Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts

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Interferon-γ Deficiency Prevents Coronary Arteriosclerosis but not Myocardial Rejection in Transplanted Mouse Hearts

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Abstract

We have hypothesized that T cell cytokines participate in the pathogenesis of graft arterial disease (GAD). This study tested the consequences of IFN-γ deficiency on arterial and parenchymal pathology in murine cardiac allografts. Hearts from C-H-2\(^{bm12}\)KhEg (bm12, H-2\(^{bm12}\)) were transplanted into C57/B6 (B6, H-2\(^b\)), wild-type, or B6 IFN-γ–deficient (GKO) recipients after immunosuppression by treatment with anti-CD4 and anti-CD8 mAbs. In wild-type recipients, myocardial rejection peaked at 4 wk, (grade 2.1±0.3 out of 4, mean±SEM, n = 9), and by 8–12 wk evolved coronary arteriopathy. At 12 wk, the GAD score was 1.4±0.3, and the parenchymal rejection grade was 1.2±0.3 (n = 8). In GKO recipients of bm12 allografts, myocardial rejection persisted at 12 wk (grade 2.5±0.3, n = 6), but no GAD developed (score: 0.0±0.0, n = 6, P < 0.01 vs. wild-type). Mice treated with anti–IFN-γ mAbs showed similar results. Isografts generally showed no arterial changes. In wild-type recipients, arterial and parenchymal cells showed increased MHC class II molecules, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 compared to normal or isografted hearts. The allografts in GKO recipients showed attenuated expression of these molecules (7–10). The present study tested the hypothesis that IFN-γ, a cytokine produced by certain T cell subsets, would presumably occupy a proximal position in this sequence by directly activating macrophages, and by enhancing the expression of MHC products, other components of the antigen presentation pathway, and adhesion molecules (7–10). The present study tested the hypothesis that IFN-γ contributes to the development of graft arterial disease (GAD) in vascularized cardiac allografts, using mice with an IFN-γ deficiency due either to targeted disruption of the IFN-γ gene, or treatment with neutralizing antibody.

Methods

Animals. Inbred mice of several strains were used. C-H-2\(^{bm12}\)KhEg (bm12, H-2\(^{bm12}\)) were used as allograft donors. C57/B6 (B6, H-2\(^b\)) and B6 IFN-γ knockout mouse (B6GKO, H-2\(^b\)) were used as recipients. B6 to B6 isografts were used as controls. The original GKO mice were generated by disrupting the IFN-γ gene, and the second set amplifies a band of 220 bp on the intact IFN-γ gene, and the second set amplifies a band of 375 bp on the region of the targeted mutation (neo\(^−\)). The PCR was

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Abbreviations used in this paper: ECM, extracellular matrix; GAD, graft arterial disease; GKO, IFN-γ knockout; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
run for 40 cycles (thermocycler; Perkin Elmer Corp., Norwalk, CT) using Taq DNA polymerase (BRL, Burlington, Ontario, Canada) at 55°C annealing temperature. The PCR products were run on an ethidium bromide gel; the GKO homozygous mice show a single (neo') band, the wild-type mice show a single IFN-γ band, and the heterozygotes showed both bands. The heterozygotes were intercrossed, and the homozygous GKO mice were identified by tail DNA testing. Homozygosity of the offspring was confirmed by further tail testing, and by testing each mouse for IFN-γ mRNA expression by reverse transcriptase PCR on spleen RNA at the time of experimental use. All GKO mice used were confirmed homozygous, and were from the eighth generation (or greater) backcross into the B6 parental strain. B6 with wild-type IFN-γ genes and bm12 mice aged 8–12 wk were obtained from Taconic Farms, Inc. (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the Harvard Medical School animal facilities on acidified water. Sentinel animals surveyed serologically for viral pathogens were negative in the room in which these mice were housed. All experiments conformed to approved animal care protocols approved by the institutional review group.

