major histocompatibility class II on the endothelium, and focal disruption of lymphatics. In contrast, long-surviving heart grafts from the Lewis orthotopic liver allograft pretreated group are near normal and freedom from chronic rejection in this group was associated with persistence of donor major histocompatibility class-II-positive hematolymphoid cells, including OX62$^+$ donor dendritic cells. This study offers insights into two different aspects of chronic rejection: 1) possible mechanisms underlying the persistent immunological injury and 2) the association between immunological injury and the development of obliterative arteriopathy. Based on the findings, it is not unreasonable to raise the testable hypothesis that direct presentation of alloantigen by donor antigen-presenting cells is required for long-term, chronic-rejection-free allograft acceptance. In addition, chronic intermittent lymphatic disruption is implicated as a possible mechanism for the association between chronic interstitial allograft inflammation and the development of obliterative arteriopathy. (Am J Pathol 1997, 150:563–578)

Obliterative arteriopathy (OA), the pathognomonic lesion of chronic rejection (CR),[1–4] is thought to be the result of direct immunological injury to the artery. It has been proposed that attachment of lymphocytes to the allogeneic arterial endothelium and subsequent endothelial damage create abnormalities that trigger intimal myofibroblast proliferation. This eventually progresses to arterial luminal narrowing.[1–3]

Supported by a grant from the American Heart Association, Pennsylvania Affiliate.

Accepted for publication October 19, 1996.

Address reprint requests to Dr. A. J. Demetris, Department of Pathology, Division of Transplantation, BMST E1548, University of Pittsburgh, Pittsburgh, PA 15213.

As CR and OA are difficult to study in clinical samples, much of the research comes from small experimental animal models that rely on minor major histocompatibility (MHC) mismatches between the donor and recipient.[5,6] These animal models and some clinical observations[7] generally support the above sequence of events. However, a number of basic clinical and some experimental observations about OA are not readily explained by that scenario: 1) OA preferentially involves medium-sized intra-organ muscular arteries over veins, despite an antigenically similar endothelium, 2) inflammatory arteritis does not always precede OA, 3) in general, pulmonary arteries are less severely affected and pulmonary veins are more severely affected than corresponding coronary vessels in heart-lung transplant recipients,[8,9] 4) the endothelium of affected vessels is frequently intact,[2,8] 5) foam cell OA is most common in hepatic allografts,[2] 6) mechanical factors in medium-sized muscular arteries are not ideal for lymphocyte attachment and exocytosis, except during severe acute rejection, and 7) the reasons for reversible, antigen-dependent and irreversible antigen-independent phases of OA are not readily apparent.[10] Several of these observations suggest that, in addition to immunological injury, mechanical factors are important in the genesis of OA. Furthermore, mechanisms underlying the continued immunological reactivity of CR are poorly understood.

The discovery of microchimerism in successful organ allograft recipients[11–13] and its disappearance in recipients with CR[13–15] offered possible insights into immunological mechanisms underlying this often indolent form of allograft injury. This contention was based on the considerable evidence that natural hematopoietic chimerism observed in freemartin cattle[16] or iatrogenically induced hematopoietic chimerism in neonates or adult experiment animals[17–20] results in a robust form of graft acceptance. Although other laboratories have confirmed our findings, they have questioned whether the microchimerism observed in patients might be an epiphenomenon of graft acceptance by some other mechanism.[21–23]

In an effort to address questions related to microchimerism, an experimental model of CR was inadvertently developed.[24] The current analysis of this model led us to propose pathogenetic mechanisms of CR that contribute to an understanding of the association between chimerism and graft acceptance and current inconsistencies in the pathogenesis of OA.

Materials and Methods

Animals and Transplant Procedures

Details of the operative procedures and immunosuppression protocol were described elsewhere.[24] Briefly, 200- to 300-g male Lewis (LEW, $RT1^l$) and Brown Norway (BN, $RT1^n$) rats (Harlan Sprague Dawley, Indianapolis, IN) were used as donors and recipients, respectively, for all experiments. Liver allografts were placed orthotopically after recipient hepatectomy.[25] Heart grafts were vascularized heterotopically in the abdomen.[26] Bone marrow cells (2.5×10^8 cells; >90% viable by Trypan blue exclusion) were washed from the medullary cavity of tibias and femurs, processed in RPMI 1640 supplemented with 25 mmol/L Hepes buffer, 2 mmol/L L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco, Grand Island, NY) and given by intravenous injection into the penile vein.[24]

Experimental Design

BN recipients were pretreated with a LEW orthotopic liver allograft (OLTx) or an infusion of LEW bone marrow cells (BM) on day 0 and given intramuscular injections of 1.0 mg/kg/day Tacrolimus (FK506; dissolved in HCO-60 and D-mannitol, Fujisawa Pharmaceutical Co., Osaka, Japan) for 14 days starting on the day of transplantation and supplemented with single injections at the same dose on days 20 and 27. This treatment regimen after BM or OLTx produces hematolymphoid microchimerism for at least 100 days. Subsequent challenge with a heterotopic LEW but not a third-party heart allograft on day 100 results in graft acceptance for more than 100 days without immunosuppression.[24] LEW cardiac grafts transplanted into recipients given the brief Tacrolimus regimen alone are rejected.[24] However, CR is observed in the heart allografts after 100 days of survival of BM-pretreated rats but not those pretreated with OLTx.[24]

To serially investigate the changes leading to CR in the BM-pretreated group in comparison with the OLTx-pretreated recipients, at least three rats from each group with a beating heart allograft were sacrificed 5, 10, 15, 30, 60, and 100 days after placement of the heart. Controls consisted of heterotopic LEW or BN heart grafts transplanted into syngeneic LEW or BN recipients, respectively. At the time of sacrifice, the challenging cardiac grafts and the native hearts were serially sectioned in a transverse plane across both ventricles. Separate pieces were appropriately triaged

Table 1. *Panel of Immunohistochemical and Histochemical Reagents Used to Characterize the Chronic Rejection Model*

Histochemical stain or monoclonal antibody	Supplier or method	Labeling specificity
R7.3	Sera-Lab*	T lymphocytes with $\alpha\beta$-receptors
OX62	Sera-Lab	T lymphocytes with $\gamma\delta$-receptors and dendritic cells
W3/25 (CD4)	Sera-Lab	T helper subset, all macrophages
OX8 (CD8)	Sera-Lab	T cytotoxic-suppresser subset
OX33	Sera-Lab	B cells
ED1	Sera-Lab	Circulating macrophages
ED2	Sera-Lab	Tissue macrophages
L21-6	Gift of Y. Iwaki	Invariant chain of MHC class II of most rat strains, except BN
OX6 (HLA-DR)	Sera-Lab	Ia common part determinant
alpha smooth muscle actin	Dako†	α-Smooth muscle isoform of actin found in smooth muscle
5' nucleotidase	Kato et al[30]	Lymphatic endothelium and lymphocyte subsets

*Sera-Lab, Crawley Down, UK.
†Dako, Carpinteria, CA.

for routine histopathology, immunohistochemistry, and cytokine mRNA analysis (see below).

Routine Pathology Studies

All slides generated from the heart grafts were read without knowledge of the pretreatment group or timing of the sample. The overall severity of inflammation in the endocardium, pericardium, interstitium, and periarterial spaces was semiquantitatively graded on a scale of 0 to 4 as none, minimal, mild, moderate, or severe. Arterial alterations, including the presence of inflammation, edema, fibrosis, and vacuolization of the intima, media, and adventitia were graded in the same fashion. All of the arteries >80 μm in shortest external diameter present in one cross section of the heart allografts at 60 and 100 days after transplantation were evaluated for the severity of OA. The grading of OA was as follows: none, grade 0; minimal, grade 1, < 10% luminal narrowing; mild, grade 2, 10 to 25%; moderate, grade 3, 25 to 50%; moderate/severe, grade 4, 50 to 75%; and severe, grade 5, > 75% luminal narrowing.

Immunohistochemical Studies

Immunohistochemical staining with a routine indirect avidin-biotin complex method[27] was used to determine the phenotype of inflammatory cells infiltrating the heart allografts (Table 1). At least two animals from each group were examined at each time point. An immunoglobulin class-matched nonimmune antibody was substituted for the primary antibody in the negative controls. As with the routine histopathological studies, the immunostained slides were reviewed without knowledge of the pretreatment regimen or timing of the sample. The relative number of positively stained cells in endocardium, pericardium, interstitium, and perivascular spaces was semiquantitatively scored for each antibody on a scale of 0 to 4, as described above.

The monoclonal antibody L21-6, which reacts with donor (LEW), but not recipient (BN) MHC class II antigen, was used to differentiate donor from recipient cells.[28] The number of donor MHC class II^+ cells was determined by selecting a representative field and counting the L21-6^+ cells at ×40 magnification. Double immunofluorescent labeling with Cy3 (indocarbocyanine)-conjugated L21-6 and ED1, ED2, or OX62 reacted with fluorescein isothiocyanate was used to determine the phenotype of donor MHC class II^+ hematolymphoid cells present in the heart grafts.[29]

The number of cells in the arterial wall undergoing DNA synthesis was determined by pulsing the rats 1 hour before sacrifice with an intravenous injection of 60 mg/kg bromodeoxyuridine (BrdU) and localizing the labeled cells with anti-BrdU monoclonal antibody, as above. Separate counts were recorded for the intima, media, and adventitia of all intramyocardial arteries for at least two animals at each time point. Only vessels cut in cross section were included in the counts.

