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LYMPHOID/NONLYMPHOID COMPARTMENTALIZATION OF DONOR LEUKOCYTE CHIMERISM IN RAT RECIPIENTS OF HEART ALLOGRAFTS, WITH OR WITHOUT ADJUNCT BONE MARROW[1]

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Background. The role of leukocyte migration and chimerism in organ allograft acceptance has been obscured by the lack of information about the late localization of the donor cells.

Methods. Male Lewis rat→female Brown Norway abdominal heart transplantation was performed under tacrolimus immunosuppression (days 0–13, 20, and 27) with or without donor bone marrow and (in bone marrow subgroups) a 1-week postoperative course of a possibly chimerism-enhancing drug. Using rat sex-determining region-Y-specific oligonucleotide primers, we determined the donor DNA concentration by polymerase chain reaction in serial venous blood samples for 100 days and in tissue specimens when animals were killed.

Results. Chimerism was detected out to 56 days in 89% of the blood samples but in none of the samples at 100 days. However, donor DNA was detected when animals were killed in 95% of the native hearts, 80% of the skin biopsy specimens, and 23% of the spleens. The presence and quantity of early and late chimerism were strongly correlated the administration of adjunct bone marrow and with a reduction in the vasculopathy and inflammation index in the cardiac allografts. Marginally significant further increases in chimerism and/or reductions in chronic heart rejection beyond those achieved with adjunct bone marrow alone were associated with additional treatment with the growth factors Flt-3 ligand, granulocyte colony-stimulating factor, and a recombinant molecular variant of interleukin-6 (interleukin-6 mutein) but not with hepatocyte growth factor or lisofylline.

Conclusions. The previously suspected shift of early chimerism in the blood and lymphoid organs to dominance in host nonlymphoid tissues is consistent with the dual mechanisms of clonal exhaustion and immune indifference, governed by antigen migration and localization, that have been postulated elsewhere to account for organ allograft acceptance.

Widespread activation of the recipient immune system is induced peripherally by the migration of donor leukocytes

from the graft to host lymphoid organs via vascular routes (1–4), including pluripotent stem cells (5, 6). Although it was long assumed that the donor leukocytes were promptly destroyed by the recipient immune system as a prerequisite for successful organ transplantation, their more complex role was recognized with the discovery that they persisted peripherally (microchimerism) and eventually were widely dispersed to host nonlymphoid (e.g., skin [7, 8] and heart [9]) as well as lymphoid sites.

The migration kinetics involved in the transition from a lymphoid-oriented donor leukocyte traffic to a more ubiquitous distribution and the eventual proportions in lymphoid versus nonlymphoid areas have not been determined. Filling this informational void out to 100 days was the primary objective of the experiments reported herein, using the rat heterotopic heart transplantation model, with or without adjunct donor bone marrow cell infusion. In an attempt to further augment the increased chimerism, subgroups of heart recipients given adjunct bone marrow were also treated with one of four hematolymphopoietic growth factors or with the phosphatidic acid inhibitor lisofylline.

MATERIALS AND METHODS

Animals and Transplant Procedures

Heart transplantation. Male Lewis (LEW*; RT1[1]) and female Brown Norway (BN; RT1[n]) rats weighing 150–200 g (Harlan Sprague Dawley, Indianapolis, IN) were used as donors and recipients, respectively. The use of sex-mismatched allografts allowed estimation of the level of chimerism after transplantation with a probe specific for sex-determining region Y (Y chromosome). The male LEW heart grafts were transplanted heterotopically into the abdomen of female BN recipients (10).

Adjunct bone marrow infusion. Male LEW bone marrow cells were obtained by flushing the tibias and femurs. The irrigating fluid was processed with RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, and 10 µg/ml gentamicin (all from Life Technologies, Grand Island, NY). Bone marrow cells (2.5×10^8 cells/animal) with >95% viability in the trypan blue exclusion test were injected intravenously into the jugular vein of female recipients on the day of heart transplantation.

Immunosuppression and Hematopoietic Growth Factors

Tacrolimus. All recipients received 1.5 mg/kg intramuscular tacrolimus per day (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan)

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* Abbreviations: BN, Brown Norway; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; LEW, Lewis; PCR, polymerase chain reaction; Sry, sex-determining region Y.

on days 0 to 13 after transplantation with additional single injections on days 20 and 27. With this model, hearts have prolonged survival, but at 100 days, the allografts frequently have histopathologic evidence of chronic rejection (*10, 11*).

