Book Chapter

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"Role of Immunosuppressive Agents in Organ Transplantation-Basic and Clinical Aspects"

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TRANSPLANT VASCULAR SCLEROSIS: FROM PATHOGENESIS TO PREVENTION

Hong Sun, M.D.[1], Vladimir Subbotin, M.D., Ph.D.[2], Abdelouahab Aitouche, Ph.D.[1]
Jin Lian Tang[1], M.D., Luis A. Valdivia, M.D, Ph.D.[1], John J. Fung, M.D., Ph.D.[1]
[redacted], Abdul S. Rao, M.D., D.Phil.[1,2]

Section of Cellular Transplantation, Thomas E. Starzl Transplantation Institute and the Departments of Surgery[1] and Pathology[2],University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania.

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Address Reprint Requests To:

Abdul S. Rao, M.D., D.Phil.
Thomas E. Starzl Transplantation Institute
E1540 Biomedical Science Tower
200 Lothrop Street
Pittsburgh, PA 15213
Telephone: (412) 648-9322
FAX: (412) 624-9493
E-Mail: raoa@a1.isd.upmc.edu

The progressive enhancements in short term organ allograft survival have largely been attributed to improved surgical and post-operative care and the advent of more effective immunosuppressive agents. However, despite these encouraging observations, the high incidence of delayed graft loss due largely to transplant vascular sclerosis (TVS) and the presence of overt complications associated with the use of nonspecific immunosuppression (IS) remain unacceptable. It has been estimated that the incidence of TVS in allografts that have survived more than five years is up to 43%, 50% and 60% in recipients of kidneys (1), lungs (2) and hearts (3) respectively. Conversely, its prevalence is markedly lower (3.6-16.8%) in long-surviving liver allograft recipients (4). These observations compounded by the fact that once established, histopathological changes characteristic of chronic rejection (CR) are irreversible and somewhat refractory to exogenous intervention, has necessitated the urgent development of therapeutic strategies that would allow for its attenuation and/or abrogation.

ETIOPATHOLOGY OF TRANSPLANT VASCULAR SCLEROSIS

While the factors that underlie the changes pathognomonic of (CR) are poorly understood, the etiology nevertheless appears to be multifactorial (Table 1; 5).

a) Non-Immune Factors:

The direct detrimental influence of various immunosuppressive agents used to prevent acute cellular rejection (ACR), on the development of post-transplant vasculopathy has been extensively documented (6, 7). The virtually indistinguishable clinical and histological presentation of the signs and symptoms of drug-induced nephrotoxicity and post-transplant vasculopathy have further compounded this conundrum. The modest and often transient improvement in graft function witnessed following withdrawal of drugs in presumptive cyclosporin or tacrolimus-induced nephrotoxicity is reminiscent of observations considered diagnostic of evolving CR. Similarly, chronic use of steroids has also been implicated in mediating progressive glomerulopathy with consequent glomerular capillary hypertension; findings considered characteristic of developing CR.

The role of systemic infection in the evolvement of CR in long-surviving organ allografts has also been documented (8, 9). Study of the effects of cytomegalovirus (CMV) infection on graft arteriosclerosis in cardiac transplant recipients suggests that its presence greatly enhances myointimal thickening with a corresponding diminution in patient and graft survival (8). Similarly, herpes virus infection both in humans and in experimental animals has been shown to accelerate chronic atherosclerosis (9). While the precise mechanism underlying the virus-induced acceleration of vasculopathy is unknown, it is nevertheless postulated that viral infections may modify lipid metabolism within the cellular components of the vessel wall resulting in overt fat accumulation; an event which may further deteriorate the ongoing intimal thickening with its corresponding luminal narrowing (10).

b) Immune Factors:

Whilst the foregoing arguments concerning the etiopathology of CR may be bewildering, the role of alloimmune responses in the evolvement of this phenomenon is nevertheless incontrovertible (11). This latter dogma has been corroborated by numerous studies suggesting a lineal correlation between the intensity and the frequency of ACR and that of the severity and the incidence of development of post-transplant vasculopathy (12, 13). Excluding the precise nature of the effector mechanisms (to be discussed later), it has been postulated that repetitive immune-mediated endothelial cell injury in vascularized allografts results in progressive intimal proliferation and hypertrophy which eventually culminates in gradually worsening luminal obliteration. In allotransplanted hearts, the eventual consequence of this low-grade "endothelialitis" is diffuse concentric tapering of the conductance vessels and gradual pruning of the distal arterioles. The piecemeal involvement of the large coronary arteries and their terminal branches results in patchy myocardial infarction and progressive fibrosis. The latter morphological aberrations translate clinically into an inexorable decline in myocardial function leading to the development of congestive heart failure, silent myocardial infarction, sustained ventricular arrhythmia, sudden cardiac arrest and in a large percentage of patients, in retransplantation.