Histochemical staining for 5'-nucleotidase activity, which reacts with lymphatic but not with arterial, venous, or capillary endothelium,[30,31] was used to highlight the lymphatic channels.

Cytokine mRNA Analysis

Total RNA was extracted from a portion of the heart allograft using the RNAzol B modified method[32] and the concentration determined by spectrophotometry. cDNA was synthesized by transcription from RNA in the presence of human placental RNAse inhibitor, 1 nmol/L deoxynucleoside triphosphates, oligo-dT primer, murine leukemia virus reverse transcriptase, and reverse transcriptase buffer. The reverse tran-

Table 2. *Primer Sequences of Cytokines Used to Detect Cytokine mRNA in Tissue Samples of Challenge Cardiac Allografts*

Primers	Primer sequence	Product size
(r)IL-2	5' ACG CTT GTC CTC CTT GTC AAC 3' CAG ATG GCT ATC CAT CTC CTC	401 bp
(r)IL-6	5' ACA GCG ATG ATG CAC TGT CAG 3' ATG GTC TTG GTC CTT AGC CAC	337 bp
(r)TNF-α	5' TGT CTA CTG AAC TTC GGG GTG 3' GAG GCT GAC TTT CTC CTG GTA	365 bp
(r)IFN-γ	5' CAA GGC ACA CTC ATT GAA AGC 3' CTC GAA CTT GGC GAT GCT CAT	297 bp
(r)IL-10	5' TTA CTT GGG TTG CCA AGC CTT 3' GTT CAC AGA GAA GCT CAG T	495 bp
(m)β-Actin	5' TTC TAC AAT GAG CTG CGT GTG 3' TTC ATC GAT GCC ACA GGA TTC	627 bp

r, rat; m, mouse; TNF, tumor necrosis factor; IFN, interferon.

scriptase polymerase chain reaction (RT-PCR) of the resulting cDNA was performed according to the method of Brenner et al[33] with some modifications[34] using [^{32}P]dCTP-labeled primers specific for the rat cytokines interleukin (IL)-2, IL-6, IL-10, interferon-γ, and tumor necrosis factor-α (Table 2). Amplification was carried out for 30 cycles on a model 480 thermal cycler (Perkin Elmer, Norwalk, CT). As an internal control for RNA quality, all samples were also assessed for β-actin. For negative controls, PCR amplification was performed with sterile water substituted for cDNA. PCR products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. The product of amplification was also electrophoresed on an 8% polyacrylamide gel, which was dried and analyzed by autoradiography. The amount of radioactivity incorporation in the PCR product was counted using a β-scan. The results were normalized to β-actin and expressed as a ratio of cytokine/β-actin.

Statistical Analyses

The results of continuous variable measurements are presented as the mean ± SD or SEM, and the Student's *t*-test was used for the statistical comparison of groups. The Mann-Whitney *U* test was used for a comparison of the ordinal noncontinuous variables, such as the severity of inflammation, OA, and immunoperoxidase staining. The level of significance for both tests was set at a *P* value of 0.05.

Results

Routine Histopathology and Lymphocytic Infiltration of the Heart Allografts

Serial routine and immunohistological studies were carried out to follow the evolution of changes leading to CR and OA in the BM-pretreated rats in comparison with the CR-resistant OLTx-pretreated recipients. For convenience of presentation, the time after transplantation was broken down into the early period extending from 5 and 30 days and the late period encompassing the 60- and 100-day time points.

During the early time period, the challenging cardiac allografts underwent an acute rejection crisis. It peaked between 5 and 15 days, regardless of the pretreatment group (Figure 1, A–C) and was characterized by mononuclear inflammation somewhat concentrated in the endocardium, pericardium, and perivascular spaces, with more patchy interstitial involvement. The quantity of inflammation was significantly less in the OLTx-pretreated rats (Figure 2) than in those primed with BM for all time points after transplantation. In general, the lymphocytic infiltrates seen early after transplantation in both groups were similar in composition. They consisted predominantly of $\alpha\beta$-receptor-positive T cells, with the CD4$^+$ subset predominating over the CD8$^+$ cells and a minimal number of OX33$^+$ B cells (data not shown). However, the number of ED2$^+$ tissue macrophages significantly decreased in the BM-pretreated rats, whereas the number of these cells remained near normal in the OLTx-pretreated rats during the early time period (see below).

The mononuclear inflammation persisted in the heart grafts from BM-pretreated recipients (Figures 1, D and E, and 2) but not in the OLTx-pretreated rats during the late phase. In the former, the inflammation eventually coalesced into nodular aggregates in the endocardium, pericardium, and perivascular spaces of blood vessels and lymphatic channels at 60 and 100 days. These had routine histopathological and immunohistochemical similarities to Quilty lesions in humans[35] (Figure 1F), which are mostly circumscribed subendocardial nodular aggregates of lym-

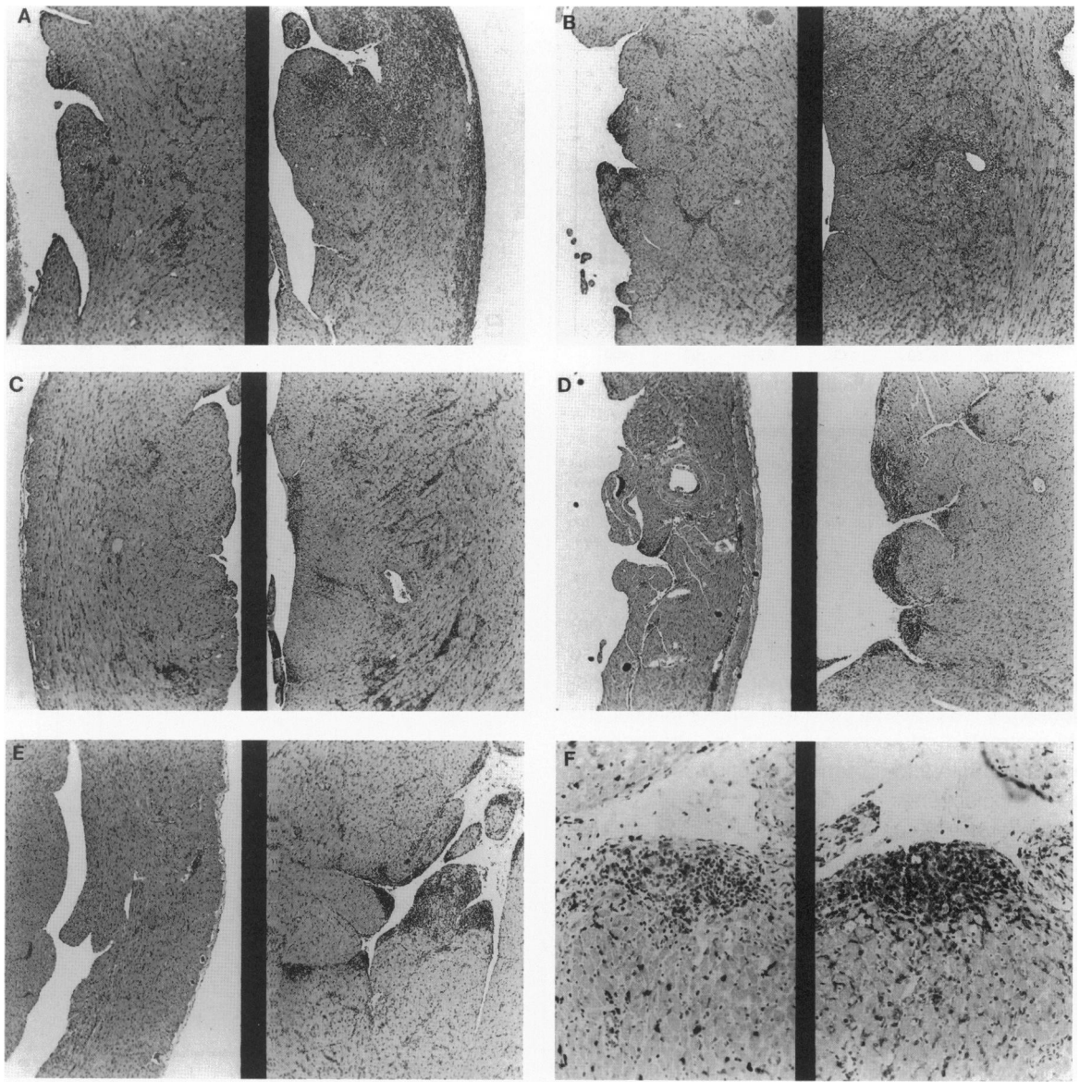

Figure 1. A *to* **E:** *Sequential histopathological appearance of the challenge heart allografts at 5, 10, 15, 60, and 100 days after transplantation, respectively. The left side of each photomicrograph is from an OLTx-pretreated recipient and the right side is from a BM-pretreated rat. Note the transient inflammation in the OLTx-pretreated rats. In the BM-pretreated rats, the inflammation coalesced into subendocardial aggregates by 60 days after transplantation.* **F:** *High power (×300) view of endocardial infiltrate in a BM-pretreated rat 100 days after transplantation, stained for CD8 (left side) and CD4 (right side) using an indirect immunoperoxidase procedure. Note the predominance of the CD4$^+$ T cells in the endocardial lymphoid aggregates and similarities to Quilty lesions in human endomyocardial biopsies. Magnification, ×18 (except for* **F**).

phocytes detected in endomyocardial biopsies. Similar to the aggregates detected in humans, the endocardial infiltrates in this study were predominantly composed of $\alpha\beta$-receptor-positive T lymphocytes, with CD4$^+$ cells outnumbering CD8$^+$ cells by 3:1 to 4:1. CD8$^+$ cells were evenly distributed throughout the lesions, and occasionally small nodules of OX33$^+$ B cells were detected. The cardiac interstitium showed an increase in the number of $\alpha\beta$-receptor-positive T cells.