Growth factors. As shown in Table 1, the conventional growth factors tested were the following: rh-granulocyte colony-stimulating factor (G-CSF, 200 µg/kg/day; Amgen, Thousand Oaks, CA), Chinese hamster ovary cell-derived rh-Flt-3 ligand (200 µg/kg/day; a gift from Immunex, Seattle, WA) (*12*), interleukin (IL)-6 mutein, a recombinant molecular variant of human IL-6 (500 µg/kg/day, a gift from ImClone System Inc., Somerville, NJ) (*13*), and rr-hepatocyte growth factor (200 µg/kg/day, a gift from Pharmaceutical Research Center, Toyobo Co., Ohtsu, Japan). In addition, the effect of the phosphatidic acid inhibitor lisofylline (150 mg/kg/day, a gift from Cell Therapeutic Inc., Seattle, WA) was determined (*14*). The daily doses, routes, and frequency of injection during the 7-day course (days 0–6) are shown in Table 1.

All reagents have been confirmed to be active in rats. Although a detailed dose-effectiveness experiment was not conducted in this study, the dosage and route for each molecule were determined from published recommendations (*15, 16*) or according to information from the supplier. In general, dosages that were confirmed to produce specific biologic effects of each molecule in rats were selected, and molecules with short half-lives were administered twice daily and those with relatively long half-lives (IL-6 and Flt-3 ligand) were administered once daily. To test their comparative biologic activity, the effects on venous hematocrit (tail vein), total leukocytes, and leukocyte subsets (Table 2) were determined 7 days after the various transplantation procedures, 1 day after completion of the treatment course. Flow cytometry was performed using monoclonal antibodies R7.3 (αβ-T cell receptor), OX1 (CD45), OX33 (B cells), 3.2.3 (natural killer cells), and ED1 (monocyte/macrophage) (all from Sera-Lab, Crawley Down, UK). The percentage of granulocytes was assessed on a cytocentrifuge preparation stained with α-naphthyl acetate esterase (Sigma Diagnostics, St. Louis, MO).

Intergroup hematocrit variances were minor or nonsignificant (data not shown). Although the BN allograft recipients receiving tacrolimus (all groups 1–7) had lower total leukocyte counts than normal nonoperated animals, this was significantly influenced by growth factor therapy only in the heart transplant/bone marrow recipients treated with IL-6. The most striking finding was an increase in the percentage of granulocytes in allograft recipients treated with lisofylline, Flt-3 ligand, and G-CSF (Table 2). Changes in the subset profile of polymorphonuclear leukocytes attributable specifically to a growth factor (as opposed to the transplant procedure or tacrolimus) were minor except for a near doubling of the percentage of natural killer cells by G-CSF treatment (Table 2).

Pathologic Studies

At autopsy, graft and native hearts were serially sectioned across both ventricles in the transverse plane. One section from the midportion of the ventricles was fixed in neutral buffered formalin for routine histopathology, and a second similar cross-section was snap-frozen in optimum cold temperature compound (Tissue-Tek, Ames Division, Miles Laboratories, Inc., Elkhart, IN) for immunohistochemical studies. A third cross-section of both donor and recipient hearts and samples from other recipient organs (including the kidney, liver, spleen, thymus, skin, cervical lymph nodes, and bone marrow) were immediately snap-frozen in liquid nitrogen for chimerism analysis with polymerase chain reaction (PCR). Special precau-

TABLE 1. Experimental groups—all transplantations were from male LEW donors to female BN recipients treated with tacrolimus[a]

Group	Transplantation[b]	Growth factor		
		Drug	Dose/day	Principal action
0[c]	None	None	NA	NA
1	H	None	NA	NA
2	H/BM	None	NA	NA
3	H/BM	Lisofylline (i.p.)	150 mg/kg[d]	Inhibits lysophosphatidic acid acyl transferase and blocks the formation of phosphatidic acid 1-α induced by the stimulation of cytokines, endotoxin, hypoxia-reoxygeneration, and cytotoxic agents; suppresses production of inflammatory and hematopoiesis-inhibiting cytokines (IL-1, IL-6, IFN-γ, TNF-α, TGF-β, macrophage inhibitory protein 1-α, and platelet factor 4) (*14*)
4	H/BM	Flt-3 ligand (i.p.)	200 µg/kg[e]	Promotes the growth and mobilization of hematopoietic stem cells and committed precursor cells for multiple myeloid and lymphoid lineages; dramatically increases the numbers of dendritic cells in both lymphoid and nonlymphoid tissues (*12*)
5	H/BM	G-CSF (s.c.)	200 µg/kg[d]	Supports the proliferation and differentiation of progenitors already committed to the neutrophil lineage; also affects all lineages derivative from stem cells
6	H/BM	HGF (i.p.)	200 µg/kg[d]	One of a functionally related group of factors that modulate hematopoiesis; is a ligand for the c-met proto-oncogene, which is expressed on epithelial cells as well as hematopoietic progenitor cells
7	H/BM	IL-6 (s.c.)	500 µg/kg[e]	Multipotential cytokine that stimulates B- and T-cell differentiation and primitive hematopoietic stem cell population; IL-6 mutein used in this study lacks 22 amino acids from the amino terminus of IL-6 and has serine replacements at positions 74 and 84; has maximum activity at a lower concentrations than native IL-6 (*13*)

[a] Intramuscular tacrolimus: 1.5 mg/kg/day on days 0–13, 20, and 27.
[b] H, heart; BM, unfractionated bone marrow 2.5×10⁸ cells/animal.
[c] Nonoperated and nonimmunosuppressed normal female BN rat.
[d] Twice daily injection of divided dose.
[e] Once daily injection.