The degree of HLA matching and its ultimate influence on the development of CR remains a controversial issue. Nevertheless, the higher incidence of long-term graft survival in living-related as compared to that to recipients of cadaveric renal allografts suggests that HLA-matching and its salutary effects on ACR may play a role in the development of CR (14). The correlation between HLA-matching and the development of post-transplant arteriopathy is however, far more nebulous in clinical heart transplant recipients. There has been conflicting evidence which has undermined the role of HLA matching in the development of progressive coronary arteriosclerosis (15).

TOLERANCE AND TRANSPLANT VASCULAR SCLEROSIS

Perhaps the most convincing evidence for an immune etiology in the evolvement of CR is provided by the observation that prior induction of donor-specific tolerance has benignant effects on the subsequent development of post-transplant vasculopathy (11, 16-24). Demetris *et al.,* (18, 19) have demonstrated the beneficial effects of donor cell chimerism-induced tolerance on CR in a vascularized model of heterotopic cardiac alloTx in rats. Using Lewis ($RT1^l$)→ Brown Norway ($RT1^n$) rat strain combination, they have demonstrated that prior donor-strain liver-induced transplantation tolerance protected the challenge donor but not third party heterotopic heart allografts from developing changes pathognomonic of CR.

In a mouse model, we have also tested this tenet by transplanting aorta across a fully allogeneic strain (C57Bl/10; H-2^b→C3H; H-2^k), combination in which the recipient was rendered tolerant by prior transplantation of liver from donor-strain (Group III) mice (11). Aorta grafted in syngeneic (Group I; C3H→C3H) and allogeneic (Group II, C57Bl/10→C3H) animals served as controls. The recipients were not immunosuppressed. No morphological changes were evidenced in the transplanted aortas in Group I animals. Contrarily, aortic allografts in Group II animals underwent a self-limiting ACR which resolved completely and was succeeded by day 30 post-transplantation,

of histopathological changes pathognomonic of CR. There was evidence for diffuse myointimal thickening, progressive concentric luminal narrowing and patchy destruction of internal elastic membranes resulting in massive vascular obliteration by day 120 post-transplantation. Interestingly, no arteriosclerotic changes were observed for the duration of follow-up (upto 120 days post-transplantation) in transplanted aortas (liver donor-type) harvested from animals in Group III (Figure 1, A, B & C). However, vasculopathy was prominent in third-party aortic grafts transplanted into tolerant recipients (Figure 1, D). These data suggest that prior induction of tolerance abrogates the development of TVS and that this protection appears to be donor-specific.

Studies in humans have also yielded encouraging observations (21-24). Keenan *et al.*, (21) have reported that the presence of microchimerism and the subsequent establishment of donor-specific hyporesponsiveness was invariably associated with lower incidence of obliterative bronchiolitis (OB) in lung allotransplant recipients. These findings have been further corroborated by Reinsmoen *et al.*, (22) who showed that in lung allograft recipients who were 2.8-5 years post-transplantation, no OB was evidenced in those exhibiting donor-specific hyporesponsiveness. Contrarily, 9/17 (53%) patients who were responsive to donor antigens in *in vitro* proliferative assays developed OB over a comparable period of follow-up (22). Similar observations have also been made in long-term renal allograft recipients (23). It is noteworthy that in our prospective clinical trial entailing concomitant perioperative donor BM infusion and organ alloTx, we have also documented the presence of increased incidence of OB in contemporaneously accrued non-marrow-augmented lung transplant recipients as compared to that to the study (marrow-augmented) patients (24).

MEDIATORS OF TRANSPLANT VASCULAR SCLEROSIS

There are three distinct host immune responses which operate in concert to orchestrate the development of TVS.