Cytokine mRNA Analysis of Heart Allografts

Analysis of the cardiac allografts for IL-2, IL-6, IL-10, tumor necrosis factor-α, and interferon-γ mRNA provided additional important functional information about the immunological milieu of the heart allografts (Figure 3). Early after transplantation, in both the OLTx- and BM-pretreated rats, increased message for interferon-γ, tumor necrosis factor-α, and IL-10 correlated with the mononuclear inflammation and

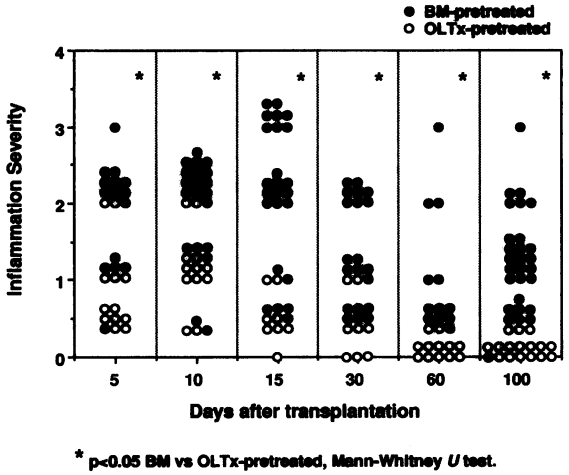

Figure 2. *A scatter plot of the overall severity of inflammation shows that the BM-pretreated challenge grafts were more severely inflamed. The severity of inflammation was scored at four sites (endocardial, pericardium, perivascular areas, and interstitium) in each graft. Two to four grafts from BM-pretreated (●) or OLTx-pretreated (○) recipients were analyzed at each time point.*

neo-expression of MHC class II on the vascular endothelial cells (see below). IL-2 and IL-6 were also increased, but to a lesser extent. Late after transplantation, the persistent allograft inflammation in the BM-pretreated rats correlated with the presence of cytokine mRNA and up-regulation of class II MHC on the vascular endothelium. In contrast, cytokine mRNA largely disappeared from the cardiac allografts of OLTx-pretreated recipients by 60 days and remained undetectable throughout the period of follow-up.

Phenotype and Origin of Infiltrating Macrophages in the Heart Allografts

Recipient macrophages play an important role in the development of CR and OA,[5] whereas donor dendritic cells (DCs) have been detected in microchimeric recipients[13,36] with uncomplicated long-term courses. Therefore, we thought it was important to determine whether differences in the macrophage and related cell populations existed between the two groups, which might be related to the development of CR.

The changes in macrophage populations mirrored the inflammatory pathology within the heart allografts. In both groups during the early period, the number of spindle-shaped ED2$^+$ tissue macrophages decreased, whereas the number of infiltrative ED1$^+$ macrophages and OX62$^+$ DCs increased compared with the number of these interstitial cells in a normal heart. By day 10, there were significantly more ED2$^+$ tissue macrophages remaining in the

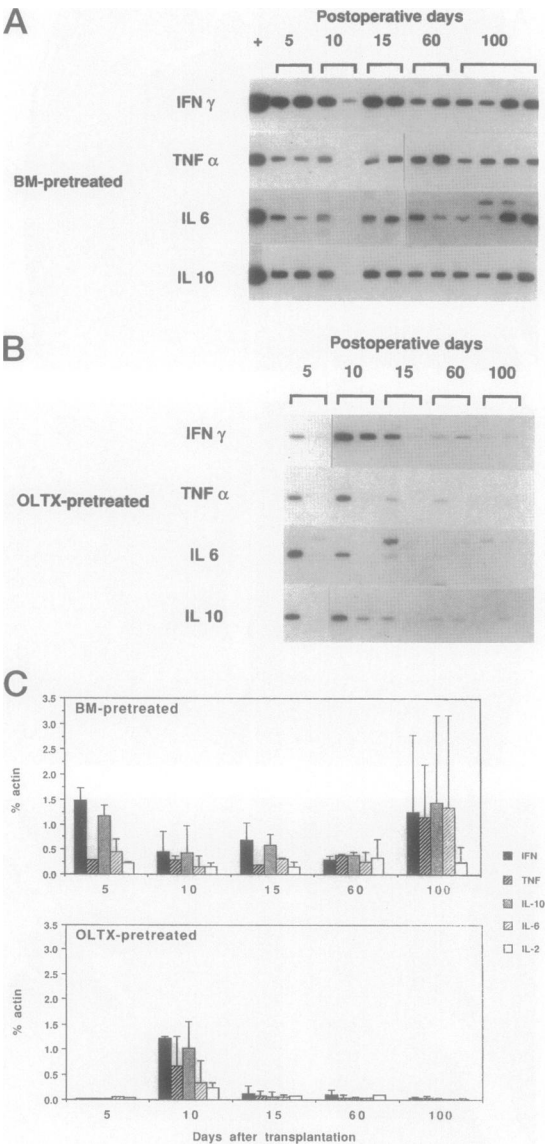

Figure 3. *Southern blot and hybridization of RT-PCR products obtained from assays for cytokine mRNA in the challenge heart allografts of BM-pretreated recipients* (**A**) *and OLTx-pretreated rats* (**B**) *at various times after transplantation. Note the waning signals for interferon-γ, tumor necrosis factor-α, IL-6, and IL-10 in the challenge heart of the OLTx-pretreated rats and the persistent message for these cytokines in the challenge heart grafts of the BM-pretreated recipients.* **C**: *Semiquantitative analysis (see Materials and Methods) of cytokine mRNA levels, based on a comparison to β-actin levels in the same tissue. The top half of the figure represents the BM-pretreated recipients and the bottom half is from the OLTx-pretreated rats. Again, note the difference in cytokine message between the two groups, particularly late after transplantation.*

heart grafts of OLTx-pretreated recipients (Figure 4) and a trend toward fewer ED1$^+$ infiltrative macrophages compared with the BM-pretreated recipients.

During the late phase after transplantation the inflammation disappeared from the heart grafts of OLTx-pretreated rats. The macrophage population in this group was similar to that seen in the interstitium

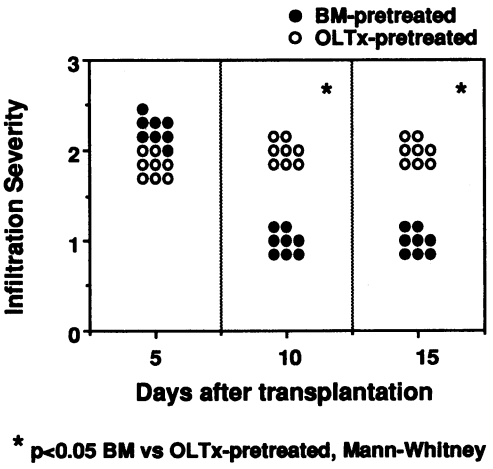

Figure 4. *A scatter plot of the relative number of ED2$^+$ interstitial macrophages present in the challenge allografts of OLTx-pretreated* (○) *and BM-pretreated* (●) *recipients during the early time period. Note the rapid decrease in the number of these cells in the BM-pretreated rats but not in the OLTx-pretreated recipients.*

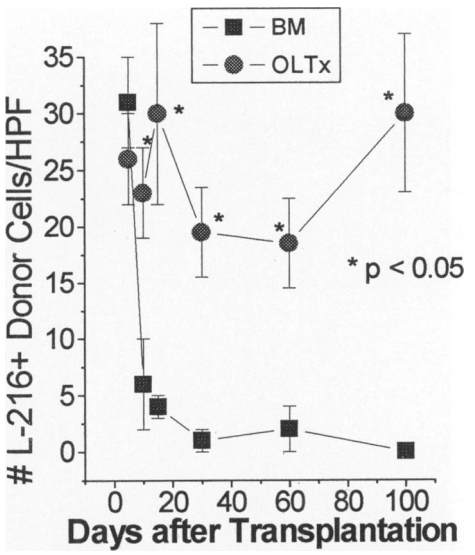

Figure 5. *The number of donor L21-6$^+$ cells/high-power field (HPF) in the cardiac allografts at various times after transplantation.* *P < *0.05 for BM* versus *OLTx-pretreated recipients (Student's t-test).*

of a normal heart. It consisted of numerous spindle-shaped ED2$^+$ tissue macrophages and fewer ED1$^+$ infiltrative macrophages. There was no arterial intimal macrophage infiltration. In contrast, there were significantly more ED1$^+$ infiltrative macrophages and fewer ED2$^+$ tissue macrophages in the heart grafts of BM-pretreated rats compared with the OLTx-pretreated rats. These ED1$^+$ infiltrative macrophages were scattered throughout the interstitium and formed cuffs around the adventitia of arteries that showed intimal thickening typical of OA (see below). The increased number of OX62$^+$ DCs in the BM-pretreated rats were concentrated amid the lymphoid aggregates. In addition, ED1$^+$ macrophages and occasional OX62$^+$ DCs also appeared in the intima of arteries affected by OA in BM-pretreated rats at 60 and 100 days.

