FRESH VENOUS ALLOGRAFTS IN PERIPHERAL ARTERIAL RECONSTRUCTION IN DOGS

Effects of histocompatibility and of short-term immunosuppression with cyclosporine A and mycophenolate mofetil

To date, no arterial substitute has been shown to be as effective as the autologous saphenous vein in peripheral revascularization procedures. In the present study, the venous allograft was evaluated as a vascular substitute in terms of patency and induction of host immune reactivity, whether used in major histocompatibility complex-incompatible, major histocompatibility complex-compatible, or immunosuppressed major histocompatibility complex-incompatible dogs. The immunosuppressive drug therapies were given for a period of 31 days, beginning 1 day before transplantation, and consisted of the use of cyclosporine A, mycophenolate mofetil, or a combination of both. All histoincompatible allografts were thrombosed at 4 or 8 weeks after transplantation with antibody development and cell-mediated cytotoxicity in the graft, whereas histocompatible allografts showed late stenosis without immunologic reactions directed toward donor cells. Given alone, neither cyclosporine A nor mycophenolate mofetil improved the overall patency of venous allografts; thrombosis occurred shortly after cessation of immunosuppression. Still, the cyclosporine A-mycophenolate mofetil combination therapy led to a 100% patency rate at 20 weeks after implantation and immune reactions were markedly reduced. This study shows that the fresh vein allograft is still an attractive and functional alternative to the autologous saphenous vein if the host immunologic reactions are controlled by cyclosporine A-mycophenolate mofetil immunosuppression. (*J THORAC CARDIOVASC SURG* 1995;110:1732-44)

Eric Wagner, PhD,^a Raynald Roy, PhD,^a Yves Marois, MSc,^b
Yvan Douville, MD, MSc,^{b,c} and Robert Guidoin, PhD,^b
Quebec, Quebec, Canada

The autologous saphenous vein and the internal mammary artery are the preferred blood conduits for coronary^{1,2} and peripheral³ arterial reconstruction. However, they can be absent or unsuitable in as many as 20% to 30% of patients. Alternative blood conduits, such as the expanded polytetrafluoroethylene prosthesis and the chemically pro-

cessed human umbilical vein graft (bioprosthesis), fail as small-caliber arterial substitutes. Indeed, they exhibit poor long-term patency rates⁴ because of thrombosis and susceptibility to bacterial colonization and lipid uptake.⁵ Only viable biologic tissue resists infection and lipid retention.⁶ Venous allografts were used in the past and have yielded interesting short-term results,^{7,8} but were later shown to be targets of immune reactions, potentially leading to allograft failure.⁹

Since the venous allograft tissue was shown to be antigenic,¹⁰ donor-recipient histocompatibility matching¹¹⁻¹³ and immunosuppressive therapies¹³⁻¹⁶ have been investigated. Furthermore, cryopreservation has also been proposed as a way to reduce antigenicity and to create a bank of readily available tissues.¹⁷⁻¹⁹ However, conflicting results have emerged from these studies so that skepticism remains about the usefulness of venous allografts in small-diameter revascularization procedures. To determine the factors that could influence venous allograft success, the roles of histocompat-

From the Rheumatology and Immunology Research Center, Laval University Medical Center,^a the Department of Surgery, Laval University, and the Biomaterials Institute, St. François D'Assise Hospital,^b and the Department of Vascular Surgery, St. Sacrement Hospital,^c Quebec, Quebec, Canada. Supported by the Medical Research Council of Canada (grant MT-7879).

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Address for reprints: Raynald Roy, PhD, Rheumatology and Immunology Research Center, Laval University Medical Center, 2705 Laurier Blvd., Room 9800, Ste-Foy, Quebec, Canada G1V 4G2.

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ibility and short-term immunosuppressive treatment were evaluated in a canine femoral artery replacement model. Cyclosporine A (CsA), a potent inhibitor of early T-cell activation, and mycophenolate mofetil (MMF), a new, powerful inhibitor of late T-cell and B-cell activation, were used either alone or in combination. Autografts and allografts were implanted and monitored in terms of patency, induction of thrombohematologic parameter modifications, cellular and humoral immune reactions, histologic changes, and appearance on scanning electron microscopy (SEM).