a) Cellular Responses: The seminal role of infiltrating effector (mainly $CD8^+$ T) cells in allograft rejection has been well documented. On the contrary, cellular infiltration in an established lesion of post-transplant vasculopathy is a relatively less prominent event (26). This latter assertion does not however, imply that cell-mediated immune responses do not play an important role in the generation of CR. On the contrary, in recipients of vascularized allografts, the primary insult to the vascular endothelium which triggers the cascade of events leading to the development of CR is envisioned to be mediated by cell-dependent cytotoxicity. It is interesting to note that regardless of the type of rejection and the degree of cellular infiltration, the phenotype of the latter population is generally comparable consisting primarily of T cells and macrophages (27). Immunohistochemical examination of chronically rejecting human cardiac allografts has revealed the presence of moderate-to-marked lymphocytic infiltration with associated endothelialitis (28). Additionally, the majority of T cells in these lesions were of $CD8^+$ phenotype which together with macrophages were primarily localized in the subendothelial space of the coronary vessels which most markedly exhibited changes characteristic of CR (28). This observation is very similar to that in lung allografts undergoing OB where the predominant infiltrating T cell population was that of the $CD8^+$ phenotype (29). For reasons not yet evident, in chronically rejecting liver allografts, preeminent infiltration of macrophages rather than that of T cells has been observed. Given the importance of cell-mediated immunity in CR, it is no surprise that a myriad of attempts are being made to abrogate and/or mitigate ACR with the assumption that its benignant effects will eventually be translated to prevent and/or reverse the evolvement of post-transplant obliterative vasculopathy.

In addition to that by previously alluded (see above) strategies of inducing donor-specific tolerance, the latter goal has also been achieved by blockade of molecular interactions considered obligatory for the generation of an efficient T and B cell-mediated effector response. For the generation of the optimal effector response, it has been postulated that at least two signals are required (30). Recognition of processed peptides on the surface of antigen presenting cells (APC) by the T cell receptor (TcR) represents the initial antigen-specific event leading to cognate

interaction. This recognition triggers the engagement of a series of accessory molecules, whose function is to stabilize the TcR-MHC interaction and to coordinate the responses of the interacting cells. Two ligand-receptor systems that exert profound influence(s) on the outcome of costimulatory interactions have been identified. The ligation of CD28 on the effector cells to B7.1 and B7.2 on the APC triggers the activated T cell to produce lymphokines and avoid apoptotic cell death and/or anergy (30). More recently, the demonstration of the expression of gp39 (CD40 ligand; CD40L) on activated T cells and its function as a ligand for CD40 expressed on various APC has unveiled as yet, another dominant costimulatory pathway for T and B cell activation (30, 31).

The availability of a wide array of suitable reagents has converged the interest of many investigators towards blocking the regulated delivery of signal two by the CD28/B7 and the CD40/gp39 costimulatory pathways and to ascertain its effect on allograft survival and on post-transplant vasculopathy. In the realm of allotransplantation, the use of CTLA4-Ig (which binds to B7.1 and B7.2 cell surface molecules) to block signaling through CD28/B7 pathway has been shown to enhance allograft survival and to prevent or else markedly reduce the evolvement of changes characteristic of post-transplant obliterative vasculopathy (32-36). Similarly, down-regulation of signaling discretely through the CD40/CD40L costimulatory pathway by the use of anti-CD40L mAb has also been shown to prolong allograft survival with albeit little or no salutary effects on CR (16, 37, 38). Perhaps the most persuasive evidence for the role of costimulatory signaling in the evolvement of post-transplant arteriosclerosis was provided by Larsen *et al.*, (16), who recently demonstrated that in a primary vascularized murine model of heterotopic cardiac allotransplantation, contemporaneous but not discrete blockade of CD28/B7 and CD40/gp39 pathway resulted in complete abrogation of CR.

In the aortic model of allotransplantation, we have also tested the efficacy of costimulatory blockage in preventing the development of TVS (39). For this purpose, aortic allografts were

transplanted across C57Bl/10J (H-2^b) → C3H (H-2^k) strain combinations. Transient or more stable blockade of signaling through CD28/B7 pathway was achieved by either a single (Group A; d2 post-transplantation) or multiple (Group B; ten doses from d2 post-transplantation and every 72h thereafter) injection(s) of CTLA4-Ig fusion protein (200μg/dose i.p.). Additionally, CD40/CD40L costimulation was blocked by anti-CD40L monoclonal antibody (Group C; 250μg i.m. on d0, 2 and 4 post-transplantation). To ascertain the effect of combined simultaneous inhibition of both CD28/B7 and CD40/CD40L pathways on CR, the aortic allograft recipients were treated with either a single (Group D; d2 post-transplantation) or multiple (Group E; d0, 2 and 4; and Group F; 10 doses from d2 post-transplantation and every 72h thereafter) doses of CTLA4-Ig (200μg i.p.) and anti-CD40L monoclonal antibody (250μg i.m.). Untreated (Group G) and recipients treated with irrelevant isotype-matched monoclonal antibody (Group H) served as controls. At d30 post-transplantation, the grafts were harvested for histopathological and immunohistochemical examination.