The number of donor MHC class II$^+$ (L21-6$^+$) interstitial hematolymphoid cells rapidly decreased during the early phase in the BM-pretreated rats. There was only a transient decrease in these same cells during the early phase in OLTx-pretreated recipients (Figure 5). During the late period in the BM-pretreated rats, donor LEW MHC class II$^+$ interstitial cells disappeared from the heart grafts. They were still easily detectable in the OLTx-pretreated recipients, even 100 days after transplantation of the heart allograft (Figure 6).

Double immunofluorescent labeling was used to determine the phenotype of the L21-6$^+$ spindle-shaped interstitial cells remaining in heart allografts at 100 days. In the OLTx-pretreated recipients, the majority of donor class II$^+$ cells were ED2$^+$ tissue macrophages (Figure 6C) with occasional ED1$^+$ infiltrative macrophages. L21-6$^+$/OX62$^+$ donor DCs were also clearly detected (Figure 6D), although they were fewer in number than the ED2$^+$ macrophages and primarily located in the adventitia of arteries and in the endocardium and pericardium. L21-6 staining of the heart allografts from the BM-pretreated recipients at 100 days showed only endothelial positivity (Figure 6A); no double labeling of ED1, ED2, or OX62 cells was detected in this group.

Donor Hematolymphoid Cells in the Recipient Skin and Liver Allografts

We next determined whether donor MHC class II$^+$ cells were also present in the skin and in the original allograft. In the liver allografts, there were usually more than ten L21-6$^+$ cells per portal triad at 200 days after OLTx, which was 100 days after heart graft placement. Three to six L21-6$^+$ cells were detected around each terminal hepatic venule. The biliary epithelium and vascular endothelium were L21-6$^-$. In the recipient skin 100 days after heart transplantation, L21-6$^+$ cells were detected around papillary dermal capillaries, in the adventitia of dermal arteries, and in the perineural space of OLTx-pretreated recipients. L21-6$^+$ cells were not detected in the skin of the BM-pretreated rats.

Sequential Arterial Pathology and Remodeling Kinetics

We next focused on the documentation of sequential arterial changes leading to CR and OA in the BM-

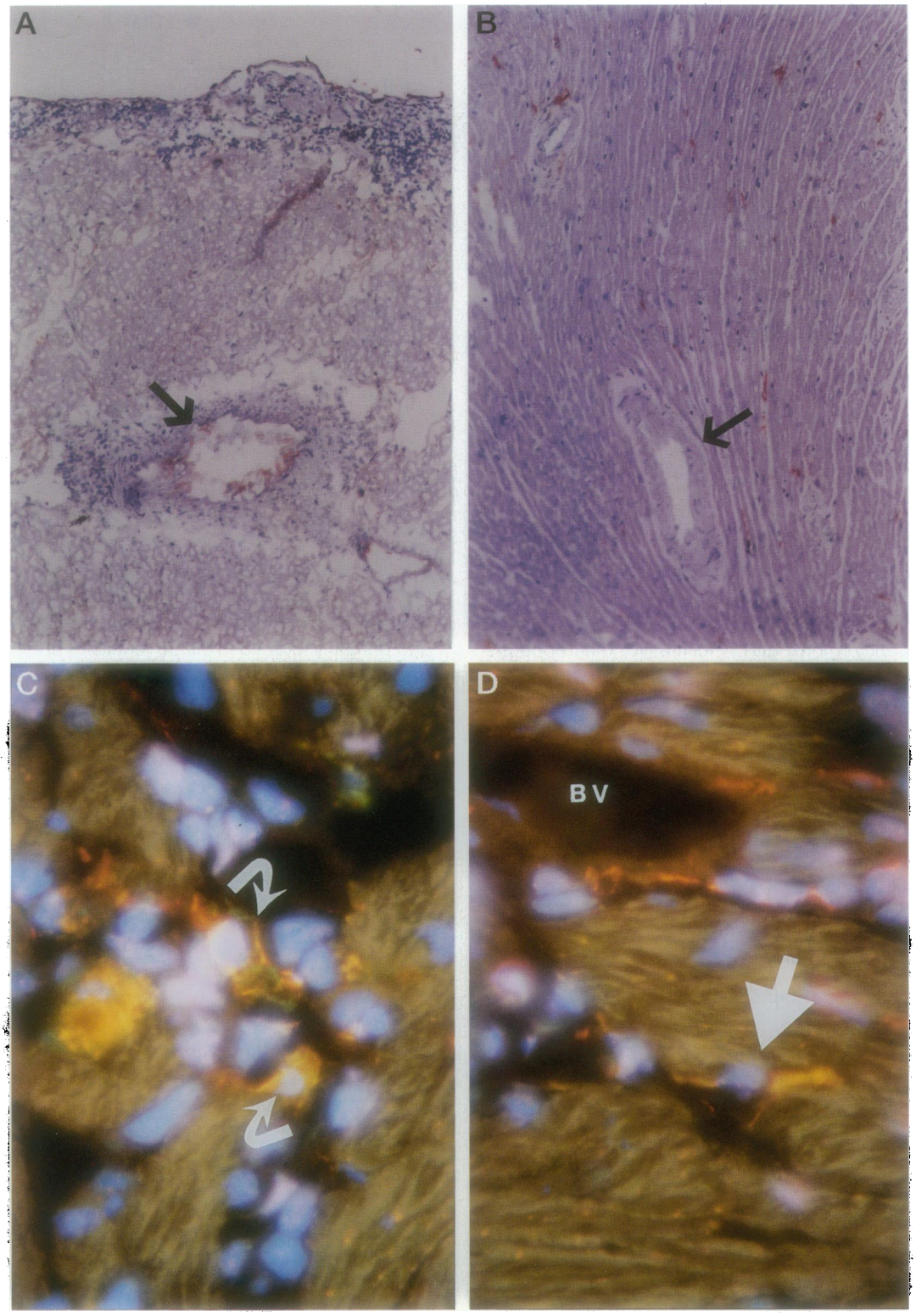
A
B
C
D
BV

Table 3. *Comparison of the Severity of Obliterative Arteriopathy in Challenge Allografts at 60 and 100 Days after Transplantation*

Treatment	Days after transplantation	Number of animals	Number of arteries scored	Average grade of lesion	Percent of arteries with disease
OLTx	60	3	22	0.27 ± 0.12*	23%
BM	60	5	37	1.19 ± 0.29	46%
OLTX	100	6	42	0.19 ± 0.09**	14%
BM	100	8	63	1.11 ± 0.18	50%

Isograft controls did not show any significant OA and therefore, are not included in this table.
*P = 0.03, Mann-Whitney U test for OLTx *versus* BM at same time point.
**P = 0.0004, Mann-Whitney U test for OLTx *versus* BM at same time point.

pretreated group. These were compared with the changes seen in the CR-resistant, OLTx-pretreated rats to determine whether the differences observed could lead to a better understanding of OA development.

The arterial pathology observed during the early phase in both the BM- and OLTx-pretreated rats was similar (Figure 7). On day 5, when focal inflammation and up-regulation of MHC class II were seen in the interstitium, edema and inflammatory cells first appeared in the adventitia of intramyocardial arteries. The media and intima were relatively unremarkable at this time, with the latter showing flattened, inactive-appearing endothelial cells. On day 10, the adventitial edema and inflammation increased, and medial edema and disintegration of individual medial myocytes appeared. The arterial endothelium became hypertrophied (Figure 7B) and MHC class II^+ at this time. By day 15, subendothelial intimal edema appeared and the media assumed an edematous, loose, disorganized appearance. In contrast to acute vascular rejection, there was no intimal inflammation observed in any of the animals.

