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ARTICLES

MARKED MITIGATION OF TRANSPLANT VASCULAR SCLEROSIS IN FasL^{gld} (CD95L) MUTANT RECIPIENTS

THE ROLE OF ALLOANTIBODIES IN THE DEVELOPMENT OF CHRONIC REJECTION[1]

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Background. **In the acute rejection of allografts, the interaction between Fas (CD95) and its ligand (FasL; CD95L) has been shown to be involved in mediating apoptotic cell death. The role, however, of these molecules in the pathogenesis of transplant vascular sclerosis is as yet undetermined. The present study was therefore designed to address this issue.**

Material. **C3H/HEJ FasL^{gld} (FasL$^-$; H2^k) spontaneously mutant mice were used either as donors or recipients of aortic allografts; wild-type C57Bl/6 (B6; H2^b) were used as corresponding recipients or donors (n=6/group), respectively. Controls included aortas transplanted across appropriate allogeneic and syngeneic strain combinations. For histopathological evaluations, the grafts were harvested at day 40 after transplantation, at which time, splenocytes and sera were also obtained for mixed leukocyte reaction and complement-mediated microcytotoxicity assays, respectively.**

Results. **Similar to aortas obtained from allogeneic controls, allografts harvested from FasL$^-$→B6 recipients had morphological evidence of chronic rejection characterized by circumferential intimal thickening with partial disruption of the elastic membranes. Correspondingly, heightened antidonor cellular reactivity was also witnessed in these recipients. On the contrary, B6 allografts harvested from the majority of C3H→FasL$^-$ recipients exhibited marked preservation of aortic morphology. Although these recipients had diminished antidonor cellular proliferation, the titers of alloantibodies were markedly elevated.**

Conclusion. **The presence of FasL-expressing functional cytotoxic T cells is required for the pathogenesis of transplant vascular sclerosis. The significant reduction and/or absence of chronic rejection with the concomitant retention of antidonor humoral response in C3H FasL$^-$ recipients of B6 aortas prompt us to suggest that perhaps posttransplantation vasculopathy is initiated by cell-mediated cytotoxicity with its perpetuation facilitated by alloantibodies.**

At a molecular level, the acute rejection of allografts by activated cytotoxic T cells (CTLs*) has been shown to be mediated by two distinct pathways: the perforin and granzyme-induced lysis and the ligand-induced apoptosis (*1*). Of the latter, perhaps the most predominant interaction is that between Fas (Apo-1 CD95) and its ligand (FasL; CD95L) (*2*, *3*). Fas, a type 1 membrane protein belonging to the tumor necrosis factor/nerve growth factor receptor family, has been documented to be ubiquitously expressed in various tissues of the body (*4*). On the contrary, expression of FasL, which is a type 2 membrane protein sharing significant homology with members of the tumor necrosis factor family, is more limited, being found primarily on activated T cells (*5*, *6*) and cells of distinct phenotype in the testis (*7*) and in the eye (*8*).

It has been postulated that FasL expressed on activated CTLs can induce apoptosis in Fas-expressing target tissue (*4*). The expression of CD95L or "death" factor on various CTL cell lines (*9*) and on activated splenocytes (*10*) has provided evidence to support the hypothesis that this molecule functions as an effector of CTL-mediated cytotoxicity. The presence of residual CTL in perforin-deficient mice, which was known to be mediated by FasL (*11*, *12*), corroborated unequivocally the earlier findings suggesting that the interaction between CD95-CD95L plays a cardinal role in allograft destruction during acute cellular rejection (ACR). The role, however, of these molecules in the pathogenesis of

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* Abbreviations: α-smA, alpha smooth muscle actin; ACR, acute cellular rejection; B6, C57Bl/6; CR, chronic rejection; CTL, cytotoxic T cells; FasL, Fas ligand; FasL$^-$, C3H/HEJ-FasL^{gld}; *gld*, generalized lymphoproliferative disease; *lpr*, lymphoproliferation; TVS, transplant vascular sclerosis; VVG, Verhoeff-van-Gieson's.

transplant vascular sclerosis (TVS) remains as yet undetermined.