Similar to that of animals in Group G (Figure 2C), aortic allografts obtained from recipients treated with either CTLA4-Ig (Groups A & B) or anti-CD40L monoclonal antibody (Group C) alone exhibited marked narrowing of the lumen primarily due to concentric intimal thickening caused by proliferation of α-smooth muscle $actin^+$ cells. Contemporaneous treatment however, with either a single (Group D) or multiple (Group E) injections of CTLA4-Ig and anti-CD40L monoclonal antibody resulted in marked diminution of intimal thickening which was largely patchy with residual evidence for endothelial denudation. Interestingly, concurrent prolonged inhibition of CD28/B7 and CD40/CD40L pathways (Group F) resulted in complete abrogation of the development of post-transplant arteriopathy (Figure 2D) suggesting that a more stable disruption of signaling through costimulatory pathways may be required to obviate the development of this vasculopathy.

b) Humoral Responses: The presence of circulating antibodies against discrete HLA epitopes or else against graft-specific antigens has been shown to precipitate early or immediate rejection of the transplanted organ allografts (40, 41). Similarly, the role of anti-HLA and anti-graft antibodies in the evolvement of CR has also been widely premised (26). In cardiac transplant recipients, the development of graft arteriosclerosis has been correlated with the presence of anti-HLA antibodies (42). Additionally, Mohanakumar *et al.,* (43) have demonstrated the presence of immunoglobulin and complement complexes within the loci of thickened intima in renal allograft recipients in whom late graft rejection was correlated with presensitization to HLA-DR antigens. Perhaps the most convincing evidence for the role of alloantibodies in the development of CR was provided by O'Connell *et al.,* (44) who successfully reproduced the histological aberration pathognomonic of post-transplant vasculopathy by intra-arterial infusion of donor-specific antisera.

What are the molecular targets for cell and antibody-mediated immunity? It is generally believed that donor MHC II antigens are the major targets for an antibody-mediated immune response. On the contrary, it is the class I antigens which are considered the key molecular targets of T cell mediated cytotoxicity. Effector responses against non-MHC antigens as targets have also been implicated in the generation of CR. Anti-B cell antibodies have been identified in patients harboring chronically rejecting renal allografts (41) as well as in long-term heart recipients in whom the allografts were undergoing CR. In addition to anti-B cell antibodies, those directed against specific antigens of the endothelial cells, tubular basement membrane and other non-MHC graft-specific antigens have also been identified.

c) Soluble Factors/Adhesion Molecules: Whilst cell and antibody-mediated immune responses play a cardinal role in initiating vascular injury, it is the soluble mediators released at the site of insult which are considered quintessential for the perpetuation of the cascade of events which eventually culminate in the establishment of changes pathognomonic of CR. Various factors including thromboxane A2, platelet-derived growth factor, leukotrienes, platelet activating factors,

tumor necrosis factor (TNF), IL-1 and IFN-γ have been documented to play either a direct or an indirect role in the evolvement of CR (26). Although once released, these factors instigate wide-ranging changes in the target tissue, they predominantly influence macrophage and monocyte infiltration and smooth muscle and mesangial cell proliferation; events that ultimately lead to the development of graft arteriosclerosis.

Additionally, the role of adhesion molecules in precise trafficking of effectors of cell-mediated immunity is also incontrovertible (45). Intercellular adhesion molecule-1, CD2 (lymphocyte function associated molecule-2; LFA-2) and LFA-3 which are expressed on the vascular endothelium play an important role in activated T cell and macrophage infiltration. These molecules, whose expression can be upregulated by numerous soluble mediators (e.g. TNF, IFN-γ, etc.), influence both the adhesion and trans-endothelial migration of effector cells thus, ensuring their optimal accumulation at the site of injury; events that presage the development of changes characteristic of chronic graft vasculopathy (26). It is anticipated that the precise understanding of the role of soluble mediators and the cell surface expressed adhesion molecules in the evolvement of CR will unravel as yet another accessible target whose interruption may help mitigate the development of post-transplant arteriosclerosis.

DIRECT VERSUS INDIRECT ANTIGEN PRESENTATION

The concept that different MHC cell surface molecules (class I and II) predominantly present either endogenously or exogenously generated peptides is well established (46, 47). In allograft recipients, this fundamental distinction by the APC is further compounded by the "origin" of the MHC molecules which are involved in presenting donor antigens to reactive T cells. It has been proposed that donor antigens can be presented to recipient's alloreactive T cells by two discrete pathways (Figure 3). Recipient's alloreactive T cells' engagement and subsequent response to allogeneic MHC molecules is referred to as "direct" antigen presentation (Figure 3A). On the

contrary, processing and presentation of allogeneic MHC molecules by recipient's APC and their subsequent presentation to self-MHC restricted alloreactive T cells is termed "indirect" antigen presentation (Figure 3B) (48). Due to the presence at a much higher frequency of non-self MHC-restricted alloreactive T cells, direct mode of alloantigen presentation is considered to be far more vigorous than that mediated by its indirect counterpart. In line with this argument, it has been suggested that the early ACR witnessed after transplantation of MHC-mismatched allografts is mediated primarily by the direct pathway, although evidence is emerging that the indirect mode of allorecognition may also play a role in this phenomenon (47-49).

