

Cyclosporin A and tissue antigen matching in bone transplantation

Fibular allografts studied in the dog

Jean F. Welter, John W. Shaffer, Sharon Stevenson, Dwight T. Davy, Gregory A. Field, LeRoy Klein, Xiao Q. Li, Jocelyn M. Zika, and Victor M. Goldberg

We studied the mechanical, metabolic, and histologic properties of short-term nonvascularized cortical bone grafts in a canine fibular graft model. Sham operated nonvascularized autotransplanted and allotransplanted bones were compared. The allografts were performed between dog leukocyte antigen (DLA) class I and II matched; DLA class I and II mismatched; and cyclosporin A (CsA) treated, DLA class I and II mismatched animals. Cyclosporin was given for 1 month, and all the animals were followed for 3 months after surgery. Mechanical properties were investigated using standard torsional tests, metabolic kinetics were assessed using isotopic pre-labeling techniques, and histomorphometric analysis of cross-sectional area properties and sequential fluorochrome labels were performed. Autografts were mechanically stronger and stiffer than all the types of allograft. CsA-treated, DLA-mismatched allografts performed better than matched allografts. These in turn were stronger than non-CsA-treated, mismatched allografts, which underwent nearly complete resorption. These relationships were preserved in the metabolic and histologic analyses. In this short-term animal study, although DLA matching resulted in a slight improvement in graft outcome, mismatched grafts in dogs receiving a short course of cyclosporin A fared even better.

Bone is one of few tissues that are transplanted routinely without any attempt at donor and recipient tissue antigen matching, and without the use of immunosuppressive drugs to prevent rejection. It has been demonstrated clinically, and, more recently, experimentally that bone allografts evoke an immune response: specifically, human leukocyte antigen (HLA) and dog leukocyte antigen (DLA) mismatched allografts elicit the production of significant levels of anti-HLA or anti-DLA antibodies (Langer et al. 1975, Friedlaender et al. 1976 and

1984, Halloran et al. 1979, Stevenson 1987). The impact of this immune response on the incorporation of clinical bone grafts remains the subject of considerable controversy (Friedlaender 1987, Czitrom et al. 1986, Muscolo et al. 1977). In general, allografts are fraught with a higher rate of complications than autografts, and the latter remain the standard by which allografts must be judged (Brown and Cruess 1982). The assumption remains that these complications are a consequence of the host's immune response. However, massive osteochondral allografting in patients with known degrees of histocompatibility mismatch to the donor did not reveal any correlation between graft incorporation and the degree of matching (Muscolo et al. 1987), nor did the appearance of anti-HLA antibodies adversely affect the outcome (Friedlaender et al. 1984). The clinical significance of the immune response therefore remains unclear.

Department of Orthopedics, Case Western Reserve University, 2074 Abington Road, Cleveland, OH 44106, U.S.A.

Tel.: (216)-844-3044
Fax: (216)-844-4876

In several previous laboratory studies, allograft incorporation has been improved by a variety of immunosuppressive techniques (Hutzschenreuter 1972, Burchardt et al. 1977, 1981, Aebi et al. 1986, Goldberg et al. 1984). However, immunosuppressive regimens including steroids, azathioprine, and anti-lymphocyte serum were associated with risks too high in relation to their potential benefits to warrant their use (Goldberg et al. 1984). The introduction of the less noxious cyclosporin A (CsA) and its derivatives has evoked renewed interest in the use of immunosuppressive regimens for the purpose of improving the incorporation of massive, cortical bone grafts (Aebi et al. 1986, 1987).

We have compared the effect of tissue antigen matching to that of a short-term CsA regimen on the incorporation of nonvascular cortical bone allografts in an established canine model.

Materials and methods

Twenty-eight purpose bred Beagle dogs of both sexes were obtained at age 4 or 8 months from a commercial breeder (Marshall Farms, North Rose, NY). They were housed in individual cages, and fed a standard dog chow (Purina, St. Louis, MO) and water diet ad libitum. American Association for Accreditation of Laboratory Animal Care (AAA-LAC) guidelines were adhered to in all aspects of this study.

All the dogs were typed for major histocompatibility complex (MHC) class I and II antigens (Stevenson 1987). Typing for class I was done by one- and two-stage microcytotoxicity tests using a battery of antisera. Dogs with identical specificities were considered to be matched. Typing for class II was by mixed leukocyte culture (MLC), performed in triplicate. Dogs with MLC stimulation indices reproducibly below three were considered matched (Stevenson 1987, Westbroek et al. 1975). In this study, matched dogs were defined as matched for class I and II antigens and were always littermates, whereas mismatched dogs were mismatched for both class I and II antigens.

The surgical model used in this study was the proximal canine fibula. Surgery was performed on the dogs at age 9 months. In all the dogs, both fibulae were studied, allowing intra-dog comparisons.

There were three groups:

In Group I (N = 12), an allograft, matched for both class I and class II was compared to a sham

operated fibula or an autograft.

In Group II (N = 6), a completely mismatched allograft was compared to an autograft.

In Group III (N = 10), as in Group II, a completely mismatched allograft was compared to an autograft. These dogs received 20 mg/kg of the commercial oral solution of CsA (Sandoz, Inc.) s.c. for the first 15 days beginning the day before surgery, then the dose was reduced to 15 mg/kg for another 15 days; at this point, CsA was discontinued. Cyclosporin blood levels were determined at 2 weeks and at 1 month postoperatively.