In the *lpr* (lymphoproliferative) mouse, the expression of CD95 has been documented to be markedly reduced (*13*, *14*). On the contrary, in the *gld* (generalized lymphoproliferative disease) mouse, the ability of CD95L to bind to Fas was shown to be abolished (*4*). The latter outcome is believed to be the result of a point mutation in the C-terminus of the coding region for FasL gene, which has been mapped to mouse chromosome 1 (*15*, *16*). In an established model of TVS (*17–19*), we have used mice bearing *gld* mutations as donors and recipients of aortic allografts to study the role of FasL in the pathogenesis of this lesion. This model also provided us with the unique opportunity to study the role of alloantibodies in the evolvement of obliterative arteriopathy.

MATERIALS AND METHODS

Animals. Seven- to 8-week-old inbred male C3H/HEJ ($H2^k$), C3H/HEJ-FasL^{gld} ($H2^k$; FasL$^-$), C57Bl/6 ($H2^b$; B6), and BALB/c ($H2^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival in Pittsburgh, they were housed in a pathogen-free facility and provided ad libitum with Purina rodent chow and tap water. For experiments detailed herewith, the mice were used at 10–12 weeks of age.

Aortic transplantation. The aorta was transplanted by a method detailed elsewhere (*19*). Using a dissecting microscope (M-32; Leica, Northvale, NJ) and under methoxyflurane (Metofane; Pitman-Moore, Inc., Munderlein, IL)-induced anesthesia, a 0.6-cm section of donor thoracic aorta was transplanted end-to-side to the recipient abdominal aorta caudal to the origin of the renal arteries. It was subsequently converted into a "jump" graft by ligation and severance of recipient aorta between the anastomotic sites.

Experimental groups. The aortic transplant recipients were divided into the following groups: group I (syngeneic)=C3H→C3H and B6→B6; group II (allogeneic)=C3H→B6 and B6→C3H; group III=C3H FasL$^-$→B6; and group IV=B6→C3H FasL$^-$. After transplantation, the recipients did not receive any immunosuppressive therapy. It is noteworthy that at the time of transplantation, the FasL$^-$ animals had no clinical evidence of *gld*, whereas the presence of mild-moderate lymphadenopathy was routinely encountered in these recipients at the time of aortic harvest (day 40 after transplantation).

Histological examination. At 40 days after transplantation, the implanted aorta was harvested en bloc and fixed by placing in 10% neutral buffered formalin (VWR, Cleveland, OH) for 48–72 hr followed by routine processing and embedding into paraffin (Sherwood Medical, St. Louis, MO). Sections (4 μm thick) were obtained and mounted onto precleaned slides. These sections were used for routine hematoxylin and eosin (Surgipath, Richmond, IL) staining and for elastic fibers (Verhoeff-van-Gieson's; VVG) (*20*), collagen (Mason's trichrome) (*20*), and alpha smooth muscle actin (α-smA) (*21*).

Morphometry. To provide a more objective assessment of the degree of intimal thickening, we resorted to undertaking morphometric evaluation in all transplanted aortas harvested at day 40 after transplantation. For this purpose, a morphometric graticle was used, and the degree of intimal thickening was ascertained under ×40 magnification. Using light microscopy, two-four sections of aorta harvested from each animal (n=4–5 animals/group) were analyzed; at a minimum, 10 measurements were obtained from each aorta. For primary analysis, VVG-stained sections were used; however, these findings were confirmed by concurrent evaluation of hematoxylin and eosin and α-smA–stained sections. The results were expressed as microns (μ; mean ± SE). For statistical analysis, a multiple intergroup comparisons (Bonferroni) test was used with significance being defined as ≤0.05.