Which class of MHC molecules predominantly presents shed donor antigens is still a controversial issue. It could however be argued, that shed antigens would prevalently be processed by the exogenous pathway resulting in its presentation by recipient's MHC class II molecules to self-MHC restricted $CD4^+$ T cells (4, 7). One could therefore appreciate the heterogeneity of epitopes that will be engendered by the processing and indirect presentation predominantly of donor MHC molecules thus, providing an explication for the potential generation of a powerful rejection response even by the indirect mode of antigen presentation. In addition to other as yet undefined justifications, the observed intransigence of chronic allograft rejection and of that of the "indirect" mode of antigen presentation to exogenously administered immunosuppression has raised the possibility of the involvement of the latter process in the development of post-transplant vasculopathy (50, 51). Perhaps the most lucid evidence for the role of indirect pathway in CR emanated from the recently presented (XVIth Annual Meeting of the American Society of Transplant Physicians, May 10-14, 1997, Chicago, IL) and as yet unpublished work of Sayegh *et al.,* (52), who by using as donors, a congenic strain of WF rats [(WF1L LEW/Gut ($RT1^l$)] which express the same major but different minor MHC antigens as LEW ($RT1^l$) recipients, argued that the allorecognition in this model would be restricted to that to indirect antigen presentation providing a unique opportunity to study its influence on CR. It is noteworthy that hearts heterotopically transplanted across congenic WF→ LEW rats developed changes pathognomonic of

CR which were abrogated by a single injection of CTLA4-Ig given at d2 post-transplantation. While these findings are important, it could nevertheless be argued that contrary to the authors' claim, the presentation of the minor MHC antigen in this model may have been mediated via the direct pathway of antigen presentation. Until this conundrum is resolved, it would be difficult if not impossible to decipher the role of indirect pathway in CR in this particular model.

How does the indirect pathway of antigen presentation facilitate the evolvement of changes characteristic of CR? While the mechanism(s) are as yet largely indeterminate, it has however, been argued that generation of alloreactive self-MHC restricted $CD4^+$ T cells by indirect mode of antigen presentation evokes predominantly a delayed type hypersensitivity (DTH) response with resultant recruitment and activation of macrophages and the generation of a chronic inflammatory response (Figure 4). Additionally, there is growing evidence that primed self-MHC restricted $CD4^+$ T cells provide the appropriate help to B cells resulting in their generation of alloantibodies. Interestingly, both DTH-type responses and alloantibodies have been implicated as possible mediators of the afferent limb of the effector response culminating in the development of CR (Figure 4).

CONCLUSION

Considerable progress has been made in the last decade to further our understanding of the pathogenesis of TVS. Numerous therapeutic strategies have been reported which are known to prevent the onset and/or reversal of this lesion in preclinical models of CR. However, as yet no therapeutic intervention is available that would allow for successful prevention or reversal of vascular changes pathognomonic of CR in clinical organ transplant recipients. The progressive nature of this lesion and its profound detrimental impact on long-term patient and graft survival has mandated urgent evolvement of clinically applicable strategies that would mitigate and/or abrogate its development. It is towards the achievement of this latter objective that we believe all of our future endeavors should be converged.

Table 1: Factors Influencing the Pathogenesis of Chronic Rejection

a) nonimmune:

- ischemia-reperfusion injury
- immunosuppressive drugs
- donor age
- systemic infections
  - cytomegalovirus
  - herpes virus
- hyperlipidemia and hypercholesterolemia

b) immune:

- histoincompatibility
- incidence and severity of acute cellular rejection

FIGURE 1

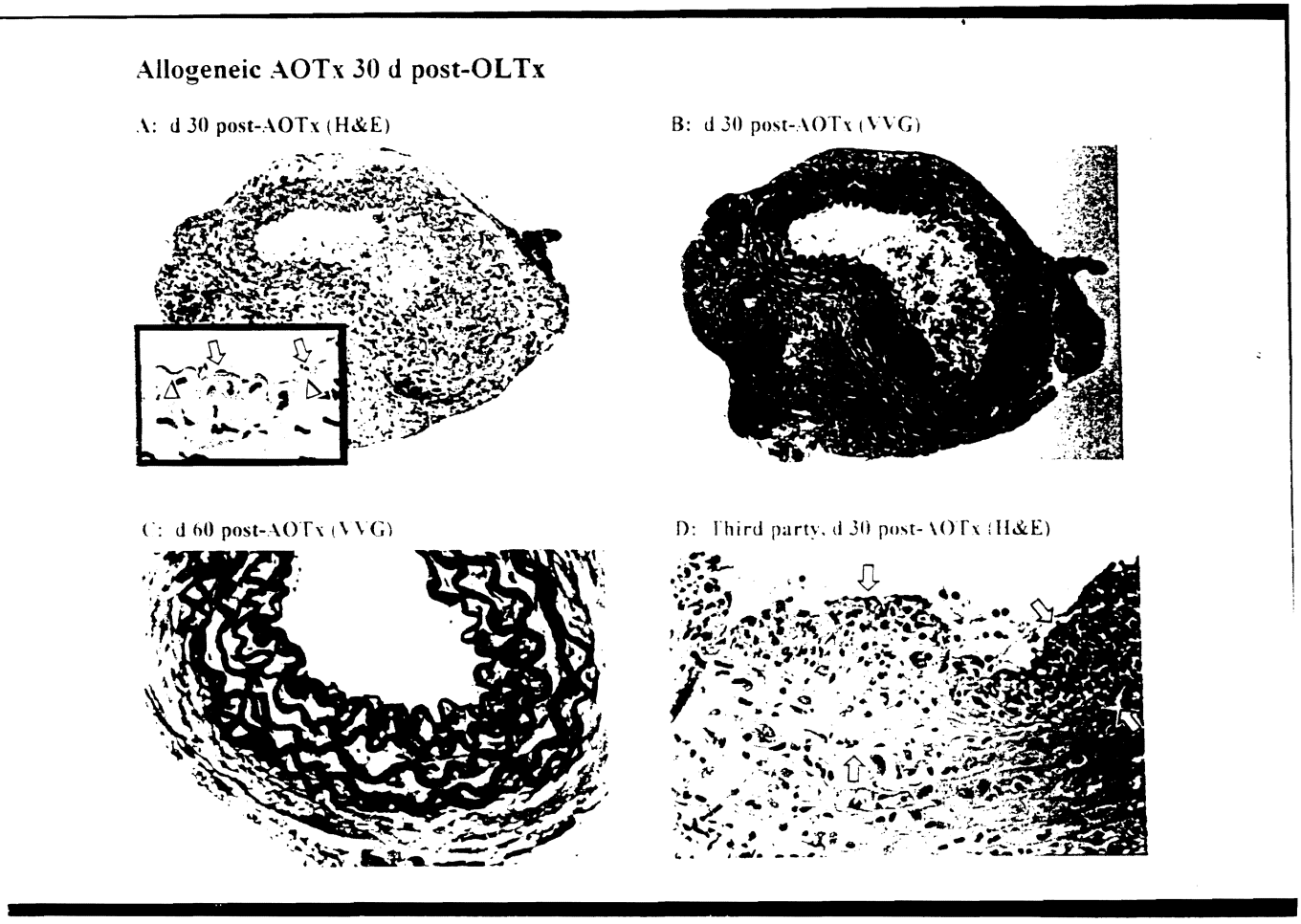

Allogeneic AOTx 30 d post-OLTx
A: d 30 post-AOTx (H&E)
B: d 30 post-AOTx (VVG)
C: d 60 post-AOTx (VVG)
D: Third party, d 30 post-AOTx (H&E)