TABLE 2. Effect of hematopoietic growth factors and lisofylline on peripheral blood leukocytes 7 days after transplantation

Group	Transplantation	Growth factor	N	Total leukocytes[a] (cell counts/mm³)	Granulocytes[b] (%)	αβ-TCR (R7.3) (%)	B cells (OX33) (%)	NK cells (3.2.3) (%)	Monocytes/macrophage (ED1) (%)
0	None	None	5	7182±1481	15.60±6.38	49.96±4.04	30.80±5.18	4.42±1.17	2.42±0.93
1	HTX	None	5	5670±1580	14.52±3.97	53.37±3.66	17.47±1.33[b]	5.30±1.59	2.13±0.25
2	HTX+BMTX	None	5	5352±983	16.56±2.06	57.17±6.34[a]	26.73±7.53[e]	4.08±0.89	2.17±0.75
3	HTX+BMTX	Lisofylline	2	5088±3189	21.38±4.82[a,e,i]	47.30±3.25[i]	32.10±1.13[f]	6.05±0.64	3.30±0.00
4	HTX+BMTX	Flt-3 ligand	5	6562±2089	24.05±5.11[c,g,j]	51.17±7.34	30.85±4.45[f]	6.80±1.31[a,j]	2.40±0.78
5	HTX+BMTX	G-CSF	3	5812±655	26.27±5.01[d,h,k]	48.60±1.47[i]	23.47±1.89	9.50±1.85[d,g,k]	1.57±0.45
6	HTX+BMTX	HGF	3	6664±2286	19.13±5.26	53.77±2.06	27.00±3.32[e]	4.17±1.10	1.17±0.15[a]
7	HTX+BMTX	IL-6	3	3997±2543[a]	17.38±5.10	55.87±6.90	18.80±6.41[b]	6.03±0.55[i]	2.67±1.11

[a] Cells were counted on a hemacytometer.
[b] Granulocytes were determined on blood smear after an α-naphthyl acetate esterase stain.
P vs group 0: $a<0.05$, $b<0.01$, $c<0.001$, $d<0.0001$
P vs group 1: $e<0.05$, $f<0.01$, $g<0.001$, $h<0.0001$
P vs group 2: $i<0.05$, $j<0.01$, $k<0.0001$

tions were taken during sampling not to contaminate female recipient tissues by contact with the male heart graft.

The formalin-fixed heart grafts were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. All slides were reviewed by one of the authors (A.J.D.) without knowledge of the treatment protocols. The overall severity of inflammation in the endocardium, pericardium, interstitium, and periarterial spaces was graded semiquantitatively on a scale of 0 to 4 as none, minimal, mild, moderate, or severe, as previously described (11). Arterial alterations, including the presence of inflammation, edema, fibrosis, and vacuolation of the intima, media, and adventitia, were graded in the same fashion. In addition, the total numbers of arteries >80 μm present in the cross-section were recorded, to ensure a similar sampling between animals. The grading of obliterative arteriopathy was as follows: none (grade 0), <10% luminal narrowing (grade 1), 10–25% (grade 2), 26–50% (grade 3), 51–75%(grade 4), and >75% luminal narrowing (grade 5) (11).

PCR and Southern blot hybridization

Genomic DNAs were prepared from blood samples and recipient tissues using a standard procedure and quantitated by spectrophotometer (17). The PCR was performed with 1.5 μg of genomic DNA in 50 μl of total reaction mixture containing 1.25 units of Taq DNA polymerase, 1 μl each of 25 μM rat sex determining region-Y (Sry)-specific oligonucleotide primers (5'-GAGAGAGGCACAAGTTGGC-3' and 5'-GCCTCCTGGAAAAAGGGCC-3'), 8 μl of 1.25 mM dNTP, and 5 μl of 10× PCR buffer (500 mM KCl, 20 mM MgCl2, 100 mM Tris HCl, and 0.1% gelatin, adjusted to pH 8.4) (18). The PCR was carried out under the following conditions: denaturation at 95°C for 60 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 2 min for 35 cycles in a DNA thermal cycler. The reaction was then extended for 7 min at 72°C to ensure the production of full-length PCR products.