A subset of the dogs (6 Group I, and 4 Group III animals) was used to study the metabolic kinetics of calcium and collagen in the grafts. These dogs were obtained at 4 months of age, and were prelabeled with 24 injections of ^3H -labeled tetracycline and ^3H -labeled proline, administered biweekly during the 5th, 6th, and 7th months. A total of 1 mCi of each of the tracers was administered. The dogs were then allowed 1 month to equilibrate before surgery was performed.

The dogs were operated on aseptically under general inhalation anesthesia. Details of the surgical procedure have been published elsewhere (Goldberg et al. 1990). Briefly, the proximal fibula was approached through a lateral incision. The tibiofibular joint was disarticulated, a transverse osteotomy was performed 8 centimeters distal to the tip of the fibular head, and the bone was removed extraperiosteally. All the soft tissues except the joint capsule were stripped subperiosteally, and the bone was replaced in situ. The joint capsule was repaired; a distal osteosynthesis was achieved by transfixing the fibula to the tibia with a 1.5-mm Kirschner wire. Allografts were harvested simultaneously from 2 dogs and exchanged between animals. All the grafts were fresh, and no pharmacologic or physical manipulations were performed to alter the graft antigenicity. The sham procedure was limited to the release and repair of the joint capsule and the placement of a K-wire. No osteotomy was performed, nor were the periosteum and muscle attachments disturbed. Postoperative analgesia was ensured by meperidine administered subcutaneously as needed. One gram of ampicillin was given postoperatively.

All the dogs received sequential bone-seeking fluorochrome markers intravenously to label active new bone formation using one of two previously published protocols (Li et al. 1990). According to the first protocol, tetracycline was given immediately after surgery and 10 days later; xylenol orange was given at 3 weeks and 10 days thereafter. Alizarin complexone was given 6 weeks after

Table 1. Radiographic scoring system

Score	Fractures	Resorption
0	No fracture	Normal bone
1	1 fracture	Slight erosion
2	2 fractures	Moderate erosion
3	> 2 fractures	Severe erosion
4		Resorbed areas
5		Complete resorption

surgery and 10 days later; finally, 2,4-bis(N,N'-di(carbomethyl)amonomethyl)-fluorescein (DCAF) was given 11 weeks after surgery and 10 days later. Following the second, alternate protocol, DCAF was given postoperatively and at 1 week, xylene orange at 3 and 6 weeks, and tetracycline at 9 and 12 weeks.

Three months after surgery, the animals were killed with a barbiturate overdose. The abdominal aorta was exposed, ligated proximally, and cannulated. A suspension of BaSO₄ was then injected into the arterial tree of the lower extremities. The fibulae were harvested in toto, stripped of all soft tissues and radiographed. The condition of the grafts in situ at the time the dogs were killed was scored using the system defined in Table 1. These data were evaluated statistically by nonparametric analysis of variance (Kruskal-Wallis *H*-test) and Mann-Whitney *U*-tests.

Mechanical tests were performed within 24 hours of harvesting. The bones were refrigerated at 5 °C until tested, and were then warmed to room temperature prior to testing. The bones were kept moist throughout. The proximal 6-cm segment of the grafts was cut off, and was potted in methylmethacrylate so that a central, 1-cm segment was left free for testing. The samples were then twisted to failure on a materials testing machine (Instron model 1230) at a rate of 4°/s. Torque vs. angular deformation plots were obtained for each test. Strength (in Nm) and stiffness (in [Nm]/[°/cm]) were computed from these results. Strength was defined as the maximum torque applied prior to failure, and stiffness as the torque twist ratio at 3° of twist. Unstable samples, i.e., samples fractured or resorbed in situ, were assigned zero values for these parameters. Grafts damaged during harvesting were treated as missing data. The data were analyzed statistically using the Wilcoxon signed rank test for matched pairs, comparing each graft with the contralateral bone from the same animal (i.e., the donor in

the case of allografts). The variability inherent in the mechanical properties made the sample size that we used too small to permit meaningful comparisons across groups.

Segments were removed for histologic studies, leaving 92 percent of the graft mass for metabolic studies (Goldberg et al. 1990). The metabolic data describes the dry weight, total calcium mass, and total collagen mass, as well as the amount (in dpm) of radioactive (³H-proline containing) collagen and radioactive tetracycline remaining in the graft. It has been shown previously that dogs prelabeled as described incorporate the isotope symmetrically in paired bones, and normally lose isotope symmetrically (Klein 1970, Klein and Jackman 1976). The isotope data therefore allows an assessment of changes in the amount of remaining old bone induced by the two experimental procedures performed in each animal. Relative losses were determined by positing the data from one of the bones equal to 100 percent and computing the percentage difference. Again, the bones were compared with the contralateral bone from the same animal. The grafts were completely decalcified by sequential extractions with 0.5 N HCl at 4 °C. The ³H-tetracycline content of the eluate was determined by liquid scintillation counting. The calcium content was determined using an automatic analyzer. Finally, the remaining organic graft matrix was hydrolyzed in 6 N HCl. Aliquots of the hydrolysate were used to determine total hydroxyproline by chromatographic separation on a Dowex-50 ion exchange column, and radioactive hydroxyproline by liquid scintillation counting (Goldberg et al. 1990, Li and Klein 1990). Statistical analysis was by paired Student's *t*-tests.

The histology specimens were fixed in 40 percent ethanol and then embedded in methyl-methacrylate. Undecalcified 100-μ-thick sections were obtained for microradiography, toluidine blue surface staining, and fluorescence microscopy (Schenk et al. 1984).

The microradiographs were used to determine cross-sectional area and porosity using a Bioquant image analyzer (Bioquant System IV, R&M Biometrics, Nashville, TN). The results from four-six sections were averaged using the general weighted average formula (Weibel 1979).