Material and methods

Dog selection and operation. Twenty-one young adult mongrel dogs (4 male and 17 female dogs) and seven Labrador retriever littermates (five male and two female dogs) approximately 1 year old and weighing 18 to 26 kg were selected for the present study. They were treated according to the guidelines of the Canadian Council on Animal Care. The animals were paired before vascular operation, depending on mutual major histocompatibility complex (MHC) compatibility as measured by a mixed leukocyte culture assay as previously described.²⁰ Because allele-specific typing sera were unavailable, the mixed leukocyte culture assay is a good and recognized indicator of mononuclear cell reactivity.²¹ Dogs were fasted for 12 hours before the operation. They were anesthetized intravenously with 20 mg/kg sodium pentobarbital (Somnotol) and the lungs were mechanically ventilated. They received additional halothane (Fluothane) when further anesthesia was required. After groin disinfection and exploration, each dog received 10 cm long femoral vein segments as substitutes for both femoral arteries: an allograft was interposed in the right femoral artery, and an autograft was used as a control in the left femoral artery. To avoid prolonged ischemia, the grafting procedure was done immediately after vein excision from the donor with an end-to-end anastomotic technique. For the performance of open biopsies after operation, to thus avoid stretching of the graft, grafts were slightly looped. After operation, the dogs were returned to their cages and fed an unrestricted standard diet.

Experimental groups. The control group consisted of nine mongrel dogs and one Labrador, all paired as histoincompatible (MHC incompatible). These dogs received a fresh venous autograft and an allograft without any treatment. The second group consisted of six untreated Labrador littermates paired as histocompatible (MHC compatible). The third group consisted of four histoincompatible graft recipients that were given an olive oil solution of CsA orally at a dosage of 10 mg/kg per day for 31 days, beginning the day before transplantation. The fourth group comprised four histoincompatible dogs that received MMF in gelatin capsules orally at a dosage of 20 mg/kg per day, also for a total of 31 days beginning 1 day before implantation. Finally, the fifth group was composed of four histoincompatible dogs that received both

CsA and MMF in the same dosages previously described and for the same period.

Thrombohematologic testing and follow-up. Changes in blood coagulation parameters were measured for each dog before transplantation, at the time of the first biopsy, and when the dogs were killed. Serum and heparinized blood samples were collected periodically after operation for donor-specific antibody screening and graft-infiltrating lymphocyte culture, respectively. Hematocrit concentration was determined by a capillary method, leukocyte and platelet counts were done by hematology, and clotting time was done by hemochrometry. In addition, platelet aggregation was evaluated by aggregometry, and hemostasis was assessed by thromboelastography. To assess blood susceptibility to clot formation, the thrombogenic index (TI) was calculated as $TI = Emx/k$, where Emx is the clot elasticity factor as obtained by the conversion of Am (clot stiffness, millimeters) according to a thromboelastograph conversion table provided by the company (Hellige, Freiburg, Germany) and k is the start of clot formation (minutes).

Graft patency was determined at periodic intervals by Doppler ultrasonographic evaluation. The dogs were killed 5 months after transplantation or when an allograft showed no indication of blood flow.

Vascular endothelial cell culture. To measure host immune reactions directed toward the allograft, a segment of jugular vein was excised from each dog the same day the transplantation procedure took place, and vascular endothelial cells (ECs) were isolated according to the method of Jaffe and associates.²² They were cultured in medium 199 supplemented with heat-inactivated fetal bovine serum, EC growth supplement, heparin, fresh glutamine, and antibiotics, as previously described.¹⁸ Cultures had to reach the third passage before the cells could be used.

Donor-specific antibody screening and cytotoxicity testing. The development of posttransplant antibodies was measured by indirect immunofluorescence with use of a flow-cytometric assay as previously described.²⁰ Vascular graft recipients were screened for antibody formation with serum samples collected at 0, 2, 4 or 5, 8, 12, 16, and 20 weeks after operation. Sera were tested against donor, recipient, and third-party ECs and splenocytes (obtained at the animal's death). A reaction was considered positive when a serum exhibited a shift of at least 10 mean fluorescence channels compared with findings in the control serum (pooled normal dog serum).

The cytotoxic activity of flow-cytometric reactive sera was assessed by a cytotoxic flow-cytometric assay. In brief, ECs were incubated with the corresponding serum and washed thoroughly. They were then incubated with rabbit complement to which propidium iodide was added, washed thoroughly, and fixed in paraformaldehyde before being analyzed on a flow cytometer (FACSsort, Becton Dickinson, San Diego, Calif.). A positive cytotoxic reaction was observed when a shift of at least 10 mean red fluorescence channels was measured for a given serum, compared with findings in the control serum.