During the late phase, obvious differences appeared in the arterial pathology between BM- and OLTx-pretreated recipients (Table 3). In BM-pretreated rats, the mild intimal abnormalities noted during the early phase worsened or progressed to severe OA by 60 and 100 days (Table 3), with continual inflammation of the adventitia. Arteries 100 to 300 μm in diameter were most often and most severely affected. In general, there were two types or stages of OA lesions in the BM-pretreated rats, which were similar to those seen in humans.[2] One consisted of an intimal accumulation of $ED1^+$ macrophages that was intermixed with α-smooth muscle actin-positive myofibroblasts, and some $CD4^+$ and $CD8^+$ T cells (Figure 7, F and G). In addition, rare $OX62^+$ dendritic cells could also be detected, similar to humans.[1] The neointimal cells were usually underlying an intact but hypertrophied endothelial layer showing expression of donor LEW MHC class II antigens. The other type or stage of OA lesion consisted of intimal thickening associated with an increased number of α-smooth muscle actin-positive intimal myofibroblasts with fewer $ED1^+$ foam cells and rare T cells. In both lesions, the media was thinned and focally traversed by $ED1^+$ macrophages, presumably coming from a cuff of $ED1^+$ adventitial macrophages.

OLTx-pretreated rats did not exhibit these arterial lesions. However, occasional arteries from animals in this group showed disorganization of medial myocytes and mild intimal thickening that was more severe than the changes seen in the LEW-LEW isograft controls (Table 3).

Quantitative Analysis of Arterial Wall DNA Synthesis

Because the intimal thickening in OA involves the proliferation of intimal myofibroblasts,[2] BrdU labeling was used to sequentially study the kinetics of arterial remodeling in this model. It showed that the onset of arterial cell proliferation coincided with the mural edema and disorganization of medial myocytes detected during the early phase on routine microscopy. It occurred before there was any detectable arterial intimal inflammation (Figures 7H and 8). Compared with normal, nontransplanted hearts in which arterial $BrdU^+$ cells are rare, increased BrdU labeling was first observed in the adventitia of arteries at day 5,

Figure 6. A: *L21-6 immunoperoxidase stain of challenge allografts for donor MHC-class-II-positive cells 100 days after transplantation in a BM-pretreated recipient. Note the positive stain on endothelial cells of an artery mildly affected by OA* (arrow). *Magnification, ×120.* B: *The same L21-6 immunoperoxidase stain for donor MHC-class-II-positive cells in the challenge allograft of an OLTx-pretreated rat showed numerous positive hematolymphoid cells, whereas the arterial endothelium was L21-6 negative and unaffected by OA* (arrow). *Magnification, ×120.* C: *Double immunofluorescent labeling for L21-6 (red Cy3) and ED2 (green fluorescein isothiocyanate) in the challenge allograft of an OLTx-pretreated recipient showed that many of the donor hematolymphoid cells present in the organ were $ED2^+$ tissue macrophages* (curved arrows). *Magnification, ×1000.* D: *Double immunofluorescent labeling for L21-6 (red Cy3) and OX62 (green fluorescein isothiocyanate) showed rare double-positive (yellow) cells* (arrow). *BV, blood vessel. Magnification, ×1000.*

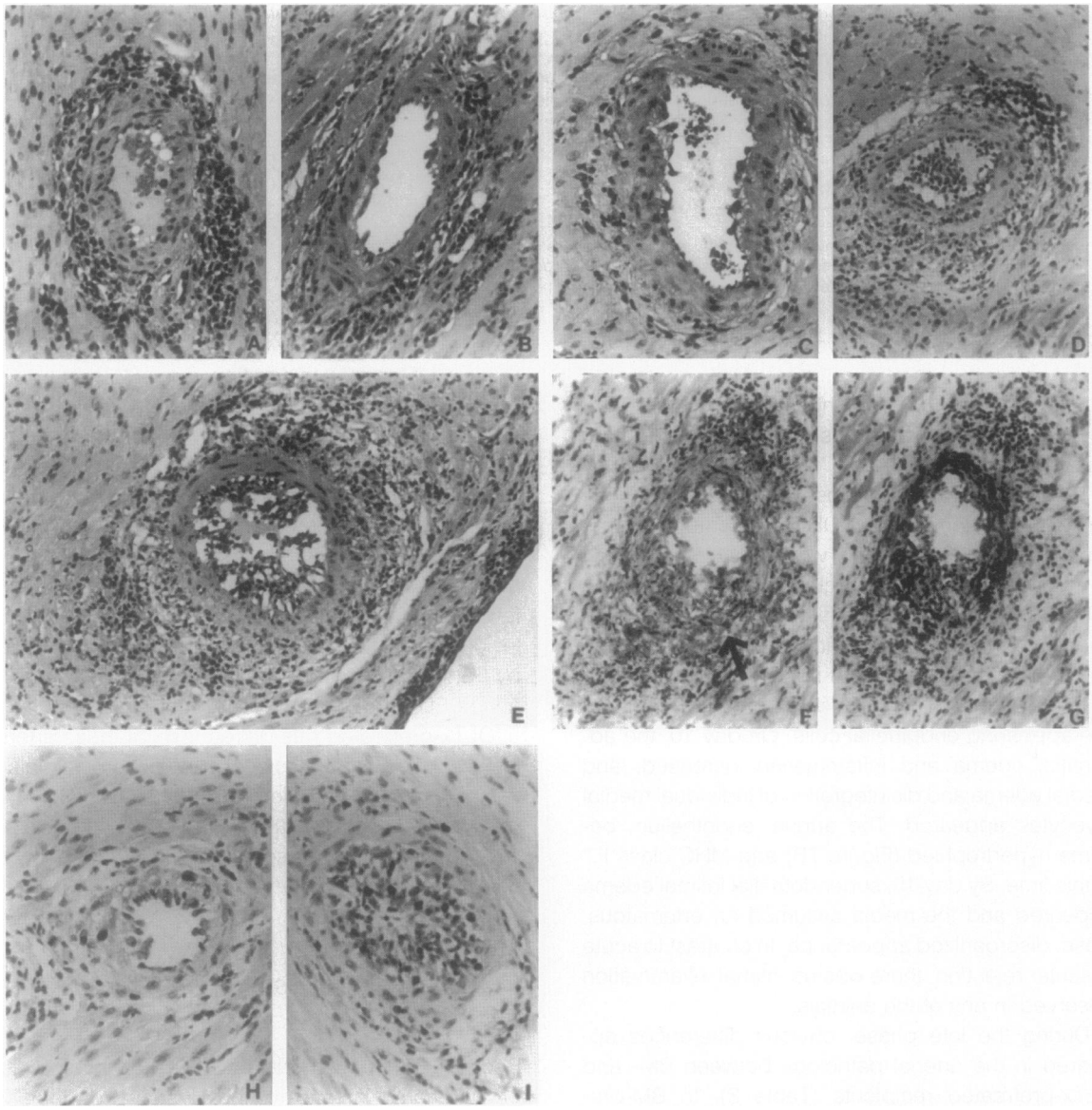

Figure 7. *Sequential arterial alterations in a BM-pretreated rat.* **A:** *On day 5, the arterial alterations were limited to adventitial inflammation and edema. Note the flattened endothelial cells.* **B:** *On day 10, the endothelial cells assumed a hypertrophied appearance and necrosis of individual medial myocytes was seen. H&E; magnification, ×200.* **C:** *On day 15, the endothelial cells became more hypertrophied, subendothelial edema appeared, and the medial myocytes showed a disorganized appearance. H&E; magnification, ×200.* **D:** *On day 30, some vacuolization of endothelial cells was seen, and the medial and adventitial changes persisted. H&E; magnification, ×100.* **E:** *On day 60, intimal infiltration by lymphocytes and macrophages became apparent. Note also the persistent adventitial changes H&E; magnification, ×100.* **F:** *On day 100, ED1$^+$ macrophages* (**arrow**) *cuffed the adventitia and traversed the media of affected arteries. IPEX for ED1; magnification, ×100.* **G:** *These infiltrative macrophages were found intermixed with α-smooth muscle actin-positive cells in the thickened intima. IPEX for α-smooth muscle actin; magnification, ×200.* **H:** *BrdU labeling of arteries from challenge grafts at 10 days after transplantation. IPEX for BrdU; magnification, ×200. Note the presence of positive cells, even without any evidence of arterial inflammation.* **I:** *By 100 days after transplantation of the challenge grafts, the number of BrdU$^+$ cells in the intima markedly increased. This increased intimal labeling was typical of the late, progressive phase of OA. IPEX for BrdU; magnification, ×200.*

which then remained at high levels for 100 days. An increase in BrdU$^+$ cells in the media did not occur until day 15, whereas those in the intima slowly increased during the early phase. Interestingly, a temporally similar pattern of increased BrdU labeling was seen in the isograft controls during the early phase. However, the overall magnitude of the response in the isografts was significantly less than seen in allograft recipients (data not shown).

