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Tolerance, Mixed Chimerism, and Chronic Transplant Arteriopathy¹,²

Paul S. Russell,³* Catharine M. Chase,* Megan Sykes,*⁺ Hiroshi Ito,*⁺ Juanita Shaffer,*⁺ and Robert B. Colvin†

Much evidence supports the conclusion that immunological responses to donor-specific incompatibilities are a major factor in producing “chronic” transplant rejection, including the arteriopathy (atherosclerosis) commonly present. Our experiments explored the effects of altered immunological responsiveness to these Ags on the formation of arteriopathy in transplanted mouse hearts. Specific immunological nonreactivity, or tolerance, was induced either by neonatal administration of allogeneic spleen cells (from F₁ donors between class I-mismatched donor and recipient strains), resulting in “classical” immunological tolerance, or by bone marrow infusion to suitably prepared adult recipients, either fully MHC mismatched or class I mismatched, yielding “mixed chimerism.” Both approaches obviated systemic graft-versus-host effects. In both groups, donor-specific skin grafts survived perfectly and donor cell chimerism persisted. Specific Abs were undetectable in all recipients. Most transplants to either group of tolerant recipients developed striking vasculopathy in their coronary arteries (12 of 15 in neonatal tolerance and 15 of 23 in mixed chimerism)." Both approaches obviated systemic graft-versus-host effects. In both groups, donor-specific skin grafts survived perfectly and donor cell chimerism persisted. Specific Abs were undetectable in all recipients. Most transplants to either group of tolerant recipients developed striking vasculopathy in their coronary arteries (12 of 15 in neonatal tolerance and 15 of 23 in mixed chimeras). Neointimal infiltrates included CD4 and CD8 T cells and macrophages. Only 2 of 29 contemporary isotransplants showed any evidence of vasculopathy. Recipients essentially incapable of T and B cell responses (C.B-17/SCID and RAG1⁻/⁻) were also used. Transplants into these animals developed vasculopathy in 16 of 31 instances. Accordingly, in this setting, vasculopathy develops in the presence of H-2 gene-determined incompatibility even with minimal conventional immune reactivity. Perhaps innate responsiveness, that could include NK cell activity, can create such arteriopathic lesions. More evidence is being sought regarding this process. The Journal of Immunology, 2001, 167: 5731–5740.

Organs transplanted between genetically diverse members of the same mammalian species frequently develop impressive obstructive changes in their arterial vessels as a prominent feature of a process that has been termed “chronic rejection”. Similar lesions can appear in a number of nontransplant settings, even following direct traumatic damage by the stripping away of the endothelial lining from arterial vessels (1). We, have, however, been persuaded that the arteriopathic lesions in transplanted organs result mainly from an ongoing immunological reaction directed against the allogeneic vessel walls as a consequence of the foreign Ags they contain. This conclusion has depended importantly on the virtual absence of such lesions from organs transplanted between members of the same highly inbred strains of animals. The freedom from involvement of isotransplants suggests that in our experimental system the effects of ischemia, and the dislocation of donor organs from their blood supply, are not, in themselves, sufficient to produce the changes of chronic rejection that we are examining. These factors have, however, been reported to be important in producing certain chronic changes in kidney transplants in rats (2). Further support for the central importance of immune responsiveness in the generation of transplant arteriopathy comes from two additional facts. First, certain maneuvers known to dampen, or intrude upon, the immunological response of recipients can reduce the prevalence and severity of such lesions and, second, the lesions concerned are confined entirely to the allogeneic structures of the transplanted organs and never visit themselves elsewhere upon the tissues of the recipient itself. In clinical practice, even though nonspecific immunosuppressive measures have made possible the survival of allogeneic transplanted organs for long periods, present forms of treatment have been insufficient to eliminate these chronic, long-term effects. It might be possible to control this immune response more completely by higher doses of available immunosuppressive agents or perhaps by new agents targeted to selected features of the inflammatory response, such as to certain cytokines, as suggested by the interesting recent experiments of Hancock et al. (3). Still, the possibility of eliminating these arteriopathic lesions and the accompanying changes of chronic rejection by inducing some form of high-grade, specific unresponsiveness to the appropriate Ags of the transplanted organ has remained an attractive one. Should this be achievable, such a state of nonreactivity, or immunological tolerance, would ideally result in the complete and long-lasting elimination of the specific immune response to all the cells of the allospecific donor tissue and should thereby eliminate all of the effects of chronic rejection and the need for continuing immunosuppression with its undesirable side effects.

This report is to describe the present status of our experiments to evaluate the efficacy of two forms of specific nonreactivity, which can be reliably induced and verified under standardized immunogenetic conditions, on the development of arteriopathic lesions in hearts transplanted between mice. As an extension of these

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² Preliminary results of these experiments were presented in brief at a combined meeting of the American Society of Transplant Surgeons and the American Society for Transplantation, Chicago, IL, May 17, 2000.

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experiments, we have also evaluated the outcome of hearts transplanted to mice nonspecifically incapable of immune responses following targeted genetic alterations.

The first example of specific nonreactivity we have selected has been “actively acquired immunological tolerance” induced by the i.v. administration shortly after birth of lymphoid cells from adult members of an allostric strain. This we have done exactly according to the original scheme of Billingham et al. (4) and by the technique subsequently described by Billingham and Brent (5). The second form of specific unresponsiveness has been created in adult recipients by the production of “mixed chimerism.” This is achieved by delivering bone marrow cells from the donor strain to adult recipients that have been prepared with a nonmyeloablative regimen that leaves recipient hemopoietic cells intact. The methods used have been studied extensively by members of our group and have undergone steady improvement, particularly in the measures required to prepare recipients for infusions of allogeneic cells (6–8). In both situations, the experiments have included the same strain combination of animals. The status of cell recipients and the outcomes of treatment in both have also been compared using the same methods.