The PCR products were then fractionated in 1.5% agarose gels and stained with ethidium bromide. DNA products amplified with Sry-specific primers were also transferred onto nylon membranes for Southern blotting and semiquantitation. The Sry-specific probe was prepared by extraction and purification of PCR product, which was prepared from male LEW spleen DNA, using the QIAEX II gel extraction kit (QIAGEN Inc., Chatsworth, CA). It was also multiprime-labeled with α-^{32}P dCTP (3000 Ci/mmol, NEN Research Products, Boston, MA) using the multiprime DNA labeling systems (Amersham Life Science, Buckinghamshire, UK). Membranes were prehybridized for 2 hr at 47°C in buffer containing saline-sodium phosphate EDTA buffer (5×), 0.1% sodium dodecyl sulfate, Denhart's solution (5×), and 0.1% SSDNA. The ^{32}P-labeled probe was added to hybridization buffer and further incubated for 16 hr at

47°C. After hybridization, each membrane was washed four times in 1× SSPE/0.1% sodium dodecyl sulfate for 5 min at room temperature and then once in the same solution for 5 min at 50°C. Membranes were exposed to Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 4 hr at room temperature, and the radioactivity on the screen was measured with the PhosphorImager (Molecular Dynamics).

The level of chimerism in each sample was calculated with a standard curve prepared using known concentrations of male DNA. DNAs were prepared from male LEW and female BN spleens and mixed at various ratios ranging from 1:10 to 1:10^5. Mixtures were amplified with PCR. After Southern hybridization, radioactivities of these mixtures were analyzed by phosphoimaging and the standard curve was created (Fig. 1), with which male DNA concentrations in blood and organs obtained from the female recipients were semiquantified. It was possible to detect the male DNA concentration up to 0.001% (donor-recipient ratio = 1:100,000). Donor DNA was considered to be nondetectable when the radioactivity after Southern hybridization of experimental samples was below the value of control females.

Statistical Analysis

One-way analysis of variance and Fisher's PLSD test were applied to assess the statistical significance of different groups. A value of $P<0.05$ was considered significant.
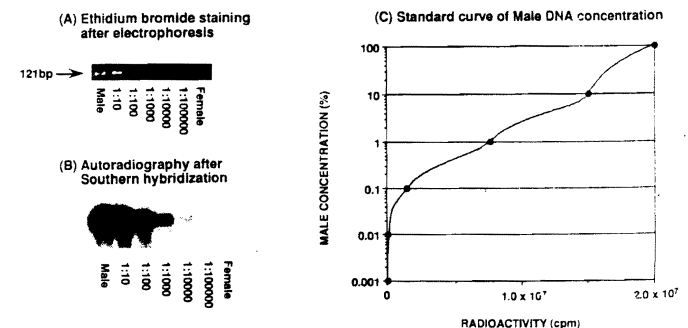

FIGURE 1. Standard curve for the semiquantitation of male DNA concentration in samples. DNAs from male LEW spleen and female BN spleen were mixed at ratios ranging from 1:10 to 1:10^5. These mixtures were amplified by PCR using Sry-specific primers. PCR products were (A) separated in 1.5% agarose gel for ethidium bromide staining and (B) transferred onto nylon membrane for Southern hybridization. After hybridization, radioactivities were analyzed by phosphoimaging and a standard curve was created (C).

RESULTS

Peripheral Blood Chimerism

Figure 2 demonstrates the frequency of detection and the concentrations of male DNA in the tail vein blood of the heart recipients 7, 14, 28, 56, and 100 days after transplantation. Adjunct donor bone marrow infusion was the only treatment variable that increased the detection rate or concentration of the chimerism, with no additional effect of growth factor treatment. In the heart/bone marrow recipients (groups 2–7), the concentration of male DNA reached 1–10% in the peripheral blood at 7–28 days after transplantation and then decreased slowly to 0.1–1% at 56 days after transplantation. Donor DNA was not detectable in the peripheral blood of any recipient by 100 days after transplantation, no matter what the treatment regimen.

Tissue Chimerism at 100 Posttransplant Days

In contrast to the disappearance of blood chimerism, male DNA was found in the following host tissues when the rats were killed: liver, native heart, skin (tongue), spleen, cervical lymph nodes, and bone marrow. The complete results from the spleen samples shown in Figure 3A are representative of the other lymphoid organs (data not shown). The results from the skin and recipient heart (Fig. 3, B and C) typify the pattern in the nonlymphoid sites. Both the frequency of positive samples and the concentrations of donor DNA were higher in the skin and native heart than in the spleen (Fig. 3).