Late after transplantation, the number of BrdU$^+$ intimal cells markedly increased in the BM-pretreated rats at 60 and 100 days (Figure 7I). The population of stained cells consisted of an admixture of endothelial cells, myofibroblasts, and inflamma-

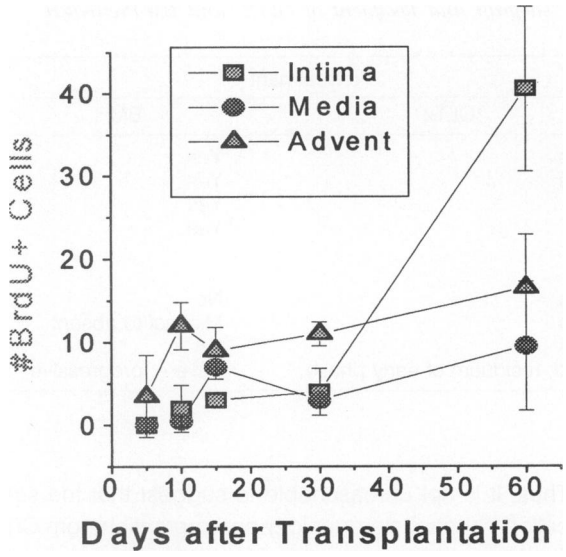

Figure 8. *Number of cells in the intima, media, and adventitia synthesizing DNA (BrdU+) at various times after transplantation in BM-pretreated rats (n = 2 at each time point).*

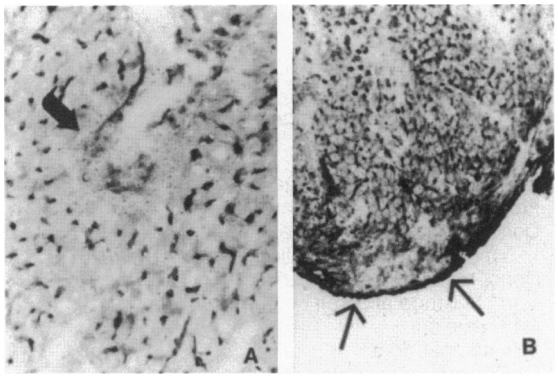

Figure 9. A: *Early after transplantation, staining for 5′-nucleotidase showed disruption of the thin-walled lymphatic channels by invading mononuclear cells* (arrow) *that formed aggregates near these vessels (10 days after transplantation in a BM-pretreated rat). 5′-Nucleotidase stain; magnification, ×300.* B: *Late after transplantation, the endocardial infiltrates displaced or focally destroyed the subendocardial lymphatic channels. Note the lack of lymphatic vessels within this lesion* (arrows) *but intense labeling just subjacent to the endocardial surface (60 days of transplantation). 5′-Nucleotidase stain; magnification, ×150.*

tory cells. In contrast, BrdU labeling decreased to isograft control levels in the OLTx-pretreated rats, which was limited to a rare positive cell.

Sequential Lymphatic and Microvascular Pathology

Some of the arterial changes described above are similar to those seen after lymphatic obstruction alone.[37] In addition, lesions similar to OA are seen in arteries at the base of ulcers and in arteries feeding carcinomas that have invaded lymphatic channels (personal observation). We therefore wondered whether lymphatic channels were disrupted in the heart allografts and whether the peculiar anatomical distribution of the lymphoid aggregates was related to the lymphatic drainage of the heart. Therefore, 5′-nucleotidase enzyme histochemistry was used to highlight the lymphatic channels.

In the normal heart, 5′-nucleotidase stained a network of small, capillary-sized lymphatic channels between cardiac myocytes. Positively stained endothelial cells also lined smaller and larger thin-walled lymphatics seen near the pericardial surface and immediately subjacent to the endocardium.

Early after transplantation, in both the BM- and OLTx-pretreated recipients, the aggregates of mononuclear cells that first appeared in the endocardium, pericardium, and adventitial space of arteries localized near larger thin-walled lymphatic channels. As the inflammation intensity increased, infiltration and damage to the wall of lymphatic channels were easily recognized (Figure 9A). The edematous adventitial space of arteries during this time also showed dilated and/or disrupted lymphatic channels. In addition, medial myocytes, intercalated disks between cardiac myocytes, and endothelial cells of arteries with medial edema (see below) acquired smudgy staining for 5′-nucleotidase. In the microvasculature, there was up-regulation of MHC class II on the endothelial cells, which was distinct, diffuse, and sustained in the BM-pretreated rats. In contrast, microvascular class II MHC up-regulation was mild, focal, and transient in OLTx-pretreated rats during this time.

The endocardial infiltrates that developed late after transplantation were situated near the 5′-nucleotidase-positive subendocardial lymphatics. The interstitium within these inflammatory cell aggregates was either devoid of lymphatic channels or showed disrupted ones (Figure 9B). The same was true for the pericardial and perivascular lymphoid aggregates. Staining for 5′-nucleotidase in the grafts from OLTx pretreated rats and LEW isografts were similar to a normal heart, except for disruption of lymphatic channels in the pericardial tissue. In addition, there was continuous up-regulation of MHC class II on the microvasculature of BM-pretreated recipients but not on those given OLTx.

A summary of the overall results comparing the OLTx- to the BM-pretreated allograft recipients is shown in Table 4.

Table 4. *Comparison of the Findings in the Challenge Cardiac Allograft and Recipient of OLTX- and BM-Pretreated Recipients*

Characteristic	Pretreatment	
	OLTx	BM
Acute rejection changes	Yes	Yes
Early-phase arterial changes	Yes	Yes
Persistent inflammation at 100 days	No	Yes
Donor reactive lymphocytes and cytokine mRNA in challenge allograft at 100 days	No	Yes
Donor hematolymphoid cells at 100 days (see text)		
In challenge allograft	Yes	No
In recipient	Yes	Minimal to absent
In original graft	Yes	NA
Obliterative arteriopathy at 100 days	Mild, residuum of early phase	Severe, progressive

NA, not applicable.

Discussion

The results presented here offer insights into two different aspects of the study of CR: 1) possible mechanisms underlying the persistent immunological injury and 2) the association between immunological injury and the development of OA. First, however, a practical observation deserves special emphasis: assessment of long-term cardiac allograft survival in experimental animals must include a detailed histopathological assessment of the graft. As this study has shown, ignoring the histology can result in an erroneous assumption of tolerance when the graft is chronically rejecting.[21,22] Functional donor specificity of graft-infiltrating lymphocytes cultured from the chronically inflamed hearts from the BM-pretreated group was shown in primed lymphocyte testing assays. Lymphocytes could not be cultured from the OLTx-pretreated heart grafts at 100 days (data not shown).

The observation that protection from CR was seen only in OLTx-pretreated rats offers some insight into the possible mechanisms underlying the persistent immunological injury in BM-pretreated rats. In isolation, this observation seems to support the contention that the allograft itself provides the most important tolerogenic signal.[21,22,38] Indeed, it would be easy to envisage the accumulation of anergic T cells produced by circulation through the large allogeneic liver devoid of antigen-presenting cells.[38] However, when alloantigen expression is limited to hepatic parenchymal cells using hepatocyte-specific promoters in transgenic mice, activation-induced lymphocyte deletion is incomplete and the livers remain chronically inflamed.[39] Furthermore, neither pretreatment with a donor heart allograft,[24] nor the heart graft itself in the BM-pretreated group, is able to provide protection from CR, despite the presence of significant nonimmunogenic parenchymal tissue. Thus, it is not unreasonable to suggest that the so-called hepatic tolerogenicity and protection from CR lies in a property other than the large component of parenchymal cells.

In this study, persistence of the donor hematolymphoid cells, including the highly immunogenic donor DCs,[40] was associated with freedom from CR. Instead of parenchymal tissue delivering a tolerogenic signal, the grafted liver may provide an especially good microenvironmental niche for delivering and/or sustaining donor hematopoietic stem cells.[41,42] Unfortunately, the experimental design of this study precludes a direct answer to the question of whether parenchyma or hematolymphoid cells is the most important factor. In addition, determining whether the liver applies other selection pressures to the recipient T cell repertoire will require additional study. However, our conclusion at this time is in agreement with Maeda et al,[43] who suggest that the tolerogenic state induced by the graft parenchyma or nonhematolymphoid tissue is less robust than that seen with hemopoietic chimerism.