In additional experiments in which allogeneic heart transplants were performed on animals with different forms of profound nonspecific immune deficiencies, no additional treatment of recipients was given. Finally, isologous heart transplants were also performed as contemporary controls beyond our previous experience already reported (9).

Materials and Methods

Plan of experiments

After the induction of specific tolerance by either of the methods under study, the nonreactivity of each treated recipient was first verified in most experiments by the application of a test skin graft from the donor strain. The strain combination selected was B10.A donors to B10.BR recipients (H-2b to H-2b, a class I Ag difference at H-2K). This combination was used for both approaches to tolerance. In addition, B10.A to C57BL/6 (H-2a to H-2d), and those with a recombinant-activating gene designation (H-2a to H-2k, a class I Ag difference at H-2D). This combination was used in experiments by the application of a test skin graft from the donor strain. In one experiment, RAG1−/− mice were also evaluated as contemporary controls beyond our previous experience (9).

In experiments using recipients with profound, but nonspecific immune deficiencies, no additional treatment of recipients was given. Finally, isologous heart transplants were also performed as contemporary controls beyond our previous experience already reported (9).

Mouse hearts were generally transplanted to a heterotopic abdominal location with appropriate microsurgical anastomoses according to our previously described technique (12). In one experiment, hearts were transplanted to a cervical site by a previously described vascular cuff technique (13).

The continuing status of transplanted hearts was determined by direct palpation at least twice weekly with the vigor of contractions of the transplants being recorded on a scale of 1–3+. Heart transplant recipients received no additional treatment after their original preparation except in one series of experiments. In these experiments, one group of C.B-17/SCID recipients of B10.BR heart transplants was treated on days −6, −3, and −1 before transplantation with anti-CD4 and anti-CD8 Abbs. This treatment was used to conform to our previous experiments with allogeneic heart transplants in which such suppression was necessary to permit long-term heart survival and the development of arteriopathy. Some isologous control transplant recipients also received this treatment. In a second group of animals, in addition to the CD4 and CD8 Abs, an Ab directed against macrophages and NK cells was given on day −1 and once weekly after transplantation for 8 wk. This Ab, anti-asialo GM-1, or ASGM-1, is a rabbit polyclonal reagent, obtained from WAKO (Richmond, VA). In some hands, this Ab has been reported to react quite selectively with NK cells (14).

Full-thickness skin grafts of flank or tail skin were placed upon recipient beds on the lateral thoracic walls preserving the panniculus carnosus muscle layer according to the standard technique described by Billingham and Brent (15). Dressing was removed at 7 or 10 days. The continuing status of skin grafts was determined by inspection, and, in some instances, confirmed by histological examination. Long-term surviving grafts were readily distinguishable from surrounding skin as they maintained their original size and sustained vigorous hair growth.

Histological techniques

The transplanted hearts were removed from their recipients, frozen in OCT compound (Arnes, Division of Miles Laboratories, Elkhart, IN), and stored at −20°C. Tissue blocks containing the hearts were placed upon the stage of a freezing microtome. Sections were cut at 4 μm, acetone or Formalin fixed, and routinely stained with H&E or elastic van Gieson. Frozen sections were also stained by immunoperoxidase using standard techniques. Immunoperoxidase-stained sections were then developed in a solution of 3-amino-9-ethyl carbazole (Aldrich, Milwaukee, WI) as described elsewhere (10). CD4 and CD8 Abs, an Ab directed against macrophages and NK cells (Becton Dickinson, Mountain View, CA), and a rabbit polyclonal anti-asialo GM-1, or ASGM-1, were used as primary Abs, and were visualized by an immunoperoxidase technique. Free-floating sections were counterstained with hematoxylin, and frozen, and routinely stained with H&E or elastic van Gieson. Frozen sections were also stained by immunoperoxidase using standard techniques. Immunoperoxidase-stained sections were then developed in a solution of 3-amino-9-ethyl carbazole (Aldrich, Milwaukee, WI) as described elsewhere (10). CD4 and CD8 Abs, an Ab directed against macrophages and NK cells (Becton Dickinson, Mountain View, CA), and a rabbit polyclonal anti-asialo GM-1, or ASGM-1, were used as primary Abs, and were visualized by an immunoperoxidase technique. Free-floating sections were counterstained with hematoxylin, and mounted in Gelvatol (Monsanto, Springfield, MA).

The severity and distribution of obstructive arterial lesions in the coronary systems of transplanted hearts was generally evaluated on frozen sections stained with the elastic tissue stain. In our experience, the proximal coronary arteries are preferentially affected. Therefore, efforts were made to include the proximal, major coronary vessels about the base of the heart in the sections to be examined. Proliferative changes in the coronary arteries were classified as stage I, in which definite increased intimal cellularity and thickening were apparent, but encroaching upon <50% of the vessel lumen, stage II, including ≥50% of the lumen, or stage III, occupying >50% of the lumen. The number of coronary vessels available for evaluation in an individual heart, and the number of these that were affected, was also recorded. This system of evaluation has been described in detail previously (9). In addition to focusing in this study upon the larger coronary vessels, we also determined the anatomical distribution of the lesions throughout the coronary systems. All vessels sectioned through their lumena were scored for the presence or absence of intimal thickening. Three levels were categorized: “proximal” denotes coronary arteries at their origin; “epicardial” refers to other coronary arteries in epicardial fat; and “intramyocardial” are all arteries within the myocardium. Controls for histological scoring were fully reactive B10.BR recipients of B10.A hearts (n = 61) (9). Statistical comparison of results, where needed, was performed using the Fisher exact test.