Adjunct donor bone marrow alone (group 2) significantly increased the frequency of detection and concentration of male DNA in all locations assayed, compared with those observed with transplantation of the heart only (group 1) (Fig. 3). Additional treatment with Flt-3 ligand (group 4) was associated with a further increase in male DNA in the spleen ($P=0.0093$, vs. group 2, Fig. 3A) and native heart ($P=0.025$, Fig. 3C). IL-6 treatment (group 7) was also associated with a significant increase of male DNA in the recipient native heart compared with group 2 ($P=0.0402$, Fig. 3C).

Histopathologic Evaluation of Heart Grafts

As expected from previous experience with this model (10, 11), all 39 heart grafts transplanted heterotopically beat for 100 days. Thirty-seven were available for histopathologic analysis and standardized grading; two grafts, one each from groups 3 and 5, were not analyzed because of an inadequate sample. The overall inflammation score reflects the general alloreactivity in the heart grafts. This score was significantly reduced in all groups in which heart grafts were transplanted simultaneously with donor bone marrow (Table 3). The greatest improvement relative to group 1 was with G-CSF, Flt-3 ligand, or IL-6 treatment (Table 3), but this advantage was not statistically significant when compared with the results with adjunct bone marrow alone (group 2).

Obliterative arteriopathy was evaluated in each sample and is expressed as the percentage of arteries showing vasculopathy and as the average grade of the disease seen in 6 to 22 arteries per heart graft. Compared with hearts transplanted alone (group 1), the cardiac allografts transplanted with adjunct bone marrow to recipients also treated with G-CSF (group 5) and IL-6 (group 7) had significant improvement in both categories (Table 3).

Histopathologic Correlations with Chimerism

The autopsy samples from all the cardiac allografts of groups 1–7 were pooled and the histopathologic changes were correlated with the pooled levels of chimerism in the native hearts of these same animals. The native hearts were selected as the chimerism reference because donor DNA was found in >90% of these specimens (see Fig. 3C).

The scattergrams in Figure 4 demonstrate the correlation between the concentration of chimerism and the average grade of arterial lesions (Fig. 4A), percentages of diseased arteries (Fig. 4B), and overall inflammation (Fig. 4C). Despite strong trends, there was no statistically significant linear relationship between the average histopathologic grades of arterial lesions and chimerism ($R^2=0.098$, $P=0.0594$; Fig. 4A) or percentages of diseased arteries ($R^2=0.070$, $P=0.1146$; Fig. 4B). However, a highly signifi-
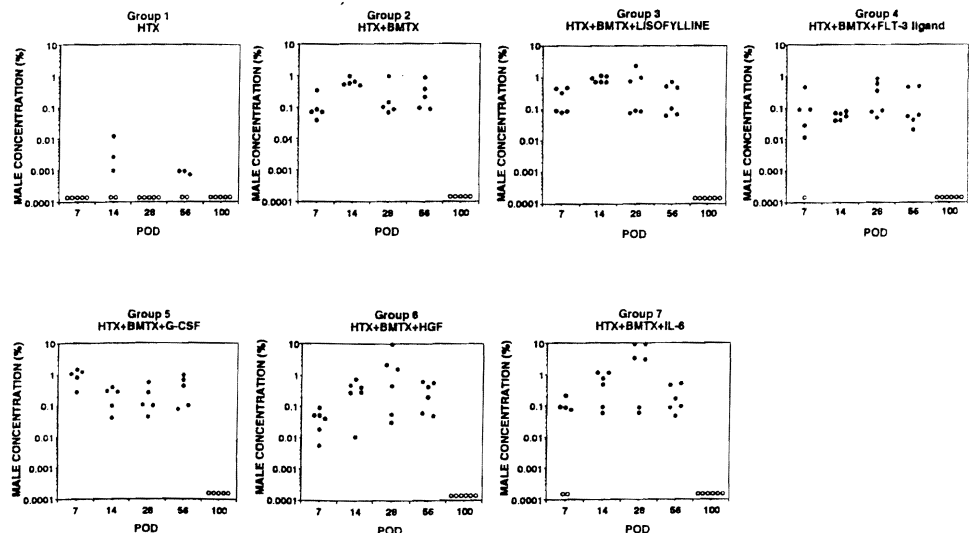