Cellular immune reactivity. Graft-infiltrating alloreactive lymphocytes were cultured from samples obtained by open autograft and allograft biopsies done at 2 and 4

Table I. Graft patency in littermates, unrelated dogs, and immunosuppressed dogs

Experimental group*	Patency rate (No. of patent grafts) by time (wk)							
	0	2	4†	5†	8	12	16	20
Histocompatible								
Littermates								
Autografts	6/6	6/6	6/6		6/6	6/6	5/6	4/6
Allografts	6/6	4/6	4/6		4/6	4/6	2/6	1/6
Histoincompatible								
Untreated								
Autografts	10/10	10/10	9/10		9/10	9/10	9/10	9/10
Allografts	10/10	7/10	3/10		1/10	0/10		
CsA								
Autografts	4/4	4/4		2/4	2/4	2/4		
Allografts	4/4	4/4		2/4	1/4	0/4		
MMF								
Autografts‡	4/4	4/4		2/3	2/3			
Allografts	4/4	4/4		1/4	0/4			
CsA + MMF								
Autografts	4/4	4/4		3/4	3/4	3/4	3/4	3/4
Allografts	4/4	4/4		4/4	4/4	4/4	4/4	4/4

*Experimental groups were as follows: *Littermates*, untreated histocompatible littermates; *Untreated*, untreated histoincompatible dogs; *CsA*, CsA-treated histoincompatible dogs; *MMF*, MMF-treated histoincompatible dogs; *CsA + MMF*, histoincompatible dogs treated with CsA and MMF.

†Graft patency was assessed at 4 weeks in untreated dogs and at 5 weeks in immunosuppressed dogs at performance of the first biopsy.

‡Only three of the four autografts could be evaluated for graft patency because of severe infection and necrosis in one dog's leg observed at 5 weeks.

weeks for dogs without immunosuppressive therapy and at 5 weeks for those receiving drug therapies. This procedure was also done when the dogs were killed. The biopsy technique was as follows. After graft exploration, a 1 cm medial vein graft segment was excised and the graft was reclosed by end-to-end anastomoses. Lymphocytes were cultured according to the method of Dal Col and associates.²³ In brief, biopsy sample fragments were divided into 10 to 15 pieces and cultured in a 96-well, round-bottomed microtiter plate in RPMI 1640 medium supplemented with 10% heat-inactivated pooled dog serum, fresh glutamine, antibiotics, 50 U/ml interleukin-2, and 10^5 irradiated (3000 rads) autologous feeder mononuclear cells. The medium was changed periodically, with the addition of feeder cells once a week until sufficient growth was noted (usually 4 weeks). Cultured lymphocytes were then tested for cytotoxic activity against donor, recipient, and third-party ECs in a 4-hour ^{51}Cr release assay. Target cells (10^6) were labeled with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ for 90 minutes at 37° C. Then they were incubated with cultured graft-infiltrating lymphocytes at effector/target ratios of 10:1 and 40:1 in a total of 200 μl of culture medium devoid of interleukin-2 in a 96-well, round-bottomed plate. After a 4-hour incubation, 100 μl of supernatant was removed and counted on a gamma counter. Spontaneous ^{51}Cr release was determined by incubating target cells alone, whereas maximal release was determined by treating the labeled target cells with 10% Triton X-100 polyoxyethylene (Union Carbide Corp., Danbury, Conn.). The percentage of specific EC lysis was calculated as follows:

Specific ^{51}Cr release =

$$\frac{\frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Maximal release (cpm)} - \text{Spontaneous release (cpm)}} \times 100$$

Graft processing and analysis. A fragment of a biopsy specimen and representative anastomotic and midportions of explanted grafts (obtained at death) were fixed in 10% formalin and later processed for light microscopy and SEM. Paraffin sections 5 μm thick were stained with Weigert's stain to reveal elastic fibers and Masson's trichrome stain to differentiate fibrin from collagen. Specimens for SEM evaluation were postfixed in carboxyhydrazide and osmium tetroxide. Dehydration was obtained by immersion of the specimens in a series of ethanol solutions, culminating in pure ethanol, followed by critical-point drying with liquid CO_2 used as the transfer medium. They were then coated with a gold-plated alloy before observation under a Jeol JSM35 SEM (Soquelec, Montreal, Quebec, Canada) at a 15 kV accelerating voltage.

Results

Graft patency. Dogs in each experimental group were evaluated periodically by Doppler ultrasonography for graft patency. The results are shown in Table I. Surprisingly, the autografts in each group exhibited different patency rates. In the group of untreated histoincompatible dogs, only one autograft thrombosed, which was attributed to a tech-

nical failure. Two of six autografts were stenosed at the end of the protocol (20 weeks) in the group of histocompatible littermates. Two autografts occluded early after implantation among the four dogs treated with CsA, one of three failed in dogs treated with MMF, and three of four remained patent at 20 weeks in dogs receiving a combined immunosuppressive therapy (CsA and MMF).