Induction of neonatal tolerance or mixed chimerism

Neonatal tolerance was instituted by the i.v. injection into the recipient’s facial vein of 5–10 × 10^6 dissociated spleen cells from adult members of the donor strain (16). In a second group of recipients, marrow strains were injected into neonatal recipients (17).

Mixed chimerism was instituted as described previously (8, 17) in age-matched (8- to 12-wk-old) mice after they had received 3 Gy total body irradiation and the i.v. injection, on the same day (day 0), of unseparated
bone marrow harvested from their MHC-mismatched donors (8–12 wk old). Mice also received a range of T cell depletion doses of rat IgG2b anti-mouse CD4 mAb GK1.5 (0.25–1.0 mg) and anti-mouse CD8 mAb 2.43 (0.02–1.0 mg) on day −1. Cell clones for GK 1.5 and 2.43 were obtained from the American Type Culture Collection (Manassas, VA). Hamster anti-mouse CD40L mAb (MR1) was injected i.p. on day 0 (0.5 mg). The MR1 hybridoma was kindly provided to us by R. J. Noelle (Dartmouth Medical School, Lebanon, NH).

Mice in both the neonatally treated group, and in most which developed mixed chimerism, underwent donor strain skin grafting to substantiate their high degrees of nonreactivity to donor Ags. Those animals that fulfilled the criterion of graft survival in perfect condition for at least 100 days were admitted further to the experiment. This occurred in >80% of mice rendered tolerant at birth and almost 100% of mixed chimeras. Frequently grafts were allowed to survive considerably longer. To evaluate the possible influence of test skin grafting upon the immunological responses of chimeric recipients, one group of mice received heart transplants in the absence of prior skin grafting, it having been confirmed that donor-specific chimeric cells were present. In all cases, potential recipient mice that had undergone treatment to induce tolerance or mixed chimerism received no further treatment to alter immune reactivity. At the conclusion of each experiment in which donor cell chimerism was expected, in addition to examining each mouse for donor cells in the peripheral blood and/or spleen, specimens of the grafted skin (where applicable) and the native and transplanted hearts were removed for histological and immunopathological analysis.

Abs and flow cytometry

Flow cytometric analysis for the presence of multilineage chimerism was performed as previously described (7). In brief, forward angle and 90° light scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in peripheral white blood cells. Two-color flow cytometric analysis was used to distinguish donor and host cells of particular lineages, and the percentage of donor cells was calculated as previously described (18) by subtracting control staining from quadrants containing donor and host cells expressing a particular lineage marker, and by dividing the net percentage of donor cells by the total net percentage of donor plus host cells expressing that lineage marker and by dividing the net percentage of donor cells by the total net percentage of donor plus host cells of that lineage. Dead cells were marked for exclusion using propidium iodide staining. Nonspecific background staining was blocked by nonimmune FcR binding to mouse FcR mAb 2.4G2 (18). FITC-conjugated mAbs included anti-CD4, anti-CD8, anti-B220 (all purchased from BD PharMingen, San Diego, CA), and anti-Mac1 (CalTag Laboratories, San Francisco, CA). Negative control mAb HOPC1-FTTC, with no reactivity to mouse cells, was prepared in our laboratory. Biotinylated anti-H-2Dα mAb 34-2-12, anti-H-2Kk mAb 36-7-5 (BD PharMingen), and a control mAb HOPC1 were developed with PE-streptavidin.

Ab determinations

Complement-dependent cytotoxic Ab titers were determined by trypan blue dye exclusion using dissociated spleen cells as targets, as described previously (19). The presence of nonspecific IgGs in the sera of recipients was assessed by a simple precipitin test (20). In this test, 20 μl of mouse serum was incubated in a microtiter plate with 5 μl of goat anti-mouse IgG1 or IgG2a serum for 1 h at 37°C. The plate was centrifuged for 1 min at 2000 rpm and allowed to stand at room temperature for 1 h. The precipitin reaction in each well was then recorded on a scale of 0–2+

Immunopathology

Immunopathological analysis was performed in similar fashion to previous studies (9). In brief, cryostat sections were stained with Abs to mouse CD3, CD4, CD8, Mac1, and ASGM1 and to donor and recipient class I-determined Ags. The distribution and intensity of the infiltrate was noted for each of the markers. Sections were also stained with H&E and for elastin to determine evidence of vascular inflammation and fibrosis and of rejection activity within the myocardium.

Results

The results from these experiments will be presented in several parts. In the first, we describe the findings in respect to hearts transplanted to recipients manifesting two types of specific unresponsiveness to the Ags of their donors described above. In the second part, we describe the findings with hearts transplanted to two classes of nonspecifically unreactive recipients. Certain supplementary experiments to begin exploring the mechanisms brought into question are then described.

Neonatally tolerant recipients

Table I (group A) includes the skin graft and heart transplant survival as well as the distribution and prevalence of coronary artery lesions in 15 mice in the neonatally induced tolerance group (B10.A to B10.BR). At the time of termination of the experiments, donor cells bearing CD8 markers were detectable in substantial numbers in the spleens of treated mice (in the 11–28% range), although few such cells were detectable in the peripheral blood (<1%: Table II, group A). Donor-derived cells of other lineages were not present in substantial numbers. Several skin grafts, each of which remained in perfect condition on inspection, were also examined histologically (example below, Fig. 1). This revealed not only that each skin graft remained completely free of infiltrating inflammatory cells, but also that viable donor skin structures had persisted normally in the graft. It will also be noted from Table I that the proximal coronary vessels of 12 of 15 recipients examined showed distinct evidence of arteriopathy, of varying degrees of severity, and that 13 of the 19 proximal coronaries examined showed advanced lesions (stages II and III), all in the presence of surviving skin grafts from the donor strain, as well as donor cell chimerism at a substantial level. In Fig. 1, we present these findings in detail for a single, representative animal to exemplify their simultaneous presence in an individual mouse.