Heart transplantation and immunosuppression. Heterotopic cardiac transplantation was performed using a modification of the method described by Corry et al. (12). In brief, donors and recipients were anesthetized with Metofan (Pittman-Moore, Mundelein, IL). Donor hearts were perfused with chilled and heparinized saline via the inferior vena cava, and were harvested after ligation of the vena cava and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava, respectively, of recipients using a microsurgical technique. Ischemic time was routinely 25 min, with a success rate of ~90%. The viability of the cardiac allograft was assessed by daily abdominal palpation. The day of graft failure was defined as the day of cessation of heartbeat.

In some experiments, transplants were performed without immunosuppression. In the remainder, immunosuppression was provided typically by a brief course of treatment with anti-CD4 (HK 1.5) and anti-CD8 (2.43) mAbs, according to a previous report (13). These were administered 6, 3, and 1 d before transplantation as intraperitoneal injections of 0.2 ml of ascites fluid containing each mAb with no subsequent treatment. Anti–IFN-γ mAb (H-22, Armenian hamster IgG; a generous gift of Dr. Abul Abbas, Harvard Medical School, Boston, MA) was also used in some experiments. This mAb (250 μg per treatment) was administered intraperitoneally every 7 d after transplantation according to a previously reported protocol (14). Polyclonal hamster IgG (PharMingen, San Diego, CA) was given to some recipients in the same amount, route, and schedule as a control for anti–IFN-γ.

Histologic techniques. The grafts were explanted at 4-wk intervals up to 12 wk. The grafts were sectioned transversely, frozen in OCT compound (Ames Co., Division of Miles Laboratories, Elkhart, IN) and stored at −80°C, and were fixed in 10% buffered formalin for morphological examination. 4-μm-thick frozen sections of heart in OCT were fixed in acetone for 10 min. For immunohistochemistry, frozen sections were stained with rat monoclonal antibodies against CD45, CD4, CD8, Mac-3, α-actin, MHC class II, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) using standard techniques. The sections were stained using rabbit anti–rat IgG by the avidin–biotin complex method (15), and were counterstained with hematoxylin. Paraffin sections were stained with hematoxylin and eosin and the elastic fiber stain using Weigert’s method.

Monoclonal antibodies. 2.43 (anti-CD4) and GK1.5 (anti-CD8) antibodies for immunosuppression were prepared from hybridoma clones (American Type Culture Collection, Germantown, MD) and used as ascites preparations, or from comparable concentrations of antibody prepared from serum-free supernatants in an artificial capillary system (Cellmax; Celluco Inc., Rockville, MD); antibodies for ICAM-1, VCAM-1, CD4, CD8, CD45, Mac-3, and MHC class II were purchased from PharMingen; antibody to α-actin (IA4) was from Sigma Chemical Co. (St. Louis, MO).

Histological evaluation and statistical analysis. Grafts were analyzed by standard hematoxylin and eosin and elastin stains, and the severity of parenchymal rejection versus GAD was scored blindly by three independent observers (H. Nagano, R.N. Mitchell, and S. Hasegawa). Scores uniformly fell within a range of one grade for all observers, and were averaged. Parenchymal rejection was graded using a scale modified from the International Society for Heart and Lung Transplantation (16) (0, no rejection; 1, mild interstitial or perivascular infiltrate without necrosis; 2, focal interstitial or perivascular infiltrate with necrosis; 3, multifocal interstitial or perivascular infiltrate with necrosis; and 4, widespread infiltrate with hemorrhage and/or vasculitis), and a GAD score was calculated from the number and severity of involved vessels (0, no or minimal [< 10%] vascular occlusion; 1, 10–25% occlusion; 2, 25–50% occlusion; 3, 50–75% occlusion;

<table>
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<td>GAD</td>
<td>PR</td>
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<td>bm12-B6</td>
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<tr>
<td>bm12-B6GKO</td>
<td>Anti-CD4 mAb</td>
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<td>0±0^6</td>
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<td>Anti-CD8 mAb</td>
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<tr>
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<td>Anti-CD4 mAb</td>
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<td>0.6±0.5^6</td>
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<tr>
<td>bm12-B6</td>
<td>Anti-IFN-γ mAb (H-22)</td>
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<td>2.6±0.2</td>
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PR, parenchymal rejection; *P < 0.01 vs. bm12-B6 with anti-CD4 and -CD8 mAb treatment; †P < 0.05 vs. bm12-B6 with anti-CD4 and -CD8 mAb treatment.