In the untreated histoincompatible group of dogs, four allografts underwent thrombosis at 4 weeks and two thrombosed at 8 weeks. Three dogs had a thrombosed allograft at 2 weeks and one had a stenosed allograft at 12 weeks. Two early thromboses at 2 weeks and three stenoses (two at 16 weeks and one at 20 weeks) occurred in the group with histocompatible allografts (littermates). Only one allograft remained patent for the 20-week implantation schedule. Allografts implanted in CsA-treated dogs thrombosed at 5 (in two dogs), 7, and 11 weeks, respectively. Treatment with MMF was also unsuccessful, with three thrombosed allografts at 5 weeks and one at 8 weeks. However, in the group of dogs treated with the combined immunosuppressive treatment composed of both CsA and MMF, all four allografts were patent at 20 weeks after implantation. Three of the explanted grafts exhibited a nonocclusive thin mural thrombus in the midportion, whereas the remaining allograft showed a thrombus-free luminal surface.

Thrombohematologic parameters. The three untreated histoincompatible dogs, which underwent allograft thrombosis at 2 weeks after transplantation, showed high TI values at operation (data not shown). However, the TI was found to be homogeneous among all other dogs before implantation and was shown to increase when thrombosis occurred. All other hematologic parameters did not differ from one blood collection to another. In the group of untreated histocompatible littermates, the hematologic parameters were not altered by the grafting procedure. One of the MHC compatible dogs had a high TI at operation and underwent allograft thrombosis at 2 weeks. Finally, within the immunosuppressed dog groups, no differences in hematologic parameters were noted except a reduction of about 50% of the circulating white blood cell counts in two dogs treated with cyclosporine at 5 weeks after operation (data not shown).

Antidonor antibody development. Recipient dogs were investigated for antidonor antibody formation with the use of donor, recipient, and third-party cultured vascular ECs and splenocytes (obtained

Table II. Posttransplant donor-specific antibodies

Experimental group*	Posttransplant antibody development†		Cytotoxicity on ECs‡
	Spleen cells	ECs	
Histocompatible			
Littermates (n = 6)	0/6	0/6	NA
Histoincompatible			
Untreated (n = 8)	8/8	8/8	8/8
CsA (n = 4)	1/4	3/4	2/3
MMF (n = 4)	3/4	1/1§	0/1§
CsA + MMF (n = 4)	1/4	1/2§	0/1§

NA, Not applicable.

*Experimental groups were as follows: *Littermates*, untreated histocompatible littermates; *Untreated*, untreated histoincompatible dogs; *CsA*, CsA-treated dogs; *MMF*, MMF-treated dogs; *CsA + MMF*, dogs treated with CsA and MMF.

†Posttransplant antibody development was measured with the use of donor splenocytes and cultured vascular ECs in a flow-cytometric assay.

‡The cytotoxic activity of posttransplant antibodies was evaluated by a fluorescent cytotoxicity assay with donor cultured vascular ECs.

§Vascular ECs could not be cultured in three dogs treated with MMF and in two dogs treated with CsA and MMF at the time of implantation or at the dog's death.

when the dogs were killed). Cytotoxic activity of developed antibodies was also measured against donor ECs. As shown in Table II, posttransplant antibodies developed in all untreated histoincompatible dogs that reacted specifically against both donor splenocytes and ECs but not against autologous or third-party cells. These antibodies appeared at 4 weeks after implantation in seven dogs and at 2 weeks in one, and remained in the recipient serum until graft occlusion occurred. Two other dogs with early allograft thrombosis (2 weeks) did not show the development of antibodies. Furthermore, all dogs had posttransplant antibodies of the immunoglobulin G isotype (data not shown) that were cytotoxic to donor ECs (Table II). In contrast, no untreated MHC-compatible littermates had posttransplant antibodies despite the low patency rates. Antibodies against donor ECs developed in three out of four CsA-treated dogs, only one of which had a cross reactivity with donor splenocytes, suggesting that in two of three cases antibodies were directed against EC-specific antigens. Moreover, two of the three dogs in which antibodies developed exhibited cytotoxic activity against donor ECs. Unfortunately, we could obtain ECs from only one dog in the group of recipients treated with MMF. Antibody-mediated reactions were noted in this case, but there was no cytotoxic activity. Still, three out of four dogs in this group formed antibodies against donor spleen cells. In addition, only one dog that was immunosup-

Table III. Graft-infiltrating alloreactive lymphocytes

Explanted grafts	Biopsy growth (No. of cultures)					
	First biopsy*		Second biopsy*		Death*	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Autografts (n = 28)†	3	18	0	8	2	26
Allografts						
Histocompatible						
Littermates (n = 6)	0	4	0	4	0	6
Histoincompatible						
Untreated (n = 10)	6	0	1	1	9	1
CsA (n = 4)	0	2			0	4
MMF (n = 4)	0	0			0	3
CsA + MMF (n = 4)	0	4			0	4

Pos., Positive; Neg., negative.

*Open biopsies were done at 2 (first biopsy) and 4 (second biopsy) weeks after operation. The culture procedure also took place when the dogs were killed using a representative midportion specimen of the entire explanted graft. In the immunosuppressive drug-treated groups, the first and only biopsy was done at 5 weeks after implantation. The results represent the number of specimens in each group in which negative or positive ($>10 \times 10^6$ cells) lymphocyte growth was observed.