Mixed leukocyte reaction. At the time of death (day 40 after transplantation), the recipient spleen was also harvested and processed to yield splenocytes. Untreated nylon wool-enriched T cells were used as responders (5×10^5 cells/well) to an equivalent number of γ-irradiated (~3Gy; Cesium^{137}; Gammacell 1000, MDS Nordion, Ontario, Canada) stimulators obtained from the splenocytes of either the donor or third-party animals. The cells were placed in triplicate in a 96-well round-bottomed microtiter plate and incubated in 5% CO_2 in air. Seventy-two hours later, the cells were pulsed with 1 μCi of]^{3}H[thymidine (NEN Dupont, Beverly, MA) and incubated for an additional 16–18 hr until harvesting. The degree of [^{3}H]thymidine incorporation was measured by scintillation counting, and the results were expressed as counts per minute (cpm; mean ± SD). Responders or γ-irradiated stimulators cultured in media were used as controls. Because T cell-mediated cytolytic activity is known to be deficient in FasL$^-$ recipients (*1*, *10–12*), these assays were not performed in this study.

Complement-dependent microcytotoxicity. Recipient serum obtained at the time of death was used to measure the titer of alloantibodies. Syngeneic and allogeneic (donor and third-party) splenocytes were suspended at 2×10^5 cells/ml; 2 μl (4×10^2 cells) of which was incubated in Terasaki microplates with an equivalent volume of decomplemented sera for 30 min at RT. To ascertain the titers of alloantibodies, the sera were used at varying known dilutions. At the end of this incubation, the cells were washed three times with phosphate-buffered saline, and 2 μl (1/10 dilution) of pretested, nontoxic, guinea pig complement (Cedarlane Labs LTD, Hornby, Ontario, Canada) was added for 20 min at 37°C in 5% CO_2 in air. To terminate further complement-mediated lysis, the plates were placed at 4°C for 5 minutes. The viability of the cells was assessed by dye exclusion, which was performed by adding 2 μl of 2% eosin (w/v) for 10 min. Viability was determined by enumerating live and dead cells in each well using an inverted phase-contrast microscope. The formula used to calculate cytotoxicity was as follows:

$$\text{cytotoxicity index} = \frac{(\%\text{ cells dead with C+serum}) - (\%\text{ cell dead with C})}{100 - (\%\text{ cells dead with C})} \times 100$$

RESULTS

Histopathological and immunohistochemical analysis. As described previously (*17–19*), aortic grafts transplanted across syngeneic (group I; B6→B6 or C3H→C3H) animals exhibited normal aortic morphology when harvested at day 40 after transplantation. There was no obvious denudation of the single-cell endothelial lining, and the elastic limiting membranes were also well preserved (Fig. 1A). Unlike that in the media, no α-smA$^+$ staining was visualized in the intima (Fig. 1B)—a finding reminiscent of that observed in the native aortas.

On the contrary, aortic allografts harvested at day 40 after transplantation from recipients in group II (C3H→B6 or B6→C3H) had morphological aberrations considered pathognomonic of chronic rejection (CR) (Fig. 1C). There was circumferential intimal thickening (Fig. 2), which was largely due to accumulation and/or proliferation of α-smA$^+$ cells (Fig. 1D). This resulted in narrowing of the lumen, which we had shown previously to be a progressively worsening event in aortas transplanted across these allogeneic strain combinations (*17–19*). It must be emphasized that the histopathological changes detailed heretofore were comparable in aortas transplanted across both C3H→B6 and B6→C3H strain combinations.