The sections were then examined by fluorescence microscopy for the presence and the number of labels; their distribution was noted as well. The total number of osteons on each of four-six sections, as well as the number of osteons on each section taking up each individual label, was counted. The number

Table 2. Radiographic score

	n	Fracture score			Resorption score		
		Mean	SD	Median (Range)	Mean	SD	Median (Range)
Auto	20 ^a	0.2	0.6	0 (0-1)	1.4	0.9	1 (0-3)
++ Allo	10	1.9	0.9	2 (0-3)	3.2	0.6	3 (2-4)
-- Allo	6	0.8	1.2	0.5 (0-3)	3.7	1.0	4 (2-5)
-- Allo+CsA	10	0.5	1.0	0 (0-3)	1.8	1.0	1.5 (1-4)

^aTwo bones were not evaluated.

Table 3. Mechanical properties

	n	Strength (Nm)			Stiffness ([Nm]/[°/cm])		
		Mean	SD	Median (Range)	Mean	SD	Median (Range)
Sham	6	0.216	0.1	0.201 (0.105-0.398)	0.019	0.01	0.017 (0.007-0.035)
Auto	22	0.085	0.064	0.073 (0.0-0.232)	0.007	0.007	0.006 (0.0-0.027)
++ Allo	11 ^a	0.006	0.013	0.0 (0.0-0.042)	0.0	0.001	0.0 (0.0-0.002)
-- Allo	6	0.0	0.0	0.0 (0.0-0.0)	0.0	0.0	0.0 (0.0-0.0)
-- Allo+CsA	10	0.056	0.073	0.011 (0.0-0.173)	0.006	0.009	0.001 (0.0-0.022)

^aOne bone was damaged during harvesting and was not tested.

of bone-forming sites at each labeling interval was thus determined to provide an index of osteogenic activity. Results are expressed as osteons taking up a given label as a percentage of the total number of osteons.

Results

All the dogs recovered uneventfully from surgery and bore weight on both hind legs within the first week after surgery. There were no deep infections. cyclosporin A blood levels averaged 1,023 ng/mL (SD 438) at 2 weeks after surgery, and 905 (SD 235) at 4 weeks after surgery. These means were not different (Paired Student's *t*-test, $P = 0.19$).

Gross anatomy

There were marked differences in the fracture and resorption scores ($H = 21.5$, 3df, $P < 10^{-4}$ and $H = 24.1$, 3df, $P < 10^{-4}$, respectively). The resorption scores (Table 2) of the autografts and the mismatched allografts with CsA (Group III) did not differ from each other ($P = 0.9$). Both of these graft

types differed from the other two ($P < 0.005$). The fracture scores behaved similarly, except that scores of the mismatched allografts without CsA (Group II) were not different from the autografts or the allografts with CsA. It must be noted, however, that, due to the extreme resorption of some of the grafts in Group II, fractures could not be identified as such, thus improving the overall score of this group.

Mechanical tests

In terms of strength (Table 3), allografts in CsA-treated animals (Group III) did not differ from autografts ($P = 0.33$). Mismatched allografts without CsA (Group II) were weaker than the autograft controls ($P = 0.028$), while the differences between matched allografts (Group I) and autografts were only marginal ($P = 0.07$). Results were similar in terms of stiffness: mismatched allografts with CsA ($P = 0.55$) did not differ from the autograft controls while the mismatched allografts without CsA ($P = 0.027$) and, albeit marginally, the matched allografts did ($P = 0.07$). One matched graft was damaged during harvesting and was excluded from evaluation.

Table 4. Metabolic kinetic data (Mean SD)

	Sham	++ Allo	Relative loss (%)	P-value
Group I (n 6)				
Dry weight (mg)	644 161	438 158	32 17	0.005
Calcium (mg)	140 40	93 36	34 16	0.003
Collagen (mg)	146 40	99 27	31 14	0.002
³ H-Tetracycline (kdpm)	58 13	30 15	48 25	0.005
³ H-Collagen (kdpm)	38 7	18 7	52 20	0.003
Group III (n 4)				
Dry Weight (mg)	853 294	747 261	12 4	0.021
Calcium (mg)	183 62	144 50	21 8	0.047
Collagen (mg)	209 62	186 57	11 8	0.069
³ H-Tetracycline (kdpm)	13 8	9 4	25 18	0.143
³ H-Collagen (kdpm)	13 6	19 8	18 14	0.061

Table 5. Histomorphometric analysis (Mean SEM)

	Sham (n 6)	Auto (n 16)	++ Allo (n 9)	-- Allo (n 4)	-- Allo + CsA (n 9)
Area (mm ²)	6.5 1.7	5.6 0.3	1.9 2.6	1 0.2	4.9 0.3
Total osteons	118 9.3	68 5.7	24 5.6	20 9.0	68 12
Porosity (%)	1 0.3	8.8 0.9	22 6.2	22 6.3	17 3.5

Metabolic kinetics

When compared with sham operated on bones, all the grafts were less massive, whether expressed as dry weight, calcium mass or collagen mass (Table 4). The resorption indices of radioactive tetracycline and radioactive collagen indicate that they conserved considerably less of the original graft substance. The differences between autografts and allografts with CsA were smaller, and only marginally significant.

It was originally planned to apply this method to the mismatched allografts without CsA. Group III grafts, however, resorbed to the point where they could not be identified with certainty at autopsy. Careful isolation of the graft from the surrounding tissue is mandatory for this technique, but proved to be impracticable; therefore, these grafts were not compatible with this methodology, and the proposed subgroup was deleted.