Table I. Distribution of coronary lesions in tolerant and chimeric heart transplant recipients

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain Combination</th>
<th>No. of Animals</th>
<th>Skin Graft Survivala (days)</th>
<th>Heart Transplant</th>
<th>Distribution and Prevalence of Coronary Lesions</th>
<th>Lesion Stage in Proximal Coronariesb</th>
<th>No. of Hearts with Proximal Coronary Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neonatal tolerance</td>
<td></td>
<td>56–270</td>
<td>16/19</td>
<td>11/22</td>
<td>4/27</td>
<td>2/15</td>
</tr>
<tr>
<td>B</td>
<td>B10.A to C57BL/6</td>
<td>14</td>
<td>273–398</td>
<td>70–123</td>
<td>Functional scorec (Prox., Epi., Intra.)</td>
<td>14/20</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>mixed chimerism</td>
<td></td>
<td>2–3</td>
<td>6/12</td>
<td>2/44</td>
<td>5/5</td>
<td>11/14</td>
</tr>
<tr>
<td>C</td>
<td>B10.A to B10.BR</td>
<td>5</td>
<td>180–204</td>
<td>89–135</td>
<td>Functional scorec (Prox., Epi., Intra.)</td>
<td>2/7</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>mixed chimerism</td>
<td></td>
<td>2–3</td>
<td>0/6</td>
<td>0/5</td>
<td>2/0</td>
<td>0/0</td>
</tr>
<tr>
<td>D</td>
<td>B10.A to B10.BR</td>
<td>4</td>
<td>Not done</td>
<td>30–91</td>
<td>Functional scorec (Prox., Epi., Intra.)</td>
<td>2/6</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>mixed chimerism</td>
<td></td>
<td>2–3</td>
<td>0/16</td>
<td>0/3</td>
<td>2/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

a Skin graft survival includes total period from application of primary grafts to end of experiment. All skin grafts were in perfect condition at the end of the experiment.

b Contractile activity of transplants.

c Severity score for affected coronaries (see Ref. 2).
The serum of representative mice in both tolerant groups was also examined by complement-dependent cytotoxicity for the presence of humoral Abs to donor strain lymphoid cells at the termination of each experiment. No such Abs could be detected in any specimen.

**Recipients with mixed chimerism**

Similar results regarding evidence for a specific lack of reactivity to donor Ags were obtained in the case of mice of the two strain combinations made tolerant according to the mixed chimerism protocol (B10.A to C57BL/6 and B10.A to B10.BR, see Table I, groups B and C). Thus, all of the mice that received skin grafts supported their survival in perfect condition throughout the duration of the experiment. Their levels of donor cell chimerism were generally higher than in mice rendered neonatally tolerant (see Table II, groups B and C), and a broader range of cell types was present.

Hearts were transplanted to a total of 19 fully tolerant recipients in the two strain combinations in which mixed chimerism had been established. In these mice, tolerance had been proved by the long-term survival of a donor strain skin graft. The findings from these heart transplants are summarized in Table I. Among recipients of fully MHC-mismatched marrow (B10.A to BL/6), hearts transplanted to the abdominal location in five of eight mice developed vasculopathy of varying severity throughout their coronary systems. Among six hearts transplanted to the neck, all six showed vasculopathy. In the class I-only mismatched B10.A to B10.BR combination, two of five hearts showed evidence of vasculopathy which was mild (stage I) in severity and lesions in epicardial and intramyocardial portions of the coronary system were absent.

Of four B10.A hearts transplanted to B10.BR mixed chimeric recipients, which had previously been shown to contain donor-specific cells by flow cytometric analysis, but had not undergone skin grafting, two manifested typical arteriopathic lesions when their heart transplants were examined at 30–91 days. Persistent donor cell chimerism was also confirmed at this time (Tables I and II, group D). These results were reassuring in regard to the possible perturbation of the system by the presence of skin grafts in some chimeric recipients before the placement of heart transplants.

Coronary lesions in the arteries of transplants to recipients manifesting either neonatal tolerance or mixed chimerism revealed a cellular infiltrate in the intima and adventia consisting of CD3⁺, CD4⁺, Mac1⁺, IA⁺, and ASGM1⁺ cells, with few CD8⁺ cells (Fig. 2). More commonly, intimal fibrosis with a scant cellular infiltrate and luminal narrowing was evident, presumably representing a later stage of the inflammatory process. There was little or no inflammation in the myocardium. The cellular and fibrotic lesions were more frequent in the proximal graft coronary arteries near their ostia and in the epicardial zone in all groups, similar to the results found in previous studies using fully reactive recipients (9) (see Fig. 4). The distal intramyocardial arteries were affected in similar frequency in the neonatally tolerant (14%), mixed chimeric (4%), and fully reactive mice (13%).

**Table II. FACS analysis of neonatally tolerant and mixed chimeric heart transplant recipients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain Combination</th>
<th>No. of Animals</th>
<th>Skin Graft Survival⁶ (days)</th>
<th>Heart Transplants</th>
<th>FACS Analysis (range, % donor cells)</th>
<th>Peripheral blood</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Survival (days)</td>
<td>Functional score</td>
<td>B cells (%)</td>
<td>Granulocytes (%)</td>
<td>CD4 (%)</td>
</tr>
<tr>
<td>A</td>
<td>B10.A to B10.BR</td>
<td>16</td>
<td>155–378</td>
<td>56–270</td>
<td>2–3</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>D</td>
<td>B10.A to B10.BR</td>
<td>4</td>
<td>Not done</td>
<td>30–91</td>
<td>2–3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁶ Skin graft survival includes total period from application of primary grafts to end of experiment. All skin grafts were in perfect condition at the end of the experiment.