These results are also consistent with allogeneic tolerance being a multi-level phenomenon.[44,45] There is no question that many manipulations can prolong allograft survival.[21,22] However, the difference in the *in vivo* level of unresponsiveness seen with chronic exposure to donor parenchymal *versus* hematolymphoid cells may account for the persistent inflammation that leads to CR. Therefore, it is not unreasonable to raise the testable hypothesis that direct presentation of alloantigen by mobile donor antigen-presenting cells, which have direct access to the entire recipient lymphoid system, are required for long-term tolerance with freedom from CR. Considering the complexity of the immune system, it is unlikely that allo-tolerance will ever be attributable to a single cell type. Nev-

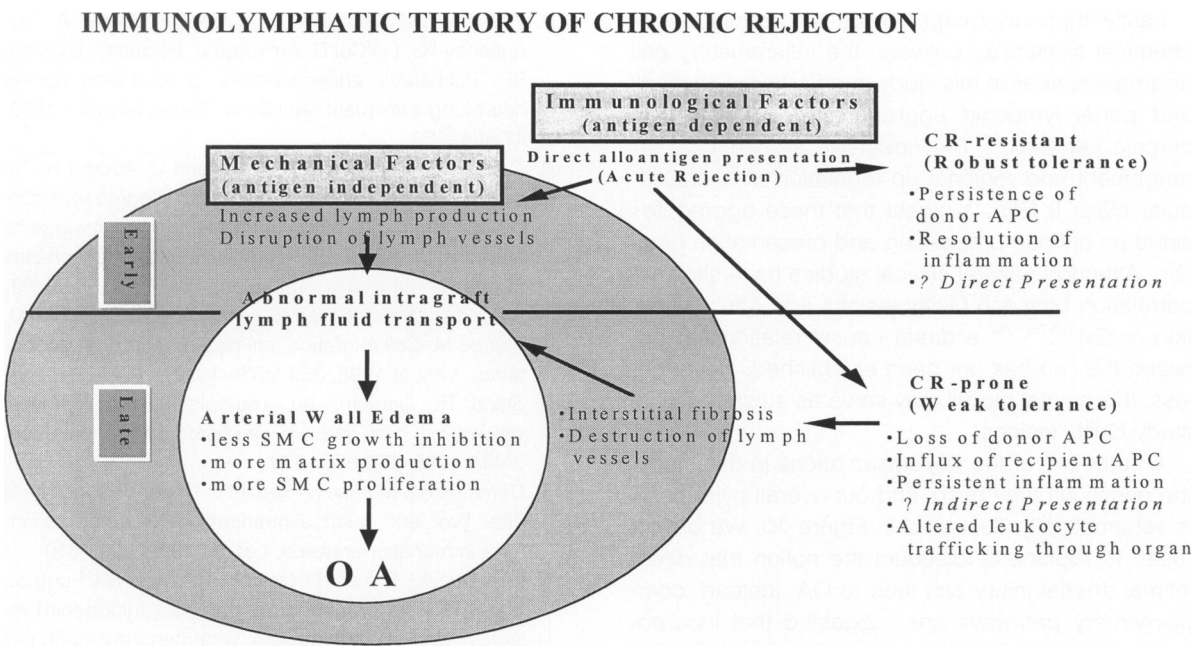

Figure 10. *Schematic representation of the immunolymphatic theory of chronic rejection. This is a hypothesis, which was raised as a result of the observations made in this experimental model.*

ertheless, these observations would explain why immunologically active hematolymphoid cells have historically been the best source of tolerogenic alloantigen.[19,20,43,46] Future studies will have to closely monitor for the development of CR and simultaneously exclude the possibility of donor hematolymphoid cell involvement.[21,22]

Two important inter-related observations in this study provide a potential insight about the association between immunological injury and the development of OA: 1) arterial remodeling begins before there is any noticeable intimal inflammation and 2) OLTx-pretreated rats experience the early phase, which results in mild intimal thickening, but they are spared from the later phase when OA worsens or progresses. These observations suggest that factor(s) associated with acute rejection, other than direct lymphocytic damage to the allogeneic endothelium, can contribute to the development of OA.

Based on the following evidence, we would like to raise the possibility that alterations in the transport of cytokine-rich lymph fluid within the graft can importantly contribute to the development of OA (Figure 10). Studies done long ago have shown that lymphatics from a renal allograft eventually reconnect to recipient lymph vessels within 10 to 14 days,[47] unless disrupted by rejection. During acute rejection, there is an increased production of lymph fluid and disruption of the lymphatic microvascular endothelial junctions, which retards lymphatic flow.[47–50] The same is true of recurrent[47] or late rejection episodes; Ruggiero et al[51] showed total cessation of lymph flow from late-rejecting lung allografts. In this study, lymphatic disruption is observed in both groups during the early stage. The arterial wall changes during this time are reminiscent of those seen after lymphatic obstruction alone.[37] Thus, impaired transmural migration of lymph formed by high-pressure arterial pulsation[52] may account for the arterial wall edema seen in this study.

The worsening or progression of OA seen during the late phase in the BM-pretreated recipients was most clearly associated with infiltration of the allograft by T lymphocytes and ED1$^+$ macrophages. Interestingly, the inflammatory cell aggregates observed in the allografts with CR during the late phase localized near draining lymphatics, which were also focally disrupted in areas of fibrosis. In addition, the inflammatory cells present in the arterial intima appeared to invade from the adventitia *via* the media. These findings, along with continued segmental medial edema of affected arteries, suggests that interstitial fibrosis and subsequent focal and/or intermittent lymphatic disruption also contributes to the late phase of OA progression.

Lastly, there are morphological and immunohistochemical similarities between the inflammatory cell aggregates seen in this study and Quilty lesions[53–56] and portal lymphoid aggregates in patients with chronic hepatitis.[57] The nodal-like architectural arrangement and regional up-regulation of microvascular class II MHC suggest that these aggregates serve an antigen processing and presentation function. Although several clinical studies have shown a correlation between Quilty lesions and acute rejection or CR,[54,58–62] a direct causal relationship between the two has not been established. Nevertheless, this animal model may serve as a useful tool to study Quilty lesions.

A summary of the key observations in this study, the questions they raise, and our overall hypothesis is schematically illustrated in Figure 10. We do not mean to replace or discount the notion that direct intimal arterial injury can lead to OA. Instead, complementary pathways are suggested that incorporate both immunological and mechanical factors into the pathogenic scheme.

References

1. Oguma S, Banner B, Zerbe T, Starzl T, Demetris AJ: Participation of dendritic cells in vascular lesions of chronic rejection of human allografts. Lancet 1988, 2:933–936
2. Demetris AJ, Zerbe T, Banner B: Morphology of solid organ allograft arteriopathy: identification of proliferating intimal cell populations. Transplant Proc 1989, 21: 3667–3669
3. Hayry P, Isoniemi H, Yilmaz S, Mennander A, Lemstrom K, Raisanen-Solokowski A, Koskinen P, Ustinov J: Chronic allograft rejection. Immunol Rev 1993, 134: 33–81
4. Paul LC: Immunobiology of chronic renal transplant rejection. Blood Purification 1995, 13:206–218
5. Adams DH, Russell ME, Hancock WW, Sayegh MH, Wyner LR, Karnovsky MJ: Chronic rejection in experimental cardiac transplantation: studies in the Lewis-F344 model. Immunol Rev 1993, 134:5–19
6. Cramer DV, Qian SQ, Harnaha J, Chapman FA, Estes LW, Starzl TE, Makowka L: Cardiac transplantation in the rat. I. The effect of histocompatibility differences on graft arteriosclerosis. Transplantation 1989, 47: 414–419
7. van Saase JL, van der Woude FJ, Thorogood J, Hollander AA, van Es LA, Weening JJ, van Bockel JH, Bruijn JA: The relation between acute vascular and interstitial renal allograft rejection and subsequent chronic rejection. Transplantation 1995, 59:1280–1285
8. Billingham ME: Pathology and etiology of chronic rejection of the heart. Clin Transplant 1994, 8:289–292
9. Yousem SA, Paradis IL, Dauber JH, Zeevi A, Duquesnoy RJ, DalCol R, Armitage J, Hardesty J, Griffith BP: Pulmonary arteriosclerosis in long-term human heart-lung transplant recipients. Transplantation 1989, 47:564–569
10. Tullius SG, Hancock WW, Heemann U, Azuma H, Tilney NL: Reversibility of chronic renal allograft rejection: critical effect of time after transplantation suggests both host immune dependent and independent phases of progressive injury. Transplantation 1994, 58:93–99
11. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M: Cell migration, chimerism, and graft acceptance. Lancet 1992, 339:1579–1582
12. Starzl TE, Demetris AJ: Transplantation milestones: viewed with one- and two-way paradigms of tolerance. JAMA 1995, 273:876–879
13. Demetris AJ, Murase N, Starzl TE: Donor dendritic cells after liver and heart allotransplantation under short-term immunosuppression. Lancet 1992, 339:1610
14. Demetris AJ, Murase N, Delaney CP, Woan M, Fung JJ, Starzl TE: The liver allograft, chronic (ductopenic) rejection, and microchimerism: what can they teach us? Transplant Proc 1995, 27:67–70
15. Reinsmoen NL, Jackson A, McSherry C, Ninova D, Wiesner Rh, Kondo M, Krom RAF, Hertz MI, Bolman RM, Matas AJ: Organ-specific patterns of donor antigen-specific hyporeactivity and peripheral blood allogeneic microchimerism in lung, kidney, and liver recipients. Transplantation 1995, 60:1546–1554
16. Owen RD: Immunogenetic consequences of vascular anastomoses between bovine twins. Science 1945, 102:400–401
17. Billingham RE, Brent L, Medawar PB: Actively acquired tolerance of foreign cells. Nature 1953, 172:603–606
18. Billingham R, Brent L, Medawar P: Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Philos Trans R Soc Lond (Biol) 1956, 239:357–412
19. Main JM, Prehn RT: Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. J Natl Cancer Inst 1955, 15: 1023–1029
20. Ildstad ST, Sachs DH: Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. Nature 1984, 307:168–170
21. Bushell A, Pearson TC, Morris PJ, Wood KJ: Donor-recipient microchimerism is not required for tolerance induction following recipient pretreatment with donor-specific transfusion and anti-CD4 antibody. Transplantation 1995, 59:1367–1371
22. Shirwan H, Wang HK, Barwari L, Makowka L, Cramer DV: Pretransplant injection of allograft recipients with donor blood or lymphocytes permits allograft tolerance without the presence of persistent donor microchimerism. Transplantation 1996, 61:1382–1386
23. Schlitt HJ, Hundrieser J, Hisanaga M, Uthoff K, Karck M, Wahlers T, Wonigeit K, Pichlmayr R: Patterns of