†Autografts implanted in each experimental group were considered all together in this table.

pressed with both CsA and MMF showed the development of antibodies against donor ECs and splenocytes, but without cytotoxic activity. Again, we could not culture ECs from two dogs in the latter group, probably because of an effect of MMF on ECs.

Graft-infiltrating alloreactive lymphocytes. To assess the role of cell-mediated immune reactions in the outcome of venous allografts, graft-infiltrating alloreactive lymphocytes were cultured from biopsy specimens taken after operation and at the dog's death. Results relative to positive culture growth from each biopsy specimen and explant obtained at the recipient's death are shown in Table III. Growth was observed mostly with biopsy specimens taken from untreated histoincompatible dog allografts. Indeed, 9 of 10 dogs had specimens at death that showed positive lymphocyte growth within 1 month of culture. Moreover, all six first biopsy specimens and one of two second biopsy specimens showed growth of alloreactive lymphocytes, whereas two were contaminated (not shown). Cells obtained from these cultures demonstrated cytolytic activity against donor ECs (Table IV). In some cases, a certain level of cytotoxic activity was measured against third-party ECs, which suggested that the reactivity was directed against a common antigenic determinant. Among 21 first biopsy specimens,

graft-infiltrating lymphocyte growth was seen in three autografts, whereas it appeared in 2 of 28 autograft specimens obtained at death. One of the autografts with cell growth thrombosed at 4 weeks, but cytotoxic reactivity was observed against the ECs of the allograft donor. In the immunosuppressed dog groups, no growth was observed. Three first biopsy cultures and one culture obtained at death could not yield lymphocyte growth because of contamination in the group of dogs treated with MMF (data not shown). Thus the cytotoxic activity of cells infiltrating these allografts could not be evaluated.

Histologic findings. Autografts in all groups generally showed similar histologic features regardless of the group. Indeed, except for those with early failures (7 of 28), autografts showed no important morphologic changes. They exhibited smooth luminal surfaces devoid of thrombus, limited intimal hyperplasia, and a low-grade inflammatory reaction, regardless of the time of sampling (Fig. 1, A). Because no important histologic change was observed in the majority of autografts, we can suppose that the biopsy procedure did not induce events such as thrombosis or intimal hyperplasia by itself. Still, allografts demonstrated a different pattern of morphologic changes. As soon as 2 weeks after operation, untreated histoincompatible allografts showed intimal destruction with deposition of fibrin on the luminal surface. Lymphocytic infiltration was frequently noted within the intima and the adventitia of the graft (Fig. 1, B). At 4 or 8 weeks, or both times, thrombus deposition onto the luminal surface was observed with decreased lymphocytic infiltration, located mainly in the adventitia (Fig. 1, C). Intimal thickening was absent in most cases but limited in a small proportion of allografts biopsied or explanted at the time the dogs were killed.

Allografts in the group of untreated histocompatible littermates either thrombosed soon after operation or had stenosed by the time the dogs were killed (5 months), except in one case in which patency was seen. In the latter case, histologic findings were comparable to those of autografts (Fig. 2, A). However, in the two stenosed allografts, no particular histologic change was noted at 2 weeks after transplantation, but important intimal hyperplasia was observed at the time of retrieval (Fig. 2, B). Furthermore, the elastic network and vein structure were disorganized in these grafts. In the groups of dogs treated either with CsA or MMF, histologic findings were comparable. Indeed, at 5 weeks after implantation, thrombus deposition was observed on

Table IV. Cytotoxic activity of graft-infiltrating lymphocytes in untreated histoincompatible dogs

Graft recipients*	Percentage of specific EC lysis†					
	Donor			Third party		
	First biopsy	Second biopsy	Explant	First biopsy	Second biopsy	Explant
Autografts (<i>n</i> = 2/3)						
283	24.3		5.1	0		0
50	20.1		0	0		0
Allografts (<i>n</i> = 6/9)						
283	52.3		71.3	13.5		0
284	16		71.7	0		12.5
40	9.5	3.9	31.8	0	0	15.9
49	ND		18.1	ND		0
50	12.5		0	0		4.6
285	25.9		20.3	16.8		12.6

ND, Not done because of culture contamination.

*Displayed are the graft recipients for which a positive lymphocyte-infiltrating growth was observed with either biopsy or explant (obtained at death) specimens or both. Only data from dogs with positive cellular cytotoxicity are presented. The number identifying each dog is given at the left of the table.