**FIGURE 1.** Simultaneous findings in a single B10.BR mouse made tolerant of B10.A cells by neonatal injection. The second of two surviving B10.A skin grafts is shown in a at 95 days (the first was also surviving at 355 days). A section of the second graft, shown in b, is stained for H-2Dd, a donor-specific Ag. It demonstrates survival of donor skin elements and the complete lack of infiltrating inflammatory cells. c, A coronary artery of this recipient’s B10.A heart transplant at 235 days shows typical inflammatory changes of transplant arteriopathy. Weigert’s elastic tissue stain.
Hearts transplanted to immunologically nonreactive recipients

Sixteen B10.BR hearts were transplanted to otherwise untreated C.B-17/SCID recipients (shown in both Tables III and V). Of these, 11 were found to have definite proximal coronary vascular lesions when the animals were sacrificed 29–64 days later. Similar lesions were found, although less commonly, in hearts transplanted to RAG1/H11002 and SCID/beige recipients, as detailed in Table III. In most cases, the lesions were fibrotic with a scant infiltrate. Those BR allografts in SCID and RAG1/H11002 H11002/B10.BR recipients whose lesions were more cellular showed a coronary arterial intimal infiltrate including Mac1/H11001 and ASGM1/H11001 cells. Cells bearing markers for CD3/H11001, CD4/H11001, and CD8/H11001 were also detected in the intima in some hearts (Fig. 3).

Perivascular CD3 cells were present in all SCID mice. In RAG1/H11002/B10.BR animals, CD3 cells were rare in the heart except for the four animals with lesions. No evidence of rejection of the myocardium was observed, as judged by the scant inflammatory infiltrate and preservation of the cardiac myocytes. The distribution of these lesions was similar to that in nonimmunodeficient recipients, affecting predominantly, but not exclusively, the proximal coronary branches (Fig. 4).

To assess the possibility of some residual immune reactivity in SCID recipients, the sera of seven of these mice that had received heart transplants were tested for the presence of Ig. Two of these gave positive reactions. Similar tests of three SCID/beige recipients were all positive. This suggested, of course, that a degree of immunological “leakiness” had occurred in some SCID mice consistent with the presence of CD3+ cells. No relationship could be established between this finding and the presence or severity of coronary lesions. This shortcoming should not apply to RAG1/H11002/B10.BR recipients, and no trace of Ig was found in several of these mice that were tested. To establish without question that RAG1/H11002/B10.BR recipients were able to mount arteriopathic lesions in H-2-incompatible transplanted hearts, two mice in which such lesions had formed were confirmed as being bona fide RAG1/H11002/B10.BR animals by PCRs showing both the presence of the neomycin marker and the absence of the RAG1 gene (tests kindly performed by J. Bracy and J. Iacomini, Transplantation Biology Research Center, Massachusetts General Hospital, Boston, MA).

Isogeneic controls

Seven B10.A to B10.A isologous heart transplants and 16 B10.BR isografts were performed to provide concurrent controls for the

### Table III. Hearts transplanted to immunologically nonreactive recipients

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>No. of Animals</th>
<th>Heart Transplants</th>
<th>Distribution and Prevalence of Coronary Lesions</th>
<th>Lesion Stage in Proximal Coronaries</th>
<th>No. of Hearts with Proximal Coronary Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.BR</td>
<td>SCID</td>
<td>16</td>
<td>Survival (days)</td>
<td>Prox. A  Epi. Intra. I II III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10.BR</td>
<td>RAG1/H11002/B10.BR</td>
<td>15</td>
<td>29–64</td>
<td>16/22</td>
<td>3/14</td>
<td>1/65</td>
</tr>
<tr>
<td>B10.BR</td>
<td>SCID/beige</td>
<td>9</td>
<td>28–55</td>
<td>7/13</td>
<td>1/12</td>
<td>1/11</td>
</tr>
</tbody>
</table>

* Contractile activity of transplants.
* Prox., Proximal; Epi., epicardial; Intra., intramyocardial.
* Severity score for affected coronaries (see Ref. 2).
present experiments. Six isologous transplants were also performed between members of the unreactive strains (Table IV). All but two hearts were completely free of arteriopathy at all levels of scoring, and those hearts showed no more than slight evidence of vascular involvement.

A possible role for macrophages and NK cells

The participation of “innate” reactivity, and particularly NK cells, in the production of vascular lesions under these conditions was considered to be an interesting possibility. Accordingly, nine B10.BR hearts were transplanted into C.B-17/SCID/beige recipients. Five of these transplanted hearts developed proximal coronary lesions (see Table III).

In an additional experiment, 13 SCID recipients of B10.BR heart transplants received anti-CD4 and anti-CD8 Abs before transplantation and were also treated on day −1 and once a week thereafter with anti-ASGM-1 Ab. This treatment significantly reduced lesion formation in this group of recipients as compared with animals in the same strain combination treated with anti-CD4 and anti-CD8 Abs alone (p < 0.0083). In those recipients treated with ASGM1 in which transplanted hearts still manifested some coronary arteriopathy (5 of 13), a number of ASGM1+ cells had escaped elimination, whereas CD3+ cells were rare. Treatment only with anti-CD4 and anti-CD8 did not suppress lesion formation significantly compared with untreated animals, as detailed in Table V (p = NS).