Similar to grafts harvested from allogeneic strain combi-

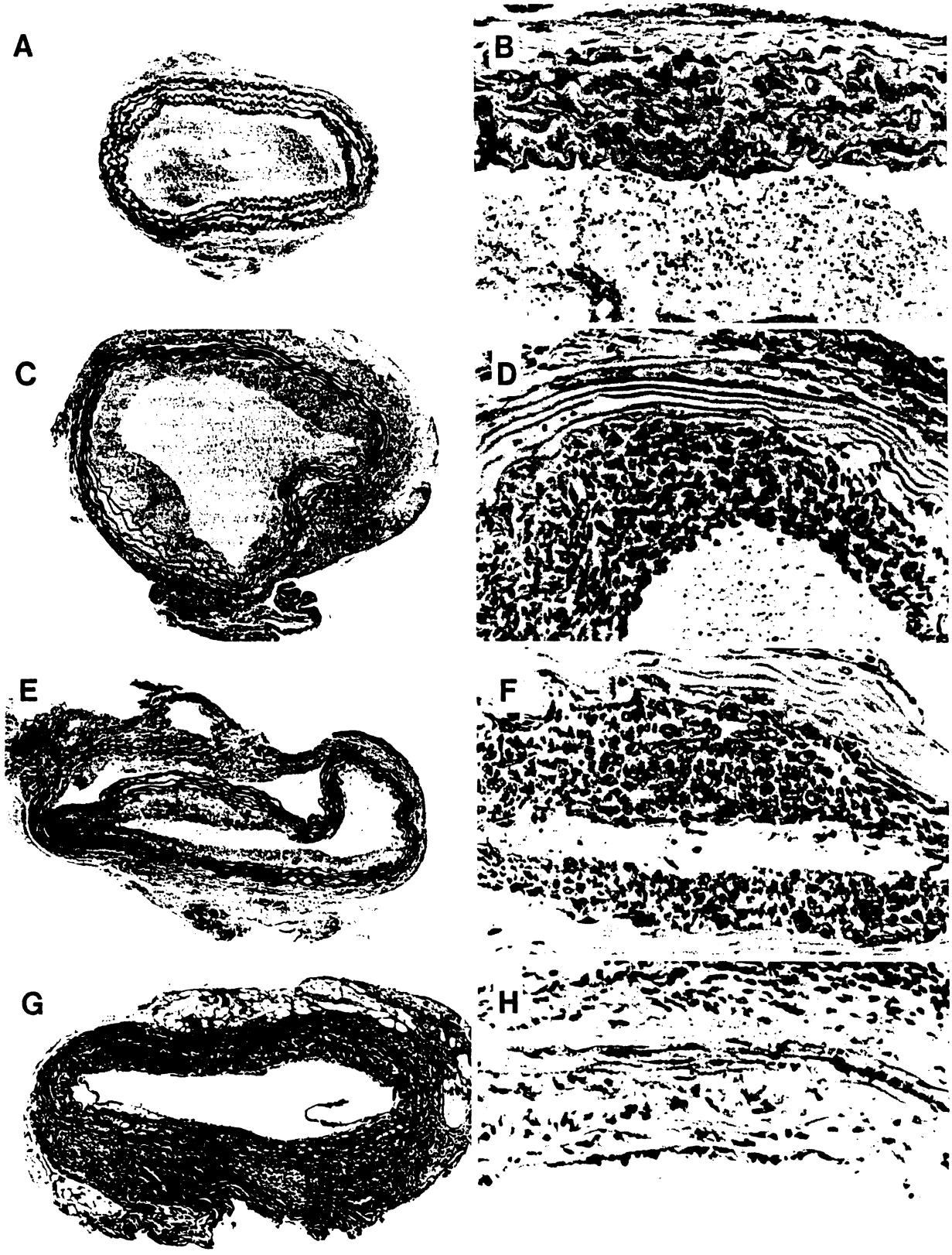

FIGURE 1. Histoimmunopathological evaluation of the morphology of aorta obtained at day 40 after transplantation. The sections were stained either with VVG (A, C, E, and G) or α-smA antibody (B, D, F, and H). Grafts were harvested from either B6→B6 (A and B); B6→C3H (C and D); C3H FasL$^-$→B6 (E and F); or B6→C3H FasL$^-$ (G and F) recipients. Original magnification: ×100 (A, C, E, and G); ×400 (B, D, F, and H).