FIGURE 2

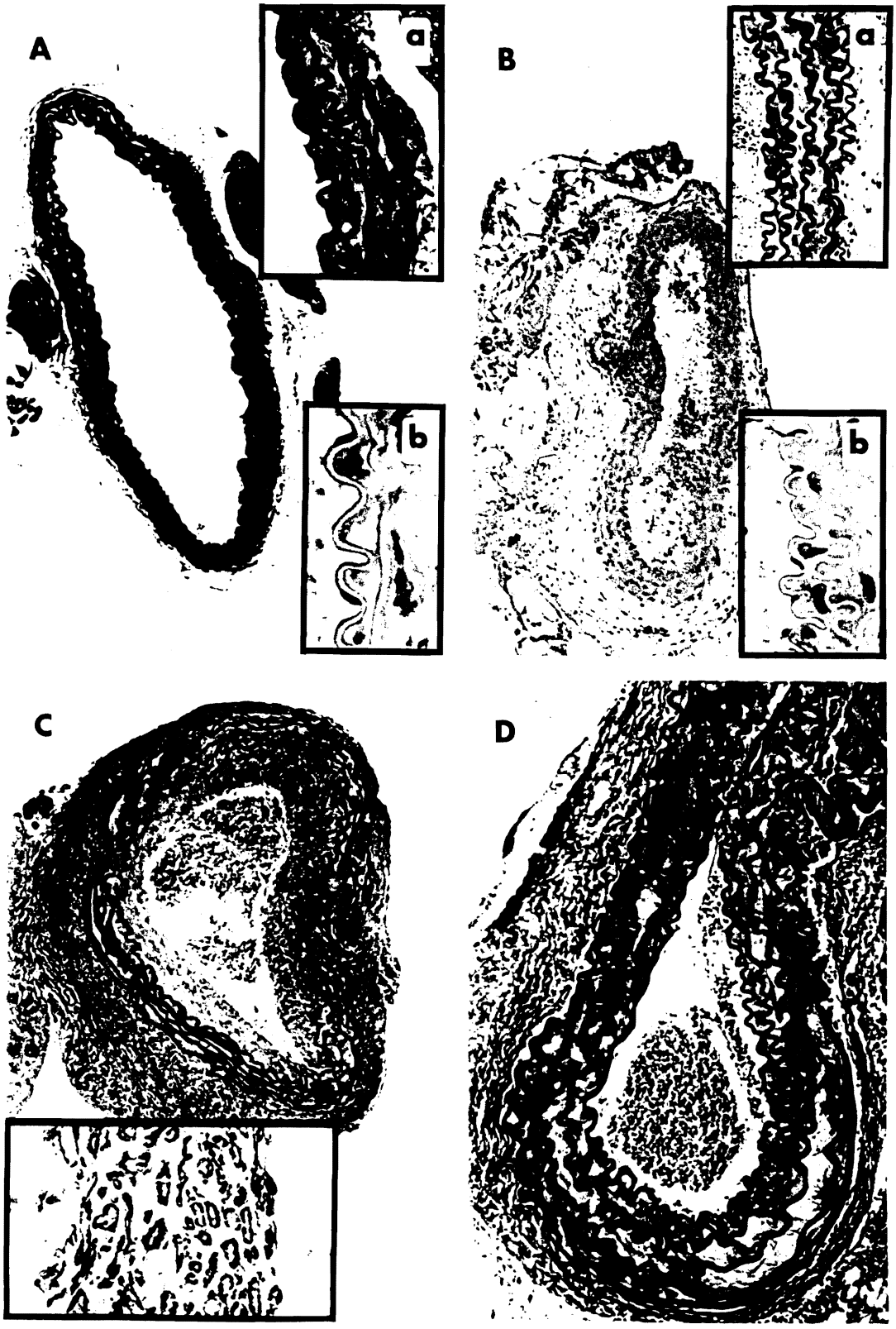


FIGURE 3

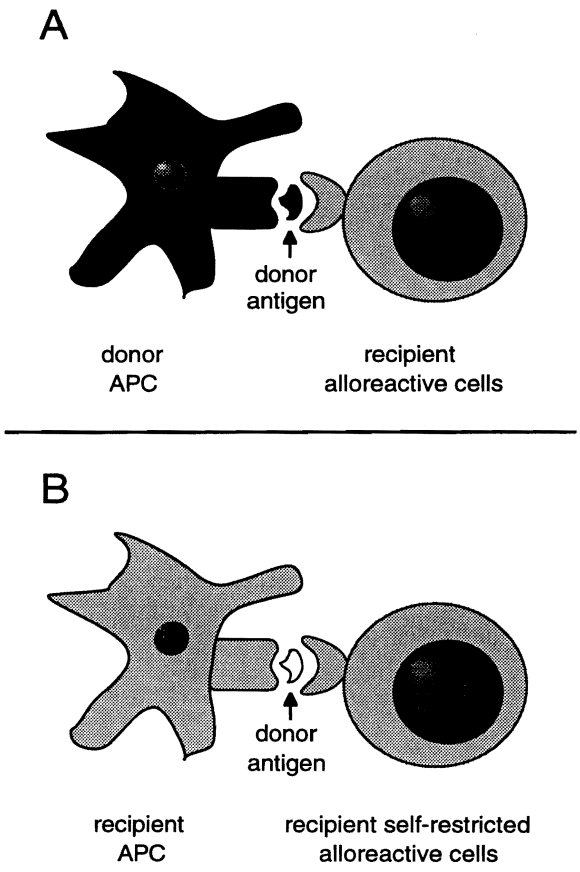
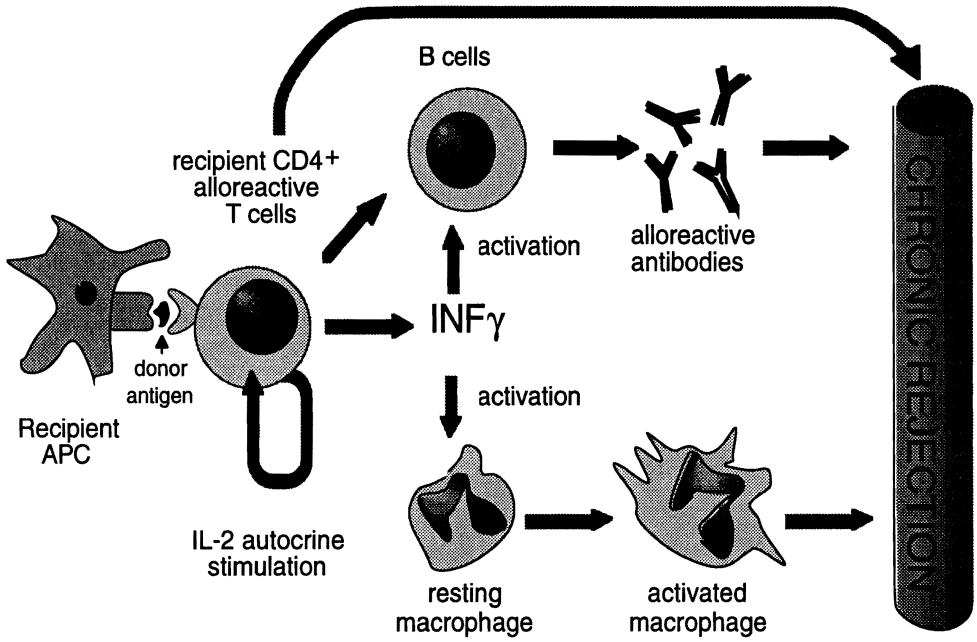