Discussion

We have examined the fate of hearts transplanted between allogeneic mouse strains after their recipients had been rendered highly, and specifically, unreactive to the foreign Ags known to be presented by the transplanted organs. In both examples of tolerance, substantial levels of donor lympho-hemopoietic cell chimerism persisted in recipients throughout the experiments, and in both cases treated animals remained entirely healthy and vigorous with no evidence of systemic graft-vs-host reactivity. This was avoided in the case of neonatally induced tolerance by delivering donor strain Ags as lymphoid cells from members of the F1 generation between the donor and recipient strains. In mixed chimeras, graft-vs-host effects were avoided since the adult donor and host cell populations became nonreactive to one another as they traversed the thymi of appropriately prepared recipients, and the first mature donor T cells present in the donor marrow graft were rendered tolerant by exposure to the same treatments that were given to the recipients (11). The profound degree of immunological tolerance achieved in mice treated in either of these ways was strongly attested to by their ability to support the indefinite survival of skin grafts from members of the donor strains, and even of second skin grafts bearing fresh complements of dendritic cells. Mixed chimeras, prepared with the regimen described here, all demonstrate specific and complete unresponsiveness to the donor in MLR and
Cell-mediated lympholysis assays, and their T cell repertoires are deleted intrathymically of cells with receptors recognizing donor Ags (8, 17). Likewise, the donor cell chimeric state was neither attributable to the donor heart, nor did the transplanted heart appear to influence it. In no treated animal did we detect the presence of Abs to donor-specific Ags. We also found no evidence that the presence of test donor skin grafts themselves might have stimulated immune reactivity to later heart transplants in these chimeric recipients.

Despite a demonstrated lack of specific immune reactivity on the part of their recipients, many transplanted hearts, in the presence of either type of tolerance, developed distinct proliferative lesions in their coronary arteries. For the purposes of this study, we have focused upon the status of the larger vessels as they are more extensively involved and more readily examined. When grades of involvement are important to an experiment, other approaches, including morphometric techniques, can clearly be helpful. Here, the mere presence of arteriopathy, in the circumstances we have examined, was taken as the main end point. All of these lesions involved the characteristic rich population of inflammatory cells in the linings of affected vessels along with proliferative and centripetal migratory responses by smooth muscle cells of the media. The coronary arteries of the allografts in both tolerance protocols showed both an advanced intimal fibrosis and a mixed mononuclear cell infiltrate, which included T cells, macrophages, and possibly NK cells, as judged by their reactivity with Abs to CD3, Mac1, and ASGM1. CD4⁺ cells and, to a lesser degree, CD8⁺ cells were present. These vascular lesions were similar in severity and distribution to those we have described in cardiac allografts in normal animals, although the cellular phase was less commonly observed in the tolerant animals and the frequency of involvement was somewhat diminished. In both the neonatal and mixed chimerism tolerance protocols, no evidence of acute rejection of the myocardium was observed as judged by a strong contractile pulse and the lack of histological injury and inflammation in the myocardium.

The finding of arteriopathy in tolerant recipients came as a surprise. Transplanted organs are usually permitted to survive in both experimental and clinical settings because of some form of continuing immunosuppressive treatment of their recipients. But this treatment may not fully suppress all immunological responses to the Ags in the donated tissue so that ongoing immune responsiveness under these conditions, and the presence of arteriopathy, is not unexpected. In our tolerant animals, arteriopathy was not anticipated because of ample evidence that chronic changes in transplanted organs are generally attributable to an ongoing specific immune response to them on the part of their recipients, as mentioned above. Evidence for this includes extensive localization of T lymphocytes and other inflammatory cells in these lesions and the dependence for their production on incompatibility between donors and recipients. Furthermore, vasculopathic lesions can be produced by the passive transfer of Abs specifically reactive with donor Ags (21) or by the transfer of appropriate immunologically competent cells (22).

In considering mechanisms for the appearance of vascular lesions in organs transplanted to tolerant recipients, several possibilities were entertained. First, the tolerant states in question, although they seemed to be quite profound by all our tests, might not have been absolute, and a small degree of continuing specific reactivity could have been spared by the treatments given. The evidence for robust and systemic tolerance described above makes

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of Animals</th>
<th>Survival (days)</th>
<th>Functional score</th>
<th>Prox.</th>
<th>Epi.</th>
<th>Intra.</th>
<th>No. of Hearts with Proximal Coronary Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.A</td>
<td>7</td>
<td>56–118</td>
<td>3</td>
<td>0/9</td>
<td>0/9</td>
<td>0/43</td>
<td>0/7</td>
</tr>
<tr>
<td>B10.BR</td>
<td>16</td>
<td>56–70</td>
<td>2–3</td>
<td>1/15</td>
<td>0/30</td>
<td>0/45</td>
<td>1/16</td>
</tr>
<tr>
<td>RAG1−/−</td>
<td>4</td>
<td>57</td>
<td>2–3</td>
<td>1/6</td>
<td>0/9</td>
<td>0/14</td>
<td>1/4</td>
</tr>
<tr>
<td>SCID</td>
<td>2</td>
<td>56–57</td>
<td>2–3</td>
<td>0/2</td>
<td>0/10</td>
<td>0/5</td>
<td>0/2</td>
</tr>
<tr>
<td>Totals</td>
<td>29</td>
<td>56–118</td>
<td>2–3</td>
<td>2/32</td>
<td>0/58</td>
<td>0/107</td>
<td>2/29</td>
</tr>
</tbody>
</table>