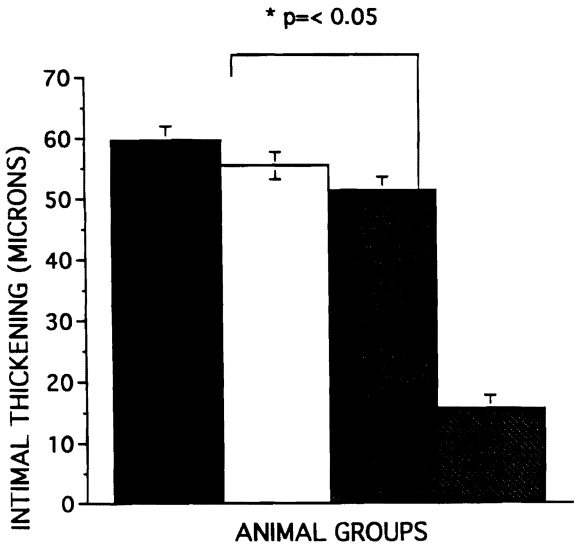

FIGURE 2. Morphometric analysis of the degree of intimal thickening in aortic allografts harvested at day 40 after transplantation. For primary analysis, VVG-stained sections were used, however, these findings were confirmed by concurrent evaluation of hematoxylin and eosin and α-smA–stained sections. Grafts were harvested from either B6→C3H (■); C3H→B6 (□); C3H FasL$^-$→B6 (▨); or B6→C3H FasL$^-$ (▧) recipients. Intimal thickening was measured using morphometric graticle at ×40 magnification (light microscopy). Ten measurements of each aorta (two to four sections/aorta/animal) were obtained (n=4–5 animals/group) for this analysis. Results are expressed as mean ± SE (μ).

nations (group II), aortas transplanted across C3H FasL$^-$→B6 (group III) mice also had comparable intimal changes when examined at day 40 after transplantation (Figs. 1E and 2). There was circumferential intimal thickening (Fig. 2) with destruction of the endothelium and the elastic membranes. Furthermore, as in the untreated allografts (Fig. 1D), the observed aberration in intimal morphology was largely due to proliferation of α-smA$^+$ cells (Fig. 1F). Contrarily, in the majority of recipients, the histology of B6 aortic allografts harvested from C3H FasL$^-$ animals was remarkably preserved (Figs. 1G, 1H, and 2). There was no evidence of circumferential intimal thickening (Fig. 2), and the staining for α-smA$^+$ cells was largely limited to the media (Fig. 1H). These observations were very similar to those encountered in native aorta as well as in grafts transplanted across syngeneic barriers (Fig. 1, A and B). It is noteworthy that similar observations were made in aortas harvested at days 70–80 after transplantation from FasL$^-$ recipients of B6 allografts (data not shown).

Mixed leukocyte reaction. As anticipated, nylon wool-enriched T cells obtained 30–40 days after transplantation from C3H recipients of B6 allografts proliferated against both donor and third-party (BALB/c) irradiated stimulators (Table 1). Similar observations were also made when responders were obtained from B6 recipients of C3H aortic allografts. Inversely, [^{3}H]thymidine uptake was relatively lower when responders were cultured in media alone or with syngeneic stimulators (Table 1). Analogous observations were also made when splenocytes from B6 recipients of C3H FasL$^-$ aortas were used as responders. Interestingly, in these recipients, the heightened donor and the third-party specific alloreactivity was associated with distinct morphological aberration in the transplanted allografts (Fig. 1, B and C), suggesting that cellular responses play a seminal role in the pathogenesis of CR. On the contrary, markedly reduced antidonor reactivity was witnessed in C3H FasL$^-$ recipients of B6, who otherwise responded adequately to third-party stimulators (Table 1). Incidentally, aortic allografts harvested from the majority of these animals had significant reduction in changes pathognomonic of CR, further corroborating our earlier contention that cell-mediated immune responses play a critical role in the initiation and perhaps perpetuation of this lesion.

Complement-dependent microcytotoxicity. Decomplemented sera obtained at days 30–40 after transplantation from either B6 or C3H recipients of syngeneic aortas (group I) exhibited no humoral responses against C3H or B6 targets, respectively (Fig. 3A). This finding was in contrast to that observed in sera of allograft recipients (group II), in whom markedly amplified antidonor cytotoxicity was evident. Similarly, sera from animals in groups III and IV also had elevated titers of antidonor antibodies (Fig. 3B).