†Cytotoxicity of graft-infiltrating cultured lymphocytes was evaluated against donor and third-party ECs and was expressed as the percentage of specific ⁵¹Cr release as detailed in the Materials and methods section. Displayed results correspond to an effector/target ratio of 40:1.

the luminal surface of most of the allografts, with destruction of the intima in all cases. In addition, mild to moderate mononuclear cell infiltration was observed in all layers of the vein wall, whereas subintimal fibrosis was either absent or limited (Fig. 2, C).

At 5 weeks, allografts from dogs treated with CsA and MMF showed a moderate inflammatory reaction within the media (Fig. 3, A) and dispersed areas of thin fibrin deposition onto the luminal surface in some cases. When the dogs were killed (20 weeks), these allografts showed a well-preserved vein structure. No mononuclear cell infiltration was observed, and a thin layer of fibrin or reorganizing thrombus (Masson's trichrome-positive stain, not shown) was observed on the luminal surface in the midportion (Fig. 3, B) but was absent from the anastomotic regions (Fig. 3, C). In addition, intimal hyperplasia was seen only in the midportions of these allografts (Fig. 3, B), and it did not appear to be concentric.

SEM evaluation. Autografts usually showed smooth luminal surfaces covered by endothelium-like cells (Fig. 4, A). Nevertheless, some denuded areas were exposed to the blood flow, but without deposition of any thrombotic material. Untreated MHC-incompatible allografts, however, showed only limited coverage by endothelium-like cells at the anastomotic regions (Fig. 4, B), whereas the midportions demonstrated complete loss of ECs with heavy deposition of thrombotic material (Fig. 4, C). This pattern of luminal covering by endothelial-like cells was also similar to that in all throm-

bosed allografts and autografts in both CsA-treated dogs and MMF-treated dogs. In contrast, allografts in dogs treated with a combination of CsA and MMF showed smooth luminal surfaces after 20 weeks of implantation. Anastomotic regions were partially covered with endothelial-like cells (Fig. 5, A), usually without any thrombotic material deposition. Interestingly, endothelial-like cells were also observed in the midportions of these allografts with thin thrombotic material present on subendothelial areas (Fig. 5, B).

Discussion

The venous allograft represents an interesting alternative to the use of the autologous saphenous vein or the internal mammary artery in small-caliber arterial reconstruction because, in contrast to synthetic prostheses and bioprostheses, it is resistant to lipid uptake and bacterial colonization.⁵ However, its antigenicity causes host immunologic reactions that can lead to graft failure.²⁴ Donor-recipient ABO compatibility was proposed in the past for improving the patency of venous allografts,²⁵ but was finally found to have no effect on graft occlusion.²⁶ With the objective of improving the patency rate of venous allografts and to determine the factors involved in graft occlusion, we used a canine model for femoral artery replacement. The effect of MHC compatibility was evaluated, as well as a short-term immunosuppressive treatment with CsA alone, MMF alone, or these two drugs in combination.

Fig. 1. **A**, Representative section of autograft explanted at 8 weeks showing normal vein wall structure (Weigert's stain, original magnification $\times 12.5$). **B**, Untreated histoincompatible allograft biopsied at 2 weeks after transplant showing heavy mononuclear cell infiltration (*M*) within intima and media, with fibrin (*F*) deposition onto the luminal surface (original magnification $\times 25$). **C**, Untreated histoincompatible allograft explanted at 4 weeks with heavy thrombus (*T*) occluding the luminal surface and markedly reduced cellular infiltration but destruction of intima and disorganization of wall structure (original magnification $\times 12.5$). *L*, Luminal surface.

As expected from previous reports,²⁷ all the allografts in the group of untreated histoincompatible dogs underwent early thrombosis, usually 4 or 8 weeks after operation. Three of these allografts thrombosed at 2 weeks but were originally implanted in dogs with a high TI value at implantation. Thus the TI could explain why graft occlusion occurred so early without signs of immune reactions in two of these three dogs. Donor-specific posttransplant antibodies developed in all histoincompatible recipients of vein allografts in the untreated group except in two with a thrombosed allograft at 2 weeks. Those antibodies appeared at 2 weeks in one case or 4 weeks in all other cases, remained present in the recipient's serum until graft occlusion was

observed, and had a cytotoxic activity on donor cultured vascular ECs (Table II). Characterization of posttransplant antibodies revealed that they were of immunoglobulin G isotype and that their cytotoxic activity was directed against antigens present on both donor splenocytes and ECs, presumably MHC antigens.²⁰ Lymphocytotoxic antibodies were also found in venous allografts implanted in the dog²⁸ and miniature swine¹³ and correlated with allograft failure. In addition, culture of graft-infiltrating lymphocytes allowed us to uncover the involvement of cytotoxic T cells in the vein allograft rejection process. Indeed, a high proportion of biopsy and explant allograft specimens in histoincompatible untreated dogs showed positive cell