Interferon-γ Deficiency Prevents Coronary Arteriosclerosis 551
4, 75–100% occlusion). The cellularity versus extracellular matrix (ECM) content of GAD was assessed blindly by one observer (R.N. Mitchell) using scanned photographic images of all GAD lesions (×66–100) and image analysis (5.2 software; Optimas Corp., Bothell, WA). Thresholds for nuclear versus nonnuclear staining were set using two-pixel averages; intimal lesions delimited by the internal elastic lamina were scored independently for percentage of area encompassed by nuclei (as an approximate measure of cellularity), versus nonnuclear staining (as an approximation of ECM). Areas of nuclear plus nonnuclear staining were normalized to 1. All results are expressed as the mean±SEM. Statistical analysis was performed by one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

All of the bm12 allografts immunosuppressed by treatment with preoperative anti-CD4 and anti-CD8 mAbs continued to beat until harvested (up to 12 wk) in both the wild-type and GKO recipients, although all exhibited substantial myocardial rejection. In the wild-type recipients, rejection was maximal at the earliest time point examined (4 wk), and diminished at later times; the coronary arteriopathy progressively increased with time (Figs. 1 and 2, Table I). When present at 4 wk, the arterial lesions in the transplanted coronary arteries predominantly consisted of accumulations of CD45+ mononuclear cells. At later time points, the lesions became markedly less cellular, with a relative increase in ECM. These later lesions consisted mainly of smooth muscle cells and MAC-3+ macrophages, and morphologically resembled typical human transplantation–associated arteriosclerosis. Some of these fibroproliferative lesions resulted in virtually complete occlusion of the coronary arteries. Many cells in the lesions displayed increased expression of MHC class II, ICAM-1, and VCAM-1 (see Fig. 4, Table II). Except for a single isograft with extensive ischemic injury, isografts showed neither myocardial rejection nor arteriopathy with (n = 13) or without (n = 9) anti-CD4 and anti-CD8 mAb pretreatment (Table I).

In comparison, and despite ongoing parenchymal rejection, allografts in IFN-γ-deficient mice developed little or no arteriopathy (Figs. 1 and 3, Table I). Even at time points up to 12 wk, hearts in the IFN-γ–deficient recipients showed negligible coronary arterial intimal thickening. In addition, the GKO recipient allografts showed markedly attenuated expression of VCAM-1 and ICAM-1 in arterial cells, and of MHC class II in arterial cells and infiltrating leukocytes (Table II, Fig. 4).

Congenital absence of the immunostimulatory cytokine IFN-γ might conceivably alter the ontogeny of the immune response in ways that could influence the development of arterial pathology. Therefore, to verify that deficiency of IFN-γ itself interrupts the development of graft vascular disease, we administered a neutralizing antibody to IFN-γ after transplants into wild-type mice. As in the IFN-γ–deficient animals, hearts of bm12 allografts in B6 wild-type recipients treated