* Contractile activity of transplants.
* Prox., Proximal; Epi., epicardial; Intra., intramyocardial.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatmenta</th>
<th>No. of Animals</th>
<th>Heart Transplants</th>
<th>Distribution and Prevalence of Coronary Lesions</th>
<th>Lesion Stage in Proximal Coronariesb</th>
<th>No. of Hearts with Proximal Coronary Lesions</th>
<th>p Value for group with Proximal Coronary Lesions</th>
<th>Proximal coronaries % Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.BR to SCID None</td>
<td>16</td>
<td>29–64</td>
<td>2–3</td>
<td>16/22 3/14 1/65 2 7 7 11/16</td>
<td>NSf  NSf</td>
<td>0.0083g  0.0056h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10.BR to SCID CD4/8(a)</td>
<td>13</td>
<td>30–78</td>
<td>2–3</td>
<td>16/18 1/8 0/36 5 9 2 11/13</td>
<td></td>
<td></td>
<td>0.0083g  0.0056h</td>
<td></td>
</tr>
<tr>
<td>B10.BR to SCID CD4/8 anti-ASGM1</td>
<td>13</td>
<td>32–79</td>
<td>2–3</td>
<td>5/16 2/8 0/29 3 2 0 5/13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Treatment with mAbs directed to CD4 and CD8 determinants has been a standard feature of our protocol for allogeneic transplants in previous experiments (2) and was continued in some groups here for uniformity.

b Contractile activity of transplants.

c Prox., Proximal; Epi., epicardial; Intra., intramyocardial.

d Severity score for affected coronaries (see Ref. 2).

e The p values calculated according to the Fisher exact Test using either the number of proximal coronary arteries with and without lesions or the number of hearts with and without lesions.

f The p value for group with no treatment vs CD4/8; p > 0.05.

g The p value for treatment group with CD4/8 vs CD4/8 and anti-ASGM1.
this explanation seem improbable. Second, the tolerant states concerned that had been instituted by lympho-hemopoietic cells might not have extended to all of the foreign Ags presented by transplanted hearts. Thus, genetically segregating Ags in the transplanted hearts, perhaps expressed especially in certain vessels, might have continued to provoke an immune response. This could have occurred even though full tolerance of all of the Ags present in the lympho-hemopoietic cells of the donor inocula and also in the skin grafts placed later in the strain combinations selected had been achieved.

In any case, either of these two possibilities would depend upon some form of continuing immune responsiveness toward the donated tissues. Accordingly, we sought evidence regarding the fate of hearts transplanted to recipients rendered profoundly incapable of all immune responses by two different genetic alterations represented by C.B.17/SCID and RAG1−/− mice. Here, again, we have regularly found proliferative coronary vascular lesions in hearts transplanted to such recipients. Isotransplants between members of these deficient strains were generally free of coronary vascular lesions. Thus, in these experiments, coronary lesions were found principally in circumstances in which the donors and recipients concerned were incompatible with one another. Some evidence of leakiness as manifested by the presence of Igs was detected in 6 of 14 SCID and SCID/beige mice. Even in these mice immune reactivity must have been quite profoundly impaired, however, and no systematic relationship between the presence of detectable Igs and the presence of lesions in transplanted hearts was evident. Arteriopathy was observed in two animals in which no Igs could be found. In addition, in vivo depletion with anti-CD4 and CD8 Abs had no impact on the incidence of these lesions. Nevertheless, these observations with RAG1−/− recipients all the more useful as no evidence for leakiness exists in this strain. The coronary artery lesions in both SCID and RAG1−/− mice were similar to those found in recipients in both tolerance protocols, even to the presence of scattered CD3+ cells. Cells reacting with Mac1 and ASGM-1 were particularly prominent in the cellular phase of arteriopathy. The origin of the cells (donor or recipient) was not determined, although the presumption is that the T cells in the RAG1−/− mouse must have been of donor origin; CD3+ cells have been described in SCID animals in small numbers (23, 24).

We do not take these findings to indicate that arteriopathy in transplanted organs generally occurs apart from specific immunological reactivity. Indeed, we continue to believe that there is convincing evidence that immunological responses to donor-specific histocompatibility Ags are an important instigating cause for these changes in most circumstances. What we consider has been raised by the above results is that conventional immunological reactivity to donor-specific Ags need not be the only cause for arteriopathy in transplanted organs between incompatible individuals. Other mechanisms could be responsible for arteriopathic lesions, either along with specific immunological responses or independent of them.

What could explain such inflammatory lesions in the absence of specific T and B cell immunity? A third pathway of primitive or innate responsiveness, such as that by the cytotoxic or cytokine release properties of NK cells, could play a part. We selected C.B-17/SCID/beige recipients for further tests because their NK cells have been demonstrated to be impaired as regards certain functions (25). The beige defect is the murine counterpart of the human Chédiak-Higashi syndrome, which is a defect in lysosomal granule exocytosis that impairs NK cell cytolytic activity. This defect would not preclude the ability of such NK cells to produce IFN-γ and other cytokines upon activation. In previous studies to address the question of whether or not donor and host NK cells are “tolerant” of one another in mixed chimeras, it was found that recipient NK cells from them are able to kill donor lymphoblast targets after stimulation in vitro with IL-2 and donor stimulator cells, yet they maintain tolerance of self-Ags (26). On the other hand, standard in vivo assays for NK cell-mediated narrow rejection in established mixed chimeras also suggest, however, that host NK cells are indeed tolerant of the donor (Y. Zhao and M. Sykes, unpublished data). Nevertheless, these cells may still be the sources of certain cytokines, including IFN-γ.