FIGURE 2. Sequential changes of chimerism (male DNA concentration) in recipient peripheral blood (○, samples with undetectable levels of male DNA). Both the frequency of detection and the concentrations of male DNA were significantly higher ($P<0.0001$, analysis of variance) in recipients with simultaneous donor bone marrow infusion (groups 2–7) than in those without infusion (group 1) at 7, 14, 21, and 56 days after transplantation.
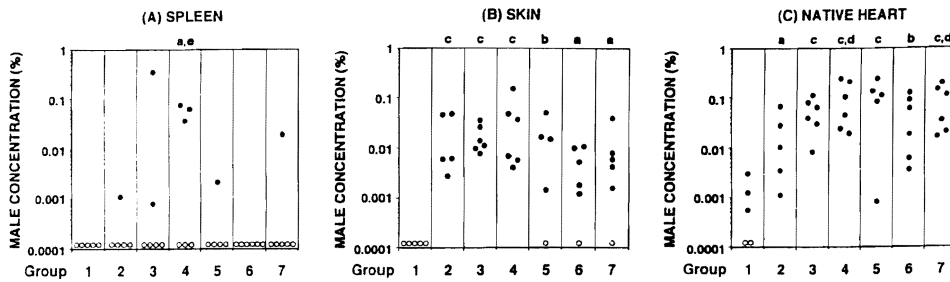
FIGURE 3. Systemic chimerism in recipient (A) spleen, (B) skin, and (C) native heart at 100 days after transplantation (O, samples with undetectable levels of male DNA). a: $P<0.01$ vs. group 1, b: $P<0.001$ vs. group 1, c: $P<0.0001$ vs. group 1, d: $P<0.05$ vs. group 2, and e: $P<0.01$ vs. group 2 (analysis of variance).

TABLE 3. Comparison of severity of obliterative arteriopathy and overall inflammation in heart allografts at 100 days after transplantation[a]

Group	Transplantation	Growth factor	n	Arterial changes			Overall inflammation score
				Total no. of arteries scored	Average grade of lesions	Percent of arteries with disease (%)	
1	HTX	None	5	60	1.12 ± 0.96	53.20 ± 22.07	2.80 ± 1.10
2	HTX+BMTX	None	5	60	0.84 ± 0.08	47.16 ± 8.44	1.30 ± 0.76^b
3	HTX+BMTX	Lisofylline	5	48	1.42 ± 0.50	60.92 ± 20.63	1.20 ± 0.57^b
4	HTX+BMTX	Flt-3 ligand	6	87	0.57 ± 0.25	43.08 ± 16.39	0.75 ± 0.27^c
5	HTX+BMTX	G-CSF	4	63	0.38 ± 0.18^a	26.84 ± 12.64^a	0.63 ± 0.25^c
6	HTX+BMTX	HGF	6	83	1.05 ± 0.71	51.00 ± 24.22	1.50 ± 1.27^a
7	HTX+BMTX	IL-6	6	86	0.44 ± 0.23^a	30.17 ± 13.86^a	0.92 ± 0.59^c

[a] P-value vs group 1: $a<0.05$, $b<0.01$, $c<0.001$.

cant inverse correlation was found between the levels of overall inflammation and chimerism ($R^2=0.261$, $P=0.0012$; Fig. 4C).

DISCUSSION

When microchimerism was discovered in long-surviving human organ recipients, two mechanisms of allograft acceptance were proposed (7, 8): (1) reciprocal clonal exhaustion of the co-existing donor and recipient immunocyte populations, and (2) reduced organ graft immunogenicity of the transplanted organ due to depletion of its peripheralized passenger leukocytes. Bishop et al. (19, 20) reported experimental evidence confirming a role of acute clonal exhaustion, attributed by Qian et al. (21) to apoptosis. However, skeptics of the significance of persistent late microchimerism (22) have focused on the inconsistency with which donor leukocytes can be found in the blood (or limited tissue samples) from patients (7–9, 23–30) and animals (31–34) bearing long-surviving organ allografts. Incomplete sampling (in some reports of blood only) in both the clinical and experimental studies is one possible explanation for wide discrepancies in the frequency of donor leukocyte detection.

It was evident in the current study that both the site and timing of sampling profoundly influenced the finding of chimerism. Blood chimerism in the heart recipients was detectable during the first 2 postoperative months in 139 (89%) of the 156 samples, including 37 (95%) of 39 at 56 days. By 100 days, at which time all of the heart allografts were still beating, all 39 blood samples were negative. When the animals were killed, however, tissue chimerism was detected in 37/39 (95%) of the native hearts, 31/39 (80%) of the skin biopsy specimens, and only 9/39 (23%) of the spleens. A similar but less clear late pattern of localization in host nonlymphoid tissues has also been noted recently in a rat heart transplant model by Shirwan et al. (34).

The eventual dominance of microchimerism in the nonlymphoid compartment (Fig. 5), which has not been recognized before, extends the second originally proposed mechanism of graft acceptance, by immune indifference (i.e., loss of organ immunogenicity caused by depletion of the donor leukocytes from the allograft [7, 8]). It is now clear that chronic survival of the peripheralized donor cells may depend in part on their confinement to nonlymphoid sites. In spite of their sequestration, we have suggested that they may be critical for maintenance of the clonal exhaustion (4) which, under circumstances of transplantation across an MHC barrier, is rarely if ever complete and nonreversible (10, 35, 36). Periodic leakage of the "hidden" chimeric cells to lymphoid organs has been postulated to fill this maintenance role (4).