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**Figure 1.** (A) Comparison between parenchymal rejection grade and the grade of GAD in bm12 allografts into C57/B6 recipient mice. Parenchymal rejection peaked at 4 wk after transplantation and subsequently diminished, while GAD increased progressively. (B) The composition of GAD lesions (cellularity and ECM) in the same series of animals as A. These were assessed by image analysis of photographs of all GAD lesions with thresholds set for nuclear versus nonnuclear staining. The ordinate marks the percentage of intimal lesions occupied by nuclei as an approximation of cellularity, or by nonnuclear material, as an estimate of ECM. When present at 4 wk after transplantation, GAD consisted predominantly of mononuclear inflammatory cells. At later time points, GAD showed progressively less cellularity with a relative increase in ECM. These later lesions show many characteristics typical of late-stage human graft arteriosclerosis. (C) Comparison between parenchymal rejection and GAD in bm12 allografts into B6 GKO recipient mice. There was moderate to severe infiltration of lymphocytes in the parenchyma, but few vascular changes appeared for up to 12 wk after transplantation. (A) Parenchymal rejection, black bars; graft arterial disease, gray bars; (B) cellularity, black bars; ECM, gray bars; (C) parenchymal rejection, black bars; graft arterial disease, gray bars.
Interferon-γ Deficiency Prevents Coronary Arteriosclerosis

Figure 2. (A) A representative section of an allograft from bm12 to C57/B6, 4 wk after transplantation. The recipient was pretreated with anti-CD4 and anti-CD8 mAbs (elastin stain, ×40). Note the focal mononuclear inflammatory cell infiltration in the parenchyma. Parenchymal rejection grade: 3–4; GAD grade: 1; the cellularity is assessed as 64.4% and the ECM as 35.6%. (B) A representative section of an allograft from bm12 to C57/B6, 8 wk after transplantation (elastin stain, ×50). Extensive infiltrate expanding the intima and compromising the lumen. Parenchymal rejection grade: 3; GAD grade: 4; the cellularity is assessed as 54.8% and the ECM as 45.2%. (C) A representative section of an allograft from bm12 to C57/B6, 12 wk after transplantation (elastin stain, ×33). Marked fibrointimal expansion and luminal stenosis, morphologically resembling typical human graft arteriosclerosis. Parenchymal rejection grade: 2; GAD grade: 3; the cellularity is assessed as 31.8%, and the ECM as 68.2%.

Figure 3. (A) A representative section of an allograft from bm12 to B6 GKO, 4 wk after transplantation (elastin stain, ×40). Although a multifocal mononuclear inflammatory cell infiltration persists in the parenchyma (parenchymal rejection grade: 3), the artery did not have intimal disease (GAD grade: 0). (B) A representative section of an allograft from bm12 to B6 GKO, 8 wk after transplantation (elastin stain, ×50). There is no GAD (parenchymal rejection grade: 3–4; GAD grade: 0). (C) A representative section of an allograft from bm12 to B6 GKO, 12 wk after transplantation (elastin stain, ×33). The coronary artery lacks intimal thickening (parenchymal rejection grade: 3; GAD grade: 0).
with anti–IFN-γ mAb showed little (one of four recipients) or no (three of four recipients) arteriopathy (score: 0.6±0.5, n = 4 at 8 wk). These anti–IFN-γ antibody-treated mouse heart allografts did, however, develop significant myocardial rejection (grade: 1.4±0.3, n = 4 at 8 wk) (Table I, Fig. 5). As in GKO recipients, there was diminished MHC class II, ICAM-1, and VCAM-1 expression in transplant recipients treated with anti–IFN-γ antibodies. In comparison, grafts in mice treated with control nonspecific IgG showed typical rejection and GAD (scores of 3–4) comparable to untreated bm12 to B6 allografts.