Therefore, our findings in C.B-17/SCID/beige recipients should not weigh decisively against the participation of NK cells particularly in view of the known importance of IFN-γ in the production of arteriopathy. We found some time ago that blocking the effects of IFN-γ with a mAb specifically reactive to it would significantly inhibit the development of arteriopathic lesions (27), and others have since found that treatment of mice with IFN-γ alone can produce them (28).

The reduction in severity of arteriopathy in SCID recipients following treatment of heart transplant recipients with ASGM-1 Ab is consistent with the possibility that NK cells are involved in the process. The glycolipid ASGM-1 is expressed by NK cells, and the polyclonal anti-ASGM-1 has been reported to deplete NK activity specifically (14). ASGM-1 is also expressed, however, by dendritic cells, macrophages, and cytotoxic T cells (29, 30). Treatment with anti-ASGM-1 in vivo can suppress NK function without affecting T cell immune reactions (31), although inhibition of cytotoxic T cells has been reported (32). Paradoxically, anti-ASGM-1 increases Ig levels in SCID mice (24). The lack of precise specificity of this polyclonal Ab for mouse NK cells must leave the question open for further definition. It might seem unlikely that NK cell activity, presumably via IFN-γ, could be mainly responsible for the inflammatory lesions we have observed, but it should be borne in mind that the process of arteriopathy is a chronic one that could well occur as a consequence of quite low-grade reactivity over a considerable period of time.

In the context of possible NK cell activity in the present circumstances, how can the difference in behavior of skin grafts vs heart transplants be explained? Skin grafts have not been shown to be susceptible to attack by NK cells, and this conclusion has also been reached for transplanted organs (33). More recently, however, evidence has been advanced supporting involvement of NK cells in acute organ rejection (e.g., Ref. 34). It may also be relevant that long-term surviving skin grafts can undergo gradual replacement of endothelial cells in small vessels by recipient-derived cells (35), whereas replacement of endothelial cells in transplanted organs is usually limited (36), although some reports suggest it to be present in varying degrees (37, 38). On the other hand, considerable evidence now supports the view that the derivation of the majority of neointimal cells, bearing α-actin markers characteristic of smooth muscle cells, is from the recipient, although their origin, in different situations, may be either from bone marrow (39, 40) or from adjacent vessel walls (41).

The involvement of various infectious agents in the generation of conventional atherosclerosis has been suspected for some time, although careful recent studies have led to the conclusion that their presence should not be considered a necessary component of the process (42). Nevertheless, one could suspect that some infectious agent might have found its way into the tissues of either some of our donors or recipients and be implicated along with foreign histocompatibility factors carried by the donor animals. To explore this possibility, a group of mice, in the combination B10.A donors and C57BL/6 recipients, was housed and treated throughout in a
specific pathogen-free environment. These mice underwent the standard protocol for production of mixed chimerism. Five of six in this group of heart transplants developed coronary vascular lesions. This evidence argues against the involvement of ordinary pathogen in the process under study.

Several reports exist regarding the effects of various forms of specific unreactivity, or tolerance, on the formation of arteriopathic lesions in transplanted organs. In two, tolerant states were induced in respect to donor Ags determined by genes of the MHC between rats (43) or miniature swine (44). In the former case, tolerance followed infusion of donor bone marrow cells and a course of cyclosporine, whereas in the latter it was induced between swine differing at class I of the MHC following a short course of cyclosporine and a donor-specific kidney transplant. In both, the test organ was a heterotopic heart transplant. No evidence of arteriopathic lesions was found in either of these experiments. When immune responsiveness is impaired by interfering with the “second signal” of activation in, for example, CD40 ligand-deficient recipients, even though transplant survival is extended, arteriopathy still occurs (45). A combination of treatment with a mAb to CD40 ligand plus donor cells has, however, been reported to eliminate arteriopathic changes in transplanted mouse hearts (46).

Two reports are also available in which tolerance, induced to non-MHC-determined Ags, likewise appeared to be sufficient to prevent the formation of arteriopathic lesions in transplanted hearts. One made use of mice (AKR and C3H) differing in respect to Ms-I and multiple minor histocompatibility Ags, but not for H-2-determined Ags (47) and the other of rats of the F344, ACI, and WF strains (48). More recently, further observations have come to light from one group that arteriopathic lesions can, indeed, be found in swine hearts transplanted to tolerant recipients under the conditions of their experiments (49).

These inconsistencies in results are not easy to account for, although variability in the vigor of innate responsiveness in different settings and variations between species, along with the time it may take for these chronic changes to occur under such widely different situations, could contribute. Different forms of nonreactivity also may have different outcomes, and it remains possible that certain forms of “peripheral tolerance” can, for example, lead to different results than we have found with the profound “central” tolerance we have been investigating. Indeed, of the two forms of tolerance tested here, there is a suggestion that arteriopathic changes may be somewhat less prevalent in recipients made tolerant through mixed chimerism than by neonatal exposure to donor-specific Ags. Furthermore, the mechanisms involved in producing nonreactivity, even of neonatal tolerance, have recently come into question as evidence for the importance of a preferential Th2 response rather than a central deletion mechanism has been advanced (50, 51).

In any case, we can assert that arteriopathic lesions were clearly present in major coronary arteries under the circumstances of our experiments. Although a full understanding of the mechanisms at work in these complex settings will require additional studies, the present results make it clear that incompatibilities, at least of MHC-determined Ags, are sufficient to provoke the formation of arteriopathy in the absence of any demonstrable conventional immune activity, either cellular or humoral. As mentioned above, a number of pathways toward the formation of arteriopathic lesions are known. The experiments reported here suggest that, at least in certain circumstances, additional possibilities, including innate responsiveness, should be added to the other mechanisms already known as possible causes for transplant arteriopathy.

Acknowledgments

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References

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