In this context, persistent chimerism, no matter what its level, is only a necessary condition for, and is not synonymous with, graft acceptance or tolerance (4, 7, 8, 37). It follows that neither the development of rejection coincident with chimerism (33, 34, 38–40) nor the inability to use chimerism to guide immunosuppressive drug weaning (38, 41) contravene the key role of microchimerism in allograft acceptance and tolerance (4). Although it has been argued that tolerance can be produced in the absence of donor hematopoietic cells (31, 32), the experiments prompting this conclusion may be experimental examples of "immune indifference" in which long survival of allografts does not confer donor-specific nonreactivity (42–44).

In the studies reported herein, we determined whether there was a correlation between chronic rejection and the quantity and localization of early and late chimerism, using three different histopathologic end points: (1) percentage of arteries showing vasculopathy, (2) severity of the vascular lesions, and (3) overall inflammation score. By all three end points, chronic rejection was reduced in inverse proportion to the amount of detectable chimerism, which in turn was al-
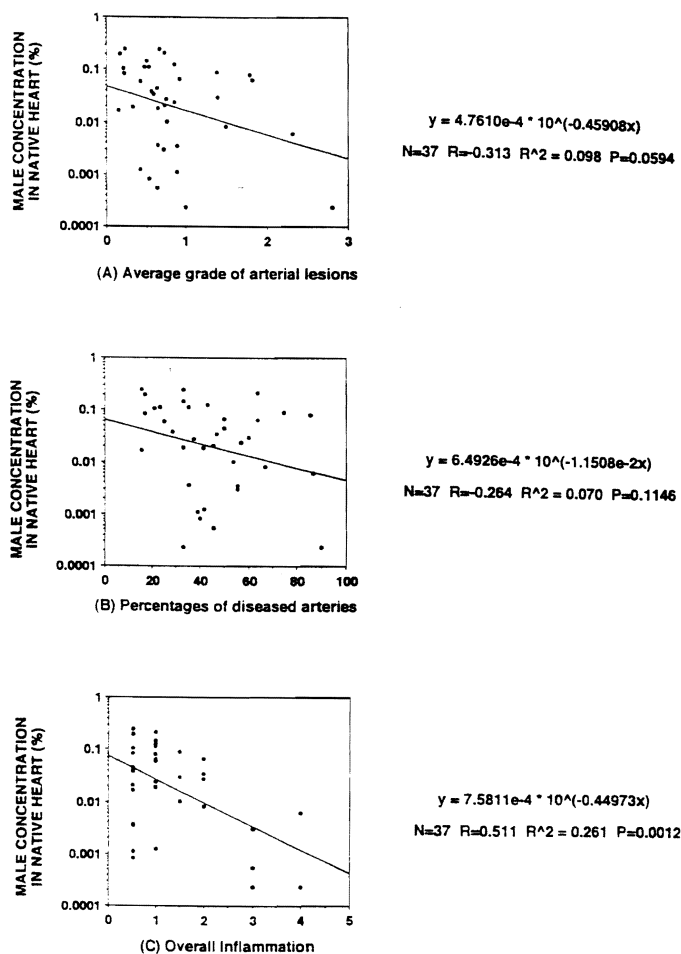
$$y = 4.7610e-4 * 10^{\wedge}(-0.45908x)$$
N=37 R=-0.313 R^2 = 0.098 P=0.0594

(A) Average grade of arterial lesions

$$y = 6.4926e-4 * 10^{\wedge}(-1.1508e-2x)$$
N=37 R=-0.264 R^2 = 0.070 P=0.1146

(B) Percentages of diseased arteries

$$y = 7.5811e-4 * 10^{\wedge}(-0.44973x)$$
N=37 R=0.511 R^2 = 0.261 P=0.0012

(C) Overall Inflammation

FIGURE 4. Correlation between histopathologic changes in heart allografts and chimerism levels (male DNA concentration) in native hearts. Hematoxylin and eosin samples were analyzed from the middle of heart allografts that were harvested 100 days after transplantation. Scattergrams demonstrate the correlation between the level of chimerism and (A) the average grade of arterial lesions, (B) percentages of diseased arteries, and (C) overall inflammation. The linear regression for each group was the following: A, $R^2=0.098$, $P=0.0594$; B, $R^2=0.070$, $P=0.1146$; and C, $R^2=0.261$, $P=0.0012$.

ways greater in animals given adjunct bone marrow. This was statistically significant with the inflammation score, in accord with numerous previous reports of the protective effect of bone marrow (45–47).