**Discussion**

This study used vascularized cardiac transplantation in mice to probe the pathogenesis of the accelerated arteriosclerosis that develops in allografted organs. In the MHC class II–disparate donor–recipient combination studied here, coronary arterial lesions develop that morphologically and immunopathologically resemble those seen in human transplantation arteriosclerosis (6, 17). Although it has not been possible to examine histologically the sequence of events during the development of GAD in human transplants, the mouse model clearly lends itself to such an analysis. 4 wk after transplantation, arteries exhibit a vasculitis characterized by perivascular cuffing and intraluminal accumulations of mononuclear leukocytes (CD45+ cells). By 8 wk, cell-rich intimal lesions have formed. At the 12-wk time point, the intimal lesions contain smooth muscle cells as well as leukocytes, and have accumulated considerable ECM. At this stage, the luminal surface of the arteries displays scant leukocytes, and perivascular leukocyte accumulation has diminished. This sequence of events suggests that an initial lymphocytic vasculitis precedes development of the fibroproliferative arteriosclerotic lesions of advanced allograft arteriopathy.

Except for a single heart (and in only 1 of over 100 vessels examined in 22 hearts) with extensive ischemic injury, no isografts in this study (with or without antibody pretreatment) showed GAD. The development of GAD in hearts transplanted across MHC barriers, but not in isografts, and the limitation of vascular lesions to the allografted vascular tree, supports an immune-mediated mechanism for the development of graft vascular disease. Nevertheless, nonimmunological mechanisms, such as ischemia/reperfusion injury, may also contribute to chronic rejection (18).

We postulated in 1989 that IFN-γ plays a central role in initiating a cytokine and growth factor cascade that leads to the development of allograft arteriopathy (19). The availability of GKO mice permitted us to test critically the contribution of IFN-γ to the development of transplantation arteriosclerosis in this model. Previous studies have established that GKO mice (a) develop normally and are healthy in the absence of pathogens, and (b) have impaired microbicidal function (11). Our GKO cardiac transplantation recipients, housed in a virus-antibody free facility, have shown no evidence of infection either before or after cardiac transplantation. In the GKO recipient mice, despite the substantial perivascular and parenchymal infiltration by mononuclear cells, no appreciable intimal thickening developed. Administration of a neutralizing antibody to IFN-γ after heart allografting into wild-type mice verified that absence of IFN-γ rather than some developmental anomaly in the immune response in genetically IFN-γ-deficient mice accounted for the attenuated arteriopathy. These results agree with previous work using anti–IFN-γ antibodies (20).

IFN-γ regulates the proliferation and function of activated T lymphocytes (21–23), and plays a pivotal role in rejection by activating macrophages (24–26). In this study, T lymphocytes and macrophages infiltrated the myocardium and coronary arteries of allografts in the wild-type recipients. In contrast, IFN-γ-deficient recipients did not develop thickening of the arterial intima, despite a T lymphocyte and macrophage infiltrate in the parenchyma. IFN-γ powerfully stimulates the increased synthesis and cell surface expression of both class I and II MHC antigens in vivo (27, 28), including bone marrow–derived immunocompetent cells, as well as vessel wall cells (19, 29–33).

Induction of local tissue injury by LPS in GKO mice revealed an essential role for IFN-γ in the induction of MHC class I and II expression in arterial endothelium (34). Similarly, in the present study, endothelial cells and smooth muscle cells of grafts in GKO recipients displayed markedly diminished MHC class II expression relative to wild-type recipients. Endothelial cells increase their expression of surface adhesion proteins, including VCAM-1 or ICAM-1, in response to inflammatory cytokines (10, 35–39). In transplanted coronary arteries, medial smooth muscle cells as well as endothelial cells can express VCAM-1 (40). Vascular endothelia, in rejecting mouse and rabbit cardiac allografts, express VCAM-1 (41, 42). IFN-γ may contribute to this process (35, 36, 38). This adhesion molecule upregulation may be important, since treatment with anti–VCAM-1 antibody induces long-term acceptance of murine cardiac allografts (43). ICAM-1 (the receptor for the leukocyte surface molecule LFA-1) may also contribute to acute and/or chronic allograft rejection. Treatment with anti–ICAM-1 and anti–LFA-1 mAbs, for example, inhibits acute cardiac allograft rejection, and induces donor-specific allogeneic skin graft acceptance in mice (44). Coronary arteriosclerotic lesions in transplanted mouse hearts show abundant ICAM-1, especially on the endothelial surface (40). Treatment with mAbs to ICAM-1 and LFA-1 limited coronary arteriosclerosis in transplanted mouse hearts (45), although the degree to which this finding results from diminished parenchymal rejection is not apparent. In the present study, endothelial and smooth muscle cells in wild-type recipients showed increased VCAM-1 and ICAM-1 expression during develop-
Interferon-γ Deficiency Prevents Coronary Arteriosclerosis