A
donor antigen
donor APC
recipient alloreactive cells
B
donor antigen
recipient APC
recipient self-restricted alloreactive cells

FIGURE 4


B cells
recipient CD4+
alloreactive
T cells
activation
alloreactive
antibodies
CHRONIC REJECTION
INFγ
donor
antigen
activation
Recipient
APC
IL-2 autocrine
stimulation
resting
macrophage
activated
macrophage

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b) immune:

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- incidence and severity of acute cellular rejection

Figure Legends

Figure 1: Aortic allografts harvested at d30 (A and B) and d 60 (C) post-transplantation across C57Bl/10→C3H mouse strain combinations. The recipients were rendered tolerant by previous (at d -30) orthotopic transplantation of liver obtained from the donor-strain animal. Aortic allografts obtained from third-party (BALB/c) animals (D) were used as controls. Unlike that into naive C3H recipients, no evidence of CR was witnessed in aortic allografts transplanted into those rendered tolerant (A, B and C). As is evident in Verhoeff-van Gieson (VVG)-stained sections (B and C), the aortic architecture is well preserved at d30 (B) and d60 (C) post-transplantation. Contrarily, at d30 post-transplantation, aortic allografts obtained from third-party donors (BALB/c) when transplanted into tolerant recipients exhibit changes pathognomonic of TVS (D). There was evidence for progressive intimal thickening (D; arrows) which was largely due to proliferation of alpha smooth muscle actin$^+$ cells. Magnification x100 (A and B); x400 (C and D); inset x1000.

Figure 2: Aortic allografts harvested at d50 post-transplantation across syngeneic (C3H→C3H) mouse strain combination (B) showed marked preservation of aortic morphology. There is no evidence of endothelial denudation (Bb) or disruption of the internal elastic membranes (Ba). Interestingly, this morphology appears very similar to that of native aorta (A). On the contrary, aortas harvested at d30 post-transplantation across untreated allogeneic (C57Bl/10→C3H) mouse strain combination (C) exhibited marked intimal thickening with associated disruption of the internal elastic membranes; the intimal thickening was due to proliferation of alpha smooth muscle actin$^+$ cells (C inset). Prolonged treatment (10 doses from d2 post-transplantation and every 72h thereafter) of the recipient with CTLA4-Ig (200 μg i.p.) and anti-CD40L

monoclonal antibody (250μg i.m.) resulted in complete abrogation of the development of changes characteristic of TVS (D). Magnification x100 (A, B and C); x200 (D); isets x400 (Aa, Ba and C); x1000 (Ab and Bb).

Figure 3: Direct (A) and indirect (B) pathways of presentation of donor antigens to recipient's alloreactive T cells.

Figure 4: Putative mechanism(s) for the development of post-transplant vasculopathy by the "indirect" pathway of antigen presentation.

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