Although the further advantage conferred by adding growth factor therapy postoperatively did not reach significance when compared with the protection of adjunct bone marrow alone, it can be concluded that none of the five tested molecules made chronic rejection worse. On the contrary, trends of reduced chronic rejection were seen with three of them (G-CSF, IL-6, and Flt-3 ligand), which suggests the need for further evaluation. Such investigations also can be justified by our previous demonstration that organ-based hematopoietic progenitor cells are increased by growth factors, such as G-CSF, that promote the growth and mobilization of hematolymphopoietic stem cells (see Table 1). G-CSF and granulocyte/macrophage CSF already have been widely used in clinical bone marrow transplant recipients and after cancer chemotherapy (48); similar trials with Flt-3 ligand are
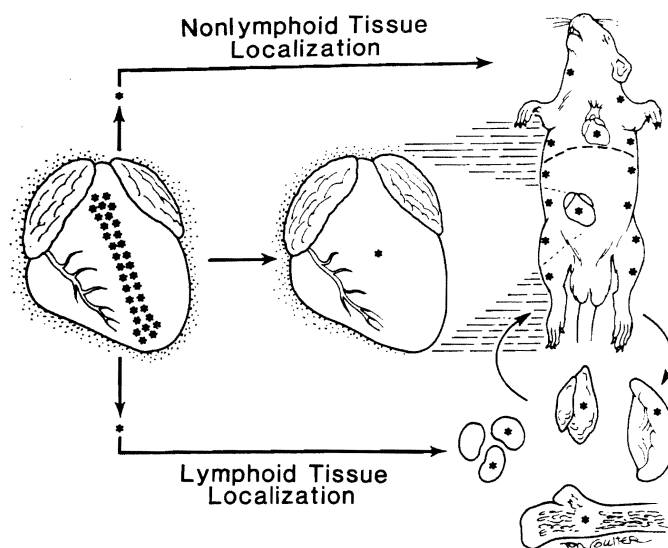

FIGURE 5. Reconstruction of events after organ transplantation, epitomized by the heart with its "passenger leukocyte" component depicted as a bone silhouette. Although these donor leukocytes are largely replaced by similar recipient cells, a small number (<5%) remain donor cells. The eventual localization of the donor migratory cells is heavily represented in nonlymphoid tissues (skin and native heart shown here), from where they presumably leak to the lymphoid organs and maintain clonal exhaustion.

underway. The administration of G-CSF and granulocyte/macrophage CSF long after transplantation to stable organ recipients has not increased the risk of either rejection or graft-versus-host disease (49–53).

In nonrandomized trials, Foster et al. (54) have reported a reduction in both infection and rejection in liver transplant recipients treated with G-CSF during the early postoperative period (54). Because these benefits have not been duplicated in multicenter randomized trials (J.W. Williams, personal communication, 1998), further preclinical laboratory studies under controlled circumstances will be doubly important.

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DIFFERENTIAL INHIBITION OF B-CELL DEVELOPMENT AND XENOREACTIVE NATURAL ANTIBODY PRODUCTION BY ADMINISTRATION OF ANTI-μ OR ANTI-δ MONOCLONAL ANTIBODIES IN ADULT RATS[1]

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Background. Given the role of xenoreactive natural antibodies (XNA) in the pathogenesis of xenograft rejection, we tested whether the administration of anti-μ or anti-δ monoclonal antibodies (mAbs) in adult rats would suppress the generation of XNA.

Methods. Adult LOU/C (Igκ-1a) rats were treated with anti-μ or anti-δ mAbs after nonlethal total body irradiation and bone marrow transplantation from congenic LOU/C (Igκ-1b) rats. The differentiation of donor bone marrow (BM)-driven Igκ-1b⁺ B cells and XNA production were analyzed.

Results. Both anti-μ and anti-δ mAbs arrested B-cell differentiation in the BM. In anti-μ-treated rats, there was a total depletion of donor-driven, peripheral Igκ-1b⁺ B cells, secreting cells, and circulating XNA of the

Igκ-1b allotype. In anti-δ-treated rats, a significant number of Igκ-1b⁺ B cells, which did not express membrane IgD, "escaped" deletion and partially repopulated peripheral lymphoid organs. This B-cell population was active in the production of XNA, as revealed by the high serum levels of XNA in these animals.

Conclusions. Anti-μ administration resulted in arrest of B-cell differentiation and in down-regulation of IgM and IgG XNA production in adult rats. These data suggest that the use of anti-μ mAbs may be a useful approach to suppress the production of XNA and prevent xenograft rejection. Furthermore, we suggest that the B-cell population responsible for the production of XNA in adult rats belongs to a B-cell lineage expressing low levels of membrane IgD and "escaping" deletion in the BM upon anti-δ treatment.

The xenotransplantation of immediately vascularized organs from pigs to humans is perceived as a potential solution to overcome the current organ donor shortage in clinical transplantation (1). The success of this approach remains limited due to the occurrence of hyperacute or delayed xenograft rejection. Humans and Old World primates have high serum levels of preformed IgM and IgG xenoreactive natural