donor-type microchimerism after heart transplantation. Lancet 1994, 343:1469–1471
24. Murase N, Starzl TE, Tanabe M, Fujisaka S, Miyazawa H, Qing YE, Delaney CP, Demetris AJ: Variable chimerism, graft *versus* host disease, and tolerance after different kinds of cell and whole organ transplantation from Lewis to Brown-Norway rats. Transplantation 1995, 60:158–171
25. Kamada N, Calne RY: Orthotopic liver transplantation in the rat. technique using cuff for portal vein anastomosis and biliary drainage. Transplantation 1979, 28: 47–50
26. Ono K, Lindsey ES: Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969, 7:225–229
27. Demetris A, Qian S, Sun H, Fung JJ, Yagihashi A, Murase N, Iwaki Y, Gambrell B, Starzl TE: Early events in liver allograft rejection: delineation of sites of simultaneous intragraft and recipient lymphoid tissue sensitization. Am J Pathol 1991, 138:609
28. Yagihashi A, Takahashi S, Murase N, Starzl TE, Iwaki Y: A monoclonal antibody (L21–6) recognizing an invariant chain expressed on the cell surface in rats with the exception of the BN ($RT1^n$): a study of tissue and strain distribution. Transplant Proc 1995, 27: 1519–1521.
29. Qian S, Demetris AJ, Murase N, Rao AS, Fung JJ, Starzl TE: Murine liver allograft transplantation: tolerance and donor cell chimerism. Hepatology 1994, 19:916–924
30. Kato S, Yasunaga A, Uchida U: Enzyme-histochemical method for identification of lymphatic capillaries. Lymphology 1991, 24:125–129
31. Werner JA, Schunke M, Tillmann B: Histochemical visualization of lymphatic capillaries in the rat: a comparison of methods demonstrated at the posterior pharyngeal surface. Arch Histol Jpn 1987, 50:505–514
32. Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979, 18:5294
33. Brenner CA, Tam AW, Nelson PA: Message amplification phenotyping (MAPPing): a technique to simultaneously measure multiple mRNAs from small number of cells. Biotechniques 1989, 7:1096
34. Keenan RJ, Zeevi A, Iacono AT, Spichty KJ, Yousem SA, Ohori PN, Paradis IL, Griffith BP: Efficacy of inhaled cyclosporine in lung transplant patients with refractory rejection: correlation of intragraft cytokine gene expression with pulmonary function and histology. Surgery 1995, 118:385–391
35. Billingham ME: Cardiac transplantation. The Pathology of Organ Transplantation. Edited by GE Sale. Boston: Butterworths, 1990, 133–152
36. Lu L, Rudert WA, Qian S, McCaslin D, Fu F, Rao AS, Trucco M, Fung JJ, Starzl TE, Thomson AW: Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. J Exp Med 1995, 182:379–387
37. Solti F, Jellinek H, Schneider F, Lengyel E, Berczi V, Kekesi V: Lymphatic arteriopathy: damage to the wall of the canine femoral artery after lymphatic blockade. Lymphology 1991, 24:54–59
38. Matzinger P: Tolerance, danger, and the extended family. Annu Rev Immunol 1994, 12:991–1045
39. Bertolino P, Heath WR, Hardy CL, Morahan G, Miller JF: Peripheral deletion of autoreactive $CD8^+$ T cells in transgenic mice expressing H-2Kb in the liver. Eur J Immunol 1995, 25:1932–1942
40. Chen-Woan M, Delaney CP, Fournier V, Wakizaka Y, Murase N, Fung JJ, Starzl TE, Demetris AJ: *In vitro* characterization of rat bone marrow-derived dendritic cells and their precursors. J Leukocyte Biol 1996, 59: 196–206
41. Murase N, Starzl TE, Ye Q, Tsamandas T, Thomson AW, Rao AS, Demetris AJ: Multilineage hematopoietic reconstitution of supralethally irradiated rats by syngeneic whole organ transplantation: with particular reference to the liver. Transplantation 1996, 61: 1–4
42. Taniguchi H, Toyoshima T, Fukao K, Nakauchi H: Presence of hematopoietic stem cells in the adult liver. Nature Med 1996, 2:198–203
43. Maeda T, Eto M, Nishimura Y, Nomoto K, Kong YY, Nomoto K: Role of peripheral hemopoietic chimerism in achieving donor-specific tolerance in adult mice. J Immunol 1993, 50:753–762
44. Arnold B, Schonrich G, Hammerling GJ: Multiple levels of peripheral tolerance. Immunol Today 1993, 14: 12–14
45. Schonrich G, Alferink J, Klevenz A, Kublbeck G, Auphan N, Schmitt-Verhulst AM, Hammerling GJ, Arnold B: Tolerance induction as a multi-step process. Eur J Immunol 1994, 24:285–293
46. Billingham R, Brent L: Quantitative studies on transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease. Philos Trans R Soc Lond (Biol) 1956, 242: 439–477
47. Malek P, Vrubel J, Kolc J: Lymphatic aspects of experimental and clinical renal transplantation. Bull Soc Int Chirurgie 1969, 28:110–114
48. Malek P, Vrubel J: Lymphatic system and organ transplantation. Lymphology 1968, 1:4–22
49. Cockett AT, Sakai A, Netto IC: Kidney lymphatics: an important network in transplantation. Trans Am Assoc Genito Urinary Surg 1973, 65:73–76
50. Eliska O, Eliskova M, Mirejovsky P: Lymph vessels of the transplanted kidney. Nephron 1986, 44:136–141
51. Ruggiero R, Fietsam R, Jr., Thomas GA, Muz J, Farris RH, Kowal TA, Myles JL, Stephenson LW, BaciewiczFA Jr: Detection of canine allograft lung rejection by pulmonary lymphoscintigraphy. J Thorac Cardiovasc Surg 1994, 108:253–258

52. Schmid-Schonbein GW: Microlymphatics and lymph flow. Physiol Rev 1990, 70:987–1028
53. Billingham ME: The postsurgical heart. Am J Cardiovasc Pathol 1988, 1:319–334
54. Costanzo-Nordin MR, Winters GL, Fisher SG, O'Sullivan J, Heroux AL, Kao W, Mullen GM, Johnson MR: Endocardial infiltrates in the transplanted heart. J Heart Lung Transplant 1993, 12:741–747
55. Kottke-Marchant K, Ratliff NB: Endomyocardial lymphocytic infiltrates in cardiac transplant recipients. Arch Pathol Lab Med 1989, 113:690–698
56. Radio SJ, McManus BM, Winters GL, Kendall TJ, Wilson JE, Costanzo-Nordin MR, Ye Y: Preferential endocardial residence of B-cells in the "Quilty effect" of human heart allografts: immunohistochemical distinction from rejection. Mod Pathol 1991, 4: 654–660
57. Mosnier JF, Degott C, Marcellin P, Henin D, Erlinger S, Benhamou JP: The intraportal lymphoid nodule and its environment in chronic active hepatitis. Hepatology 1993, 17:366–371
58. Kemnitz J, Cohnert T, Schafters H, Helmke M, Herrmann G, Schmidt RM, Haverich A: A classification of cardiac allograft rejection. Am J Surg Pathol 1987, 11:503–515
59. Forbes RDC, Rowan RA, Billingham ME: Endocardial infiltrates in human heart transplants: a serial biopsy analysis comparing four immunosuppression protocols. Hum Pathol 1990, 21:850–855
60. Kemnitz J, Cremer J, Schaefers H, Restrepo-Specht I, Haverich A, Ulysal A, Heublein B, Wirth S: Some aspects of changed histopathologic appearance of acute rejection in cardiac allografts after prophylactic application of OKT3. J Heart Lung Transplant 1991, 10:366–372
61. Pardo-Mindan FJ, Lozano MD, Contreras-Mejuto F, deAlava E: Pathology of heart transplant through endomyocardial biopsy. Semin Diagn Pathol 1992, 9:238–248
62. Joshi A, Masek MA, Brown BWJ, Weiss LM, Billingham ME: "Quilty" revisited: a 10-year perspective. Hum Pathol 1995, 26:547–557