Fig. 2. A, Histologic staining of the only histocompatible allograft that was patent at 20 weeks. Endothelium is well preserved, and moderate intimal thickening (*I*) is present (original magnification $\times 12.5$). B, MHC-compatible allograft explanted at 16 weeks because of stenosis. Thrombus (*T*) is present on the luminal surface and important intimal thickening (*I*) is observed (original magnification $\times 12.5$). C, Allograft from a dog treated with CsA explanted at 5 weeks showing moderate cellular infiltration within intima and media at 5 weeks and unorganized obstructive thrombus (*T*) lying on the luminal surface (original magnification $\times 25$). *L*, Luminal surface.

growth (Table III) and cytolytic activity toward donor ECs (Table IV). We suspect that cells grown from allograft specimens were cytotoxic T cells because, in some cases tested, no cell-mediated cytotoxicity was measured against the K562 cell line (sensitive to natural killer cell-mediated lysis), P815 cell line (resistant to natural killer cell-mediated lysis), and HL60 cell line (sensitive to lysis mediated by lymphokine-activated killer cells) (data not shown). Cell growth was observed in some autograft specimens, but cytolytic activity was directed only against ECs from the allograft donor. These results support the hypothesis according to which cellular infiltration is directly involved in the occlusion of venous allografts.²⁹ In kidney³⁰ and cardiac³¹ trans-

plantation, graft-infiltrating cytotoxic T lymphocytes are recognized as good indicators of acute rejection phenomena and show donor-specific lytic reactivity. Our results thus correlate with those obtained in other transplantation systems.

Histocompatible allografts from canine littermates exhibited a poor patency rate at 20 weeks after transplantation (1 in 6). In general, MHC compatibility only prolonged the patency of venous allografts but did not prevent late failure. This finding correlates with results reported by Stevens and colleagues,¹³ who used the miniature swine model, and Calhoun and colleagues,³² who used canine littermates. In both studies, MHC-compatible venous allografts thrombosed later than MHC-

Fig. 3. Histologic stainings of allografts from dogs treated with CsA and MMF that remained patent for 20 weeks after operation. **A**, Five-week biopsy specimen of an allograft showing normal wall organization and moderate cellular infiltration (original magnification $\times 25$). **B**, Allograft explanted at 20 weeks showing organized thrombus (*T*) over a nonconcentric area of intimal thickening (*I*) in midportion (original magnification $\times 12.5$). **C**, Almost unmodified wall structure observed at anastomotic sites of an allograft explanted at 20 weeks after operation (distal portion of graft, original magnification $\times 12.5$). *L*, Luminal surface.

incompatible grafts. However, long-term patency was not observed, as in our experiments. In our study, factors other than immunologic reactions are probably at the origin of graft failure, inasmuch as neither antibody development nor graft-infiltrating alloreactive lymphocytes could be found in the littermates. Another process could also be responsible for the uncontrolled intimal thickening that led to graft stenosis. However, one allograft remained patent at 20 weeks without major morphologic changes within the wall structure (Fig. 2, *A*). Thus, in certain cases, MHC compatibility could lead to results similar to those seen with the use of autologous tissues as was proposed in previous studies.¹²

To achieve better patency rates, we used immu-

nosuppressive therapies: CsA, MMF, and a combination of these two were assayed in a 1-month protocol, beginning the day before operation, thus avoiding the long-term side effects seen in prolonged systemic immunosuppression. CsA is a reference immunosuppressive agent that is therapeutically used to prevent solid organ transplant rejection.³³ It specifically acts on the helper T cell by inhibiting early activation and T cell-mediated functions.³⁴ MMF is a semisynthetic derivative of mycophenolic acid, which is a noncompetitive, reversible inhibitor of the *de novo* purine biosynthesis on which T and B lymphocytes strictly depend for deoxyribonucleic acid replication and thus acts on the late stage of activation.³⁵ Because MHC-incom-

Fig. 4. SEM examination. **A**, Explanted autograft showing confluent endothelial-like cell lining in midportion of graft (original magnification $\times 200$). **B**, Proximal region of a thrombosed untreated histoincompatible allograft explanted at 4 weeks with partial colonization by endothelial-like cells and exposition of subendothelium to blood flow (original magnification $\times 200$). **C**, Midportion of an allograft denuded of ECs, with deposition of thrombotic material onto the luminal surface (original magnification $\times 200$).

patible venous allografts in our study are the targets of both cell-mediated and humoral immune reactions, MMF should represent a suitable immunosuppressive agent in preventing immune-mediated allograft failure. MMF was shown to effectively reverse acute renal allograft rejection³⁶ and to be as effective as azathioprine in combined immunosuppressive therapy in heart transplantation.³⁷ It was also shown to significantly prolong dog renal allograft survival at a low dosage of 20 mg/kg per day.³⁸ Although this dosage is suboptimal, it was used in the present study, because higher dosages induce toxic side effects (gastrointestinal toxicity).