Histology

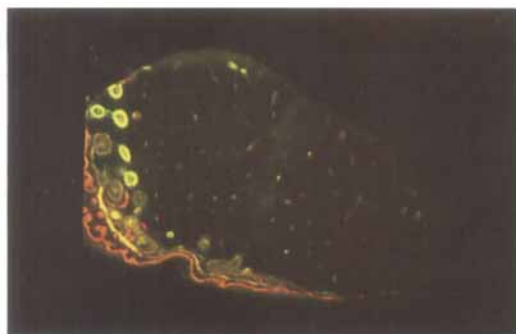
In terms of cross-sectional area, sham operated on bones were, on an average, the largest (Table 5) and the least porous of all (Figure 3). Barium was noted in nearly all the vascular spaces, and fluorochromes

were taken up at all the intervals. In these grafts, labels were generally concentrated near the graft periphery (Figure 1).

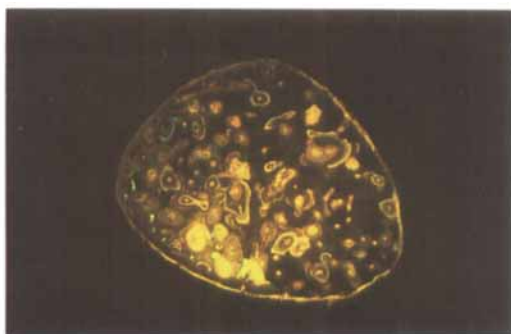
The autografts were smaller and more porous; but as indicated by the presence of barium in the vascular channels, the grafts were entirely revascularized (Figures 2 and 3). All the fluorochrome labels were present in the grafts (Figure 1).

The matched allografts demonstrated a variable pattern of repair. There was extensive resorption and remodeling from the periphery. In several grafts, much of the original bone was eroded, with very little vascular invasion (Figure 3) and minimal fluorochrome uptake within the old bone, which retained its original level of porosity. Most grafts, however, repaired in the usual fashion by creeping substitution. Porosity was the highest in this group, and blood vessels could be demonstrated in most vascular spaces. Six to 11 week fluorochrome labels were generally present, but were primarily located at the periphery of the grafts (Figure 1). In all the cases, these grafts were surrounded by a fibrous-tissue cuff, and frequently they were encased by woven bone, although the latter may have been caused by fracture repair (Figure 3).

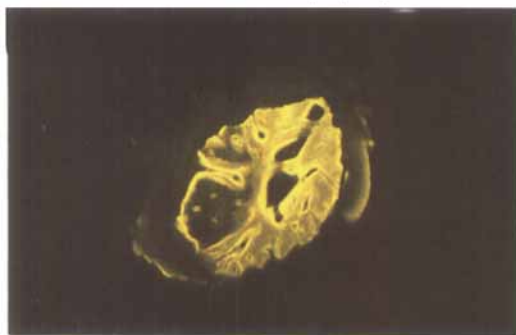
In sharp contrast, the mismatched allografts without CsA were largely resorbed. A thick fibrous tissue cuff surrounded the grafts (Figure 2), which



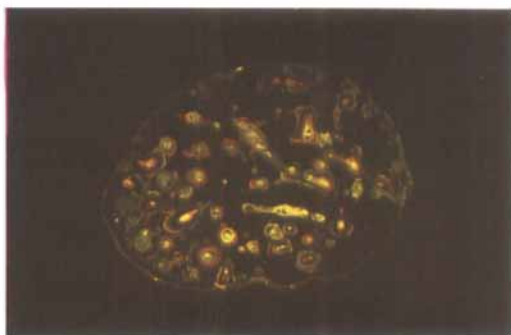
Sham operated on bone. Labeling protocol 1. All labels are present and are concentrated at the periphery of the bone.



Autograft. Labeling protocol 1. All labels are present.



DLA classes I and II matched allograft. Labeling protocol 2. Nine- and 12-week tetracycline labels are preponderant; massive graft resorption is evident.



DLA classes I and II mismatched allograft with cyclosporin A. Labeling protocol 1. The 6-week labels are the most frequent.

Figure 1. Epifluorescence photomicrographs of cross sections of the grafts 3 months after surgery demonstrating fluorochrome uptake. Colors: red = alizarin red, orange = xylenol orange, yellow = tetracycline, bright green = DCAF. Unstained, 100- μ -thick cross sections. $\times 16$.

were generally also extremely porous. Blood vessels were present, but only very few fluorochrome labels were visible, mainly the 11-week label (Figures 1 and 4).

Mismatched allografts with CsA were grossly similar in size and shape to autografts. They were fully revascularized, and fluorochrome labels given after 3 weeks were present. Quantitative analysis revealed that, although the mean cross-sectional area and total osteon counts were similar to the autografts, porosity was higher, and a drop in the percentage of labeled osteons was noted after 6 weeks (Figure 4).

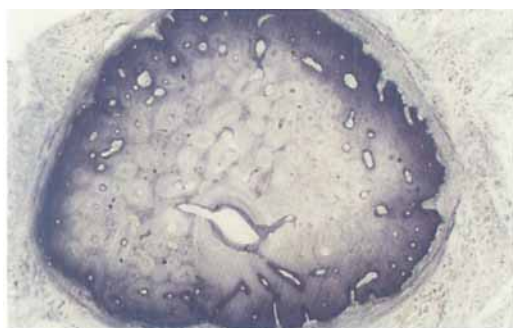
Discussion

The ultimate aim of all bone-graft procedures is to obtain a stable, mechanically sound construct. Using the mechanical properties as a benchmark in this