DISCUSSION

The use of Fas-FasL by activated CD4$^+$ and CD8$^+$ T (*11*) and natural killer cells (*22*) is believed to be one of the predominant effector mechanisms used by these cells to mediate cytotoxicity. These molecules are also involved in the establishment and maintenance of peripheral tolerance (*23*) and in the activation-induced apoptosis of mature lymphocytes (*24*). These pleiotropic functions of Fas-FasL are perhaps best exemplified in mice bearing either Fas (*lpr*) or FasL (*gld*) mutations in which failure to maintain immune homeostasis results in widespread lymphoproliferation, lymphadenopathy, lack of cytolytic activity, and production of high titers of autoreactive IgG and IgM antibodies with associated autoimmune disorders (*16*, *25*, *26*).

The constitutive expression of FasL on Sertoli cells of the testis and on tissues of the anterior chamber of the eye has

TABLE 1. Results of mixed leukocyte reaction using 5×10^5 splenocytes/well as stimulators (irradiated) or responders

Responders	Irradiated stimulators			
	Media	Donor	Recipient	Third party
B6→C3H	1405±754	8815±1544	1553±242	19682±4555
C3H→B6	1564±186	18986±1686	1556±535	19836±3366
C3H FasL$^-$→B6	1996±831	14075±991	3675±312	74232±11055
B6→C3H FasL$^-$	1617±297	4615±464	1575±376	22692±928

Splenocytes obtained from aortic recipients at day 40 after transplantation were used as responders. The results are expressed as cpm (×±SD).

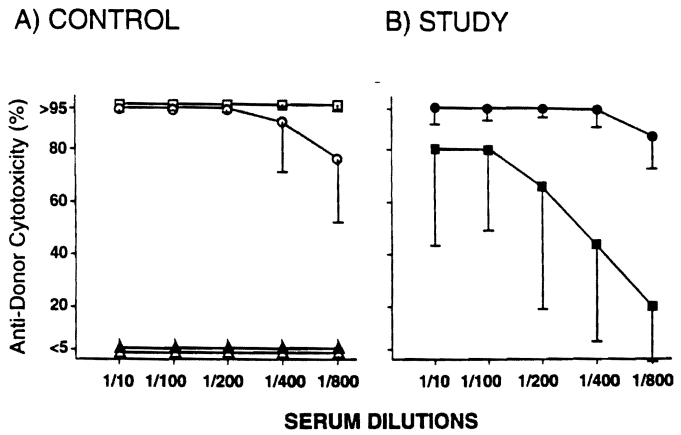

FIGURE 3. To determine the titers of alloantibodies, microcytotoxicity assays were performed using decomplemented sera obtained from recipients at days 30–40 after aortic transplantation. The transplanted groups (n=5 animals/group) were as follows: (▲) B6→B6; (△) C3H→C3H; (○) B6→C3H; (□) C3H→B6; (●) B6→C3H FasL$^-$ and (■) C3H FasL$^-$→B6. The results (from triplicate wells) are expressed as mean ± SD of antidonor cytotoxicity in (A) control and (B) study groups.

underscored its participation in maintaining an immune privilege state at these sites (*7*, *8*, *27*). This assertion has been confirmed in a murine model of islet allotransplantation, whereby coimplantation of FasL-transfected syngeneic myoblasts resulted in prolonged islet allograft survival (*2*). Similarly, significantly delayed rejection of orthotopically transplanted corneal allografts obtained from FasL$^+$ but not FasL$^-$ mice provided yet further evidence for the seminal role played by this molecule in mediating graft destruction (*3*). Using a murine model of heterotopic heart transplantation, Larsen et al. (*28*) have provided contrary evidence suggesting that the Fas-FasL pathway is not essential for mediating efficient allograft rejection. However, in light of the documented observations, this latter conclusion is difficult to interpret.