Figure 4. Immunohistochemical staining of representative sections from bm12 to B6 wild-type and bm12 to B6 GKO recipients, 8 wk after transplantation. B6 wild-type recipient grafts displayed increased MHC class II (A, ×50), ICAM-1 (C, ×50), and VCAM-1 (E, ×50). In contrast, B6 GKO recipients showed low levels of MHC class II (B, ×50), ICAM-1 (D, ×50), and VCAM-1 (F, ×50) expression.
expression in GKO mice suggests mechanisms by which lack of finding of diminished MHC class II and adhesion molecule expression of intimal lesions, while IFN-\(\gamma\)-deficient recipients showed much lower levels of these adhesion molecules. The possibility exists that the abrogation of GAD is attributable to a diminished MHC class II expression in a strain combination (bm12 to B6) where the immune response depends on class II difference. Similar results, however, were obtained in completely mismatched allografts (BALB/c (H-2\(^{b}\)) to B6; data not shown).

In terms of the contribution of IFN-\(\gamma\), our data show a clear difference in the pathogenesis of GAD versus parenchymal rejection. Although IFN-\(\gamma\) appears necessary for coronary intimal thickening, non–IFN-\(\gamma\)-dependent mechanisms suffice to generate parenchymal rejection. Indeed, in addition to the persistent diffuse mononuclear cell inflammatory infiltrate in the parenchyma, nonimmunosuppressed GKO recipients demonstrate accelerated cardiac allograft failure, indicating a more aggressive rejection (our own observation and reference 47). This mechanistic dichotomy illustrates why the term chronic rejection may be imprecise in context of cardiac allografts. Some transplantation pathologists use chronic rejection to refer to the proliferative intimal lesion of GAD, however, chronic rejection implies a mechanistic commonality with acute rejection, which may not pertain as shown herein. Moreover, acute rejection refers to a myocardial rather than to a vascular process. Because of this potential ambiguity, we advocate avoiding the term chronic rejection altogether. We prefer to use persistent myocardial rejection to describe the smoldering or indolent parenchymal process seen at endomyocardial biopsy, and graft arterial disease or graft vascular disease to describe the vascular pathology.

Our results support the view that the development of GAD results from a response of cells within the donor coronary arteries to stimuli released during a chronic, localized form of delayed-type hypersensitivity to alloantigens on graft-derived vessel wall cells. In such a reaction, host helper T cells activated by encountering foreign histocompatibility antigens secrete IL-2, IFN-\(\gamma\), and TNF. The present data identify IFN-\(\gamma\) among these cytokines as an essential mediator of GAD. IFN-\(\gamma\) secreted by the T cells, activated by contact with foreign MHC, might trigger the next wave of responses within the transplanted arteries, and stimulate expression of MHC class II, VCAM-1, and ICAM-1 on the surface of vascular endothelial cells, culminating in an accentuated local immune response. IFN-\(\gamma\) might also activate macrophages, with subsequent increased production of other fibrogenic mediators including IL-1, IL-6, PDGF, and TNF-\(\alpha\).

These data establish a critical role for IFN-\(\gamma\) in the pathogenesis of coronary arteriosclerosis in transplanted hearts, but not in myocardial rejection. These data further indicate that reduced expression of class II histocompatibility antigens and leukocyte adhesion molecules may contribute to the mechanism of attenuated coronary arteriopathy in hearts allografted into IFN-\(\gamma\)-deficient mice.

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