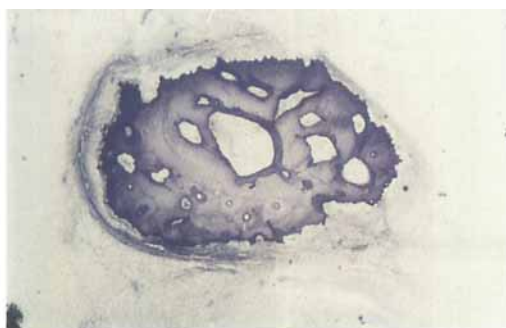
study of nonvascular cortical grafts, autografts outperformed allografts, regardless of their type. Among the allografts, mismatched allografts from dogs not receiving CsA fared the worst: all were largely resorbed, fragmented, and mechanically untestable. In terms of outcome of the grafting procedure, they must be classified as failures. Matched allografts performed only slightly better: only two were strong enough to be tested. In contrast, the mismatched allografts placed in CsA-treated hosts were nearly indistinguishable from autografts in terms of strength and stiffness.

These relationships were preserved by the other parameters studied: in general, the mechanical results and the radiographic, metabolic, and histologic examinations correlated well.

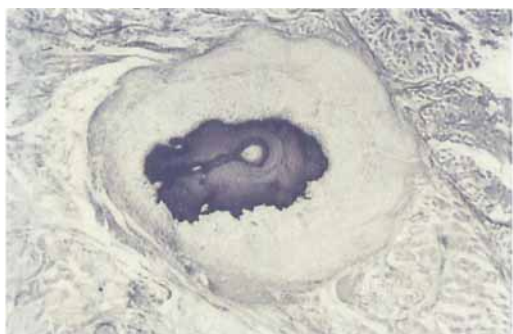
(1) Compared with mismatched, nonimmunosuppressed grafts, improvement of radiographic scores by MHC match was slight. In contrast, short-term immunosuppression with CsA lowered the scores to autograft level.



Autograft.



DLA classes I and II matched allograft. Note the large resorption spaces.



DLA classes I and II mismatched allograft showing massive resorption of the graft and thick, encasing fibrous-tissue cuff.



DLA class I and II mismatched allograft with cyclosporin A. Size is comparable to autograft, but the porosity is markedly higher.

Figure 2. Photomicrograph of cross sections of the grafts at 3 months. Toluidine blue surface stain. $\times 16$.

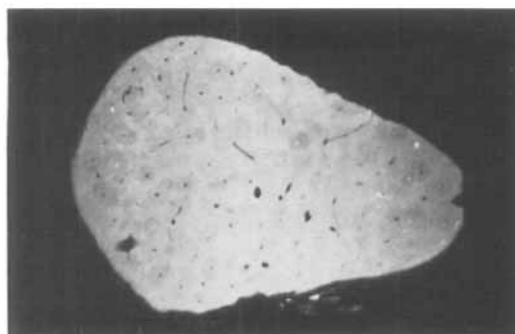
(2) In comparison with the autografts, grafts placed in CsA-treated hosts retained similar amounts of the original graft material, as indicated by the metabolic analysis. Although this retained original graft material is most likely the cause of their good performance in this study, it is also a potential liability: this material is necrotic, and thus may be susceptible to fracture at a later time. Total mass, collagen mass, and calcium mass were only slightly less (Table 4). Compared with sham operated on bone, matched allografts retained half as much of the original graft material. In animals not treated with CsA, mismatched allografts lost the bulk of the original graft material.

(3) Histologically, the grafts from CsA-treated animals were very different from non-CsA-treated matched or mismatched allografts. Cross-sectional area and total osteon count were greater, porosity was less, and revascularization appeared complete (Figure 3). Fluorochrome labeling was similar in distribution to that seen in autografts with active new bone formation throughout the graft. However,

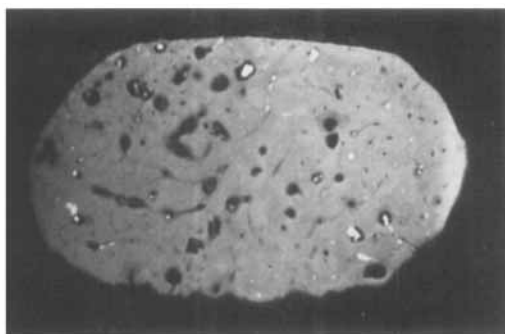
the number of bone-forming sites decreased sharply after 6 weeks (Figure 4). This decrease began 2 weeks after the discontinuation of CsA, and may be a histologic manifestation of an increasing immune response. Matched allografts showed massive resorption, but frequently augmented the grafts with peripheral callus, and remodeling was evident within the substance of the grafts. It is thus conceivable, that given adequate time, these grafts would have regained strength.

Several idiosyncrasies of this model must be considered before attempting to relate these results to clinical bone grafts. Reviewing them may help to put our findings into perspective:

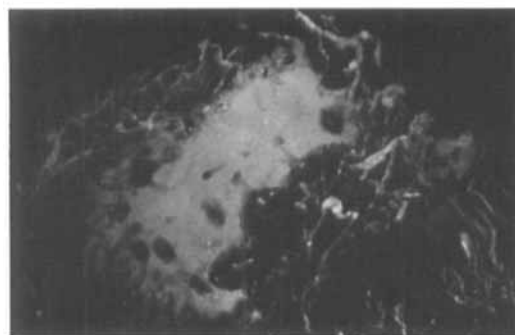
(1) The geometric properties of the canine fibula are very dissimilar to those of a clinical, typically massive, cortical bone allograft. Although almost pure cortical bone, it is very small and slender. Our model is therefore very sensitive to even small absolute amounts of surface resorption, which leads to a dichotomous behavior. Grafts that incorporate do so quickly and completely, whereas grafts that



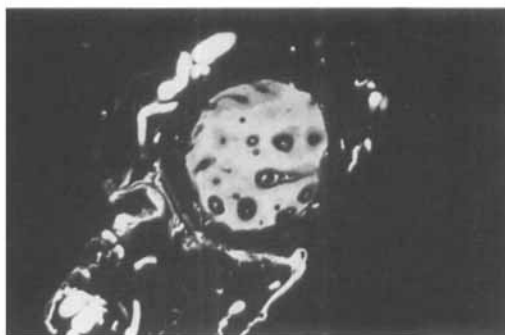
Sham operated on bone.