The multifactorial etiopathology of TVS has precluded a more comprehensive understanding of the mechanism(s) involved in the pathogenesis of this lesion (*29*). However, despite these limitations, the observed correlation of the severity of obliterative arteriosclerosis to the intensity and the frequency of ACR has underscored the importance of alloimmune responses in its evolvement (*18*, *19*, *30*). Despite the heretofore stipulated involvement of Fas-FasL in mediating tissue destruction in ACR, its role in the evolvement of TVS is as yet ambiguous. To delineate the role of FasL in the pathogenesis of TVS, we used FasL$^-$ mice as donors or recipients of aortic grafts transplanted heterotopically across untreated wild-type allogeneic animals.

As has been reported previously (*17–19*), aortic transplantation across allogeneic but not syngeneic barriers resulted, by days 30–40 after implantation, in morphological changes distinctive of progressively worsening CR. There was evidence of circumferential intimal thickening, destruction of the elastic membranes, and accumulation and/or proliferation of α-smA$^+$ cells exclusively within the intima. These animals also exhibited heightened antidonor proliferative activity confirming the importance of cellular immunity in the pathogenesis of CR.

The role of alloantibodies in the development of TVS has also been widely premised (*31–33*). In cardiac transplant recipients, ongoing graft arteriosclerosis has been correlated with the presence of antidonor HLA antibodies (*34*). Additionally, Mohanakumar et al. (*35*) 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 donor HLA-DR antigens. Perhaps a more convincing evidence for the role of alloantibodies in the evolvement of this lesion was provided by studies in which histological aberrations pathognomonic of posttransplant vasculopathy were successfully reproduced by intra-arterial infusion of antidonor sera (*36*).

To ascertain the role of alloantibodies in the pathogenesis of CR in this model, we also determined their titer using microcytotoxicity assays. The sera were obtained from recipients of both syngeneic and allogeneic aortic transplants at day 40 after implantation, at a time when changes characteristic of CR are most discernible (*18*). Unlike that in syngeneic combinations, markedly elevated titers of antidonor antibodies were detected in the sera of allogeneic recipients. This finding affirmed earlier observations (*31–36*) that in addition to antidonor cellular responses, alloantibodies may also play an important role in the development of CR. Still obscure, however, is the precise involvement of the cellular and humoral pathways in the initiation and/or perpetuation of TVS.

To ascertain the role of FasL in the development of CR, aortas were transplanted from C3H FasL$^-$ mutant mice into wild-type allogeneic (B6) recipients. Interestingly, when harvested at days 30–40 after implantation, the morphological changes in the majority of these allografts were indiscernible from those in the untreated allogeneic (C3H→B6) controls. It is noteworthy that similar observations were also made in aortas harvested at days 70–80 after transplantation from FasL$^-$ recipients of B6 allografts (data not shown); this finding precludes the argument that the observed preservation of morphology in the majority of these allografts reflects a mere alteration of the tempo of CR. Additionally, similar to allogeneic but not syngeneic controls, these recipients also exhibited heightened antidonor cellular and humoral reactivity. It must be emphasized that these were anticipated observations because unlike Fas, FasL expression is known to be limited to activated T cells (*5*, *6*) and to distinct immune-privileged sites in the eye (*8*) and the testis (*7*).

The outcome in allografts transplanted across B6→C3H FasL$^-$ mice, however, was quite different. When harvested at day 40 after transplantation, a majority of these allografts exhibited a marked reduction in changes characteristic of CR; their morphology was very similar to that of native aorta as well as to grafts implanted across syngeneic barriers. Initially, this observation seemed inexplicable given that elevated antidonor humoral responses were noted in these recipients. However, the subsequent demonstration in these recipients of reduced antidonor cellular reactivity suggested that perhaps the initiation of morphological aberrations characteristic of CR is initiated by cell-mediated cytotoxicity, with its perpetuation being facilitated by alloantibodies.

In light of these foregoing observations, it is perhaps rational to propose that the absence of a functional FasL on effector T cells results in a blunted cellular response with the

observed partial or complete freedom from changes pathognomonic of posttransplant vasculopathy. Additionally, in the absence of an effective cell-mediated cytotoxicity, the presence of high titers of alloantibodies is incapable of initiating and/or perpetuating histopathological changes of CR.

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