In our study, CsA treatment at a low dosage of 10 mg/kg per day for a period of 4 weeks did not prevent allograft occlusion beyond 11 weeks. Moreover, 50% of autografts thrombosed within this group of dogs probably because of the formation of

an angle within the graft in the immediate posttransplant period. This phenomenon could have accounted for autograft thrombosis because grafts, which were originally looped, failed before the first scheduled biopsy procedure. Although it has been reported that CsA can improve the patency of venous allografts,^{16, 39, 40} other investigators noted that short-term cyclosporine therapy did not eliminate later occlusion.^{13, 29, 41} Interestingly, posttransplant cytotoxic antibodies, in this group of dogs, reacted mostly against donor EC antigens. Because reactivity against donor splenocytes was observed in only one case without cytotoxic activity, we can suppose that MHC antigens were not involved in the sensitization of the CsA-treated hosts. Even though mild to moderate mononuclear cell infiltration was seen in CsA-treated allografts, no alloreactive lymphocytes could be cultured from the grafts. Donor-specific antibodies and other mechanisms such as

Fig. 5. Scanning electron micrographs (original magnification $\times 200$) of allograft retrieved at 20 weeks from a dog that was treated with CsA and MMF. Anastomotic regions showed almost complete covering of luminal surface with endothelial-like cells without fibrin or thrombotic matrix deposition (A), whereas midportion revealed presence of endothelial-like cells along with thin fibrin (F) deposition (B).

natural killer cell allorecognition⁴² could have accounted for graft failure in this case.

MMF monotherapy did not produce better results. Indeed, none of the allografts was patent at 8 weeks (Table I). Because graft-infiltrating lymphocytes did not grow (mostly obtained at the dog's death), we can postulate that this treatment was partially effective, in as much as antibody development was measured against donor splenocytes. Of particular interest is that ECs could not be cultured in three dogs treated with MMF and in two dogs treated with CsA plus MMF. In fact, it has recently

been reported that MMF can inhibit EC growth in vitro.⁴³

Despite the poor efficacy of CsA and MMF monotherapies, the combined immunosuppressive treatment of CsA and MMF yielded a 100% allograft patency rate at 20 weeks. Antibodies were measured in the serum of one out of four dogs, reacting with donor splenocytes and ECs but without cytotoxic reactivity. Furthermore, no graft-infiltrating lymphocytes could be cultured from the allografts, which suggests a potent effect on the immune response. Thin mural thrombotic material was observed mainly in the midportion of the explanted allografts (Fig. 3), demonstrating damage toward graft ECs or an effect of MMF, because ECs could not be cultured in 50% of these dogs either at the time of operation or when they were killed. Intimal hyperplasia was not concentric and was observed mostly in the midportions of grafts (Fig. 3, C); however, it did not lead to graft occlusion as in dogs that received an MHC-compatible allograft. Thus we can state that treatment with two powerful immunosuppressants such as CsA and MMF, acting on two different stages of immune cell activation, led to alloreactive recognition inactivation and that a certain form of immune tolerance to the graft was established. The addition of MMF to CsA certainly helped in gaining immune tolerance to the allograft because CsA alone precludes reaching allograft tolerance in dogs.⁴⁴ In addition, when used at the same dosages as in our experiments, CsA and MMF prolong the survival of hepatic allografts in dogs,⁴⁵ demonstrating the efficacy of the combined immunotherapy. A synergistic interaction between the two immunosuppressants could be responsible for the 100% patency rate observed. However, it would have been helpful to measure blood concentrations of CsA and MMF to assess this possibility. Nevertheless, immunosuppression with CsA or MMF alone was done with suboptimal dosages. Further experiments should be done to establish the optimal dosages to use in dogs and then to determine the efficacy as compared with CsA-MMF therapy. Still, in our venous allograft model, MMF seems to be an appropriate immunosuppressant, and it was recently shown to prevent intimal thickening in a rat aortic allograft model⁴⁶ by inhibiting smooth muscle cell growth.⁴⁵ This characteristic is important in arterial reconstruction with the use of venous tissues because approximately 50% of peripheral bypass grafts fail within 5 years because of intimal hyperplasia.⁴⁷ This effect of MMF on intimal hyperplasia could

explain the better results obtained with allografts from dogs treated with CsA and MMF.

Although a limited number of dogs was used, this study indicates that the use of fresh vein allografts with short-term treatment with CsA and MMF in combination could lead to long-term acceptance of this allogeneic tissue. However, further studies involving a higher number of allograft recipients and longer follow-up periods will be necessary to assess the exact role of this immunosuppressive treatment on the outcome of venous allografts used as small-diameter arterial substitutes.

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