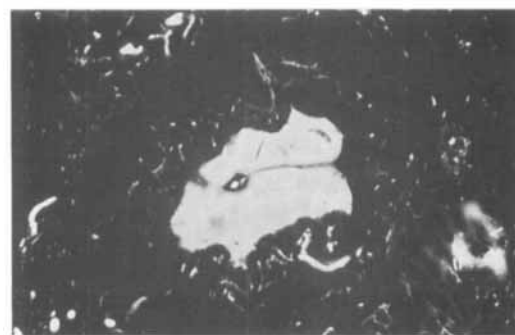
Autograft.



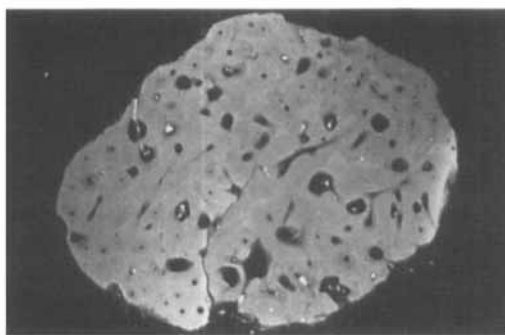
Matched allograft. Massive peripheral resorption and encasing woven bone.



Matched allograft. Repair by creeping substitution.



Mismatched allograft with surrounding highly vascular fibrous tissue cuff.



Mismatched allograft with CsA.

Figure 3. Microradiographs of 100 μ thick cross sections. Injected BaSO_4 is visible as bright white profiles of the blood vessels. $\times 16$.

resorb frequently fail dramatically. In comparison, Schwarzenbach et al. (1989) failed to detect statistically significant differences in remodeling patterns between nonimmunosuppressed and CsA-suppressed dogs using more massive tibia grafts.

(2) The dogs were killed at a single point in time, 3 months after surgery. Clearly, the CsA exerted a protective influence on the allografts, the effects of

which could still be detected 3 months after surgery, and 2 months after the CsA was discontinued. However, this advantage may merely reflect a temporal offset due to the drug: these grafts still may ultimately fail. Conversely, grafts with unhealed fractures 3 months after surgery were untestable, and accordingly received mechanical scores of zero. Had these fractures been allowed

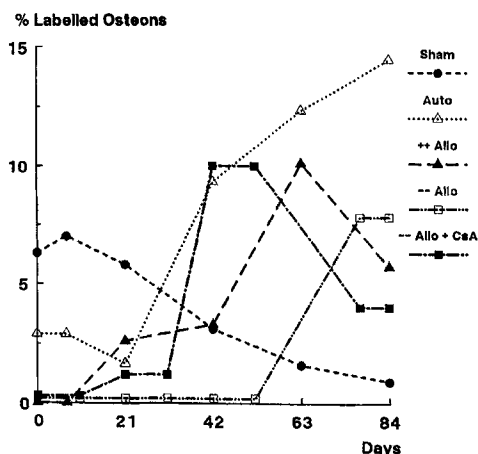


Figure 4: Percentage of total osteon count taking up fluorochrome labels. Auto = Autograft, ++ Allo = DLA class I and II matched allograft, -- Allo = DLA class I and II mismatched allograft, + CsA = with cyclosporin A

sufficient time to heal, these grafts might well have received better mechanical scores at a later date.

(3) In nonvascular grafts, any cells surviving transplantation must do so from diffused nutrients, and must thus be located near bone surfaces; revascularization and creeping substitution also proceed from the graft surfaces inward. Bone resorption, however, occurred primarily from the periphery: it is therefore conceivable that fluorochromes were taken up in these regions early in the experiment, but were subsequently lost to resorption. The massive resorption and the resulting low total osteon count seen in some of the allografts thus have two consequences: the number of early labels may be underestimated, and small absolute numbers of labeled osteons yield high percentages (Figure 4).

(4) In this model, the mechanical environment of the grafts was markedly altered, because the interosseous membrane and all the muscle attachments were stripped. Thus, a degree of adaptive remodeling, the end point of which would most likely differ from a normal fibula, is to be expected. The model also differs from the interposition grafts used by others (Dell et al. 1985, Burchardt et al. 1977) in that the grafts are longer, and only one, stably fixed osteosynthesis site was used. This limits motion at the graft-host junction, promoting union; however, the contribution of encasing callus and direct creeping substitution across the graft-host junction to the biology of the graft was also reduced

(Goldberg et al. 1990).

(5) Antigens in addition to the class I and II antigens we assessed could presumably have played an important role in bone-graft rejection in our model. Conversely, human allografts are generally not fresh; their antigenicity is probably altered by any storage procedure employed (Burwell 1976).

The nature of the advantage conferred by CsA remains unclear. Two effects of the drug are currently under investigation: immunosuppression and a direct or mediated effect on bone-remodeling dynamics.

Results similar to ours have been achieved by short-term, non-CsA immunosuppression: in studies of other immunosuppressive regimens in dogs receiving mismatched allografts (i.e., azathioprine, steroids, or anti-lymphocyte serum), treated allografts were radiographically and histologically indistinguishable from autografts (Goldberg et al. 1984). Burchardt et al. (1981) and Burchardt (1983) also concluded that short-term immunosuppression was effective in improving graft incorporation, and they suggested two mechanisms for this effect:

(1) The immunosuppression allows sufficient time for the ingrowth of vessels and the initial adequate creeping substitution, which leads to a sequestration of the antigenic material.

(2) The elapsed time under immunosuppression is sufficient to allow a degradation of the immunogenicity of the graft to the point where it can be tolerated by the host.

Recent evidence implies that neither of these hypotheses can be completely correct. It appears that after CsA is discontinued, antibodies are generated against the graft donor (Stevenson et al. 1989). Similar results in a rat model confirm that the immunosuppression is only temporary (Rodrigo et al. 1989). The decrease in the number of labeled osteons in the mismatched allografts with CsA seen in this study after the CsA was discontinued indirectly supports this theory as well (Figure 4).

Other authors have studied the influence of CsA on bone remodeling. Orcel et al. (1989) recently reported that therapeutic doses of CsA inhibit bone resorption and stimulate bone formation in vivo in a rat cancellous bone model. They suggest either a direct or a cytokine-mediated effect on the bone cells. The latter effect has been demonstrated in vitro, as a modulation of the effects of interleukin-1 (Skjodt et al. 1985), and as an inhibition of osteoclast activating factor synthesis (Horowitz et al. 1985); these factors are known to mediate bone resorption (Gowen et al. 1983, Horowitz et al. 1985, Lorenzo et al. 1987).

It is therefore tempting to conclude that the improved incorporation of bone allografts is due to a synergistic modulation of both the immune response and the bone remodeling mechanisms by CsA. However, it should be noted that other authors have reported results at variance with the previously cited investigations, although not in canine cortical bone. Markedly increased bone resorption and depressed bone formation following CsA *in vivo* have also been described in the rat tibia and in human bone biopsies (Movsowitz et al. 1988, Aebi et al. 1988, Movsowitz et al. 1989, Schlosberg et al. 1989). In addition, regardless of its direction, the effect of CsA on bone resorption was reversible within days if CsA was withdrawn in several other studies (Stewart et al. 1986, Klaushofer et al. 1987, Schlosberg et al. 1989,).

Acknowledgements

This study was supported in part by grants from the National Institutes of Health (Grant # AM-30833) and from the Veterans Administration.

References

- Aebi M, Regazzoni P, Perren S M, Harder F. Microsurgically revascularized bone allografts with immunosuppression with cyclosporine. Preliminary report of the effect in an animal model CSC. *Transplantation* 1986; 42 (5): 564-8.
- Aebi M, Schwarzenbach O, Regazzoni P. Long-term versus short-term immunosuppression in experimental bone allotransplantation. *Trans Ortho Res Soc* 1987; 12: 89.
- Aubia J, Masramon J, Serrano S, Lloverans J, Marinoso L. Bone histology in renal transplantation patients receiving cyclosporin. *Lancet* 1988; 1: 1048.
- Brown K L, Cruess R L. Bone and cartilage transplantation in orthopaedic surgery. A review. *J Bone Joint Surg (Am)* 1982; 64 (2): 270-9.
- Burchardt H, Glowczewskie F P, Enneking W F. Allogeneic segmental fibular transplants in azathioprine immunosuppressed dogs. *J Bone Joint Surg (Am)* 1977; 59 (7): 881-94.
- Burchardt H, Glowczewskie F P, Enneking W F. Short-term immunosuppression with fresh segmental fibular allografts in dogs. *J Bone Joint Surg (Am)* 1981; 63 (3): 411-5.
- Burchardt H. The biology of bone graft repair. *Clin Orthop* 1983; 174: 28-42.
- Burwell R G. The fate of freeze dried bone allografts. *Transplant Proc (Suppl 1)* 1976; 8: 95-111.
- Czitrom A A, Langer F, McKee N, Gross A E. Bone and cartilage allotransplantation. A review of 14 years of research and clinical studies. *Clin Orthop* 1986; 208: 141-5.
- Dell P C, Burchardt H, Glowczewskie F P Jr. A roentgenographic, biomechanical, and histological evaluation of vascularized and non vascularized segmental fibular canine autografts. *J Bone Joint Surg (Am)* 1985; 67 (1): 105-12.
- Friedlaender G E. Bone grafts. The basic science rationale for clinical applications. *J Bone Joint Surg (Am)* 1987; 69 (5): 786-90.
- Friedlaender G E, Strong D M, Sell K W. Studies on the antigenicity of bone. I. Freeze dried and deep frozen bone allografts in rabbits. *J Bone Joint Surg (Am)* 1976; 58 (6): 854-8.
- Friedlaender G E, Strong D M, Sell K W. Studies on the antigenicity of bone. II. Donor specific anti HLA antibodies in human recipients of freeze dried allografts. *J Bone Joint Surg (Am)* 1984; 66 (1): 107-12.
- Goldberg V M, Bos G D, Heiple K G, Zika J M, Powell A E. Improved acceptance of frozen bone allografts in genetically mismatched dogs by immunosuppression. *J Bone Joint Surg (Am)* 1984; 66 (6): 937-50.
- Goldberg V M, Stevenson S, Shaffer J W, Davy D, Klein L, Zika J, Field G. Biological and physical properties of autogenous vascularized fibular autografts in dogs. *J Bone Joint Surg (Am)* 1990; 72 (6): 801-10.
- Gowen M, Wood D D, Ihrie E J, McGuire M K, Russell R G. An interleukin 1 like factor stimulates bone resorption *in vitro*. *Nature* 1983; 306 (5941): 378-80.
- Halloran P F, Lee E H, Ziv I, Langer F, Gross A E. Orthotopic bone transplantation in mice. II. Studies of the alloantibody response. *Transplantation* 1979; 27 (6): 420-6.
- Horowitz M, Baron R, Mart J, Andreoli M, Vignery A. Osteoclast activating factor is distinct from the monokine IL 1. *Br J Rheumatol (Suppl 1)* 1985; 24: 162-4.
- Huttschenreuter P. Beschleunigt Einheilung von allogenen Knoche Knochentransplantaten durch Präsensibilisierung des Empfängers und stabile Osteosynthese. *Langenbecks Arch Chir* 1972; 331 (4): 321-43.
- Klaushofer K, Hoffmann O, Stewart P J, Czerwenka E, Koller K, Peterlik M, Stern P H. Cyclosporine A inhibits bone resorption in cultured neonatal mouse calvaria. *J Pharmacol Exp Ther* 1987; 243 (2): 584-90.
- Klein L. Hydroxyproline in urine and tissues. *Stand Methods Clin Chem* 1970; 6: 41-56.
- Klein L, Jackman K V. Assay of bone resorption *in vivo* with ³H tetracycline. *Calcif Tissue Res* 1976; 20 (3): 275-90.
- Langer F, Czitrom A, Pritzker K P, Gross A E. The immunogenicity of fresh and frozen allogeneic bone. *J Bone Joint Surg (Am)* 1975; 57 (2): 216-20.
- Li X Q, Stevenson S, Klein L, Davy D T, Shaffer J W, Goldberg V M. Differential patterns of incorporation and remodeling among various types of bone grafts. *Acta Anatomica* 1990. Submitted.

- Li X Q, Klein L. Decreasing rates of bone resorption in growing rats in vivo: comparison of different types of bones. *Bone* 1990; 11 (2): 95-101.
- Lorenzo J A, Sousa S L, Alander C, Raisz L G, Dinarello C A. Comparison of the bone resorbing activity in the supernatants from phytohemagglutinin stimulated human peripheral blood mononuclear cells with that of cytokines through the use of an antiserum to interleukin 1. *Endocrinology* 1987; 121 (3): 1164-70.
- Movsowitz C, Epstein S, Fallon M, Ismail F, Thomas S. Cyclosporin A in vivo produces severe osteopenia in the rat: effect of dose and duration of administration. *Endocrinology* 1988; 123 (5): 2571-7.
- Movsowitz C, Epstein S, Ismail F, Fallon M, Thomas S. Cyclosporin A in the oophorectomized rat: unexpected severe bone resorption. *J Bone Miner Res* 1989; 4 (3): 393-8.
- Muscolo D L, Kawai S, Ray R D. In vitro studies of transplantation antigens present on bone cells in the rat. *J Bone Joint Surg (Br)* 1977; 59 (3): 342-8.
- Muscolo D L, Caletti E, Schajowicz F, Araujo E S, Makino A. Tissue typing in human massive allografts of frozen bone. *J Bone Joint Surg (Am)* 1987; 69 (4): 583-95.
- Orcel P, Bielakoff J, Modrowski D, Miravet L, de Vernejoul M C. Cyclosporin A induces in vivo inhibition of resorption and stimulation of formation in rat bone. *J Bone Miner Res* 1989; 4 (3): 387-91.
- Rodrigo J J, Schnaser A M, Reynolds H M Jr, Biggart J M, Leathers M W, Chism S E, Thorson E, Grotz T, Yang Q M. Inhibition of the immune response to experimental fresh osteoarticular allografts. *Clin Orthop* 1989; 243: 235-53.
- Schenk R K, Olah O J, Herrmann W. Preparation of calcified tissue for light microscopy. In: *Methods of Calcified Tissue Preparation* (Ed. Dickson G R). Elsevier Press, Amsterdam 1984: 1-56.
- Schlosberg M, Movsowitz C, Epstein S, Ismail F, Fallon M D, Thomas S. The effect of cyclosporin A administration and its withdrawal on bone mineral metabolism in the rat. *Endocrinology* 1989; 124 (5): 2179-84.
- Skjodt H, Crawford A, Elford P R, Ehrie E, Wood D D, Russel G G. Cyclosporin A modulates interleukin-1 activity on bone in vitro. *Br J Rheumatol (Suppl 1)* 1985; 24: 165-9.
- Schwarzenbach O, Regazzoni P, Aebi M. Segmental vascularized and non vascularized bone allografts. In: *Bone Transplantation* (Eds. Aebi M, Regazzoni P). Springer Verlag, Berlin 1989: 78-81.
- Stevenson S. The immune response to osteochondral allografts in dogs. *J Bone Joint Surg (Am)* 1987; 69 (4): 573-82.
- Stevenson S, Goldberg V M, Shaffer J, Field G, Davy D, Klein L. Interactions among immunosuppression, immune response and histomorphometry in canine fibular vascularized and nonvascularized segmental allografts. *Trans Orthop Res Soc* 1989; 14: 272.
- Stewart P J, Green O C, Stern P H. Cyclosporine A inhibits calcemic hormone induced bone resorption in vitro. *J Bone Miner Res* 1986; 1 (3): 285-91.
- Weibel E R. Stereological methods. Academic Press, London 1979; 1.
- Westbroek D L, Vriesendorp H M, van den Tweel J G, de Gruyl J, van Urk H. Influence of SD and LD matching on kidney allograft survival in unrelated mongrel dogs. *Transplant Proc* 1975; 7 (3): 427-9.