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THE EFFECTS OF CYCLOSPORINE ON BONE VOLUME AND BONE FORMATION RATE: A DOSE RESPONSE HISTOMORPHOMETRIC ANALYSIS IN THE RAT MODEL

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Abstract

The Effects of Cyclosporine on Bone Volume and Bone Formation Rate: A Dose Response Histomorphometric Analysis in the Rat Model

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Cyclosporine (CsA) is an immunosuppressive agent that has been associated with improved survival of vascularized solid organ and bone marrow transplantation, and recently has promise for the support of conventional or fresh, revascularized osteochondral allografts. The purpose of this study was to examine the effects of CsA on intact bone with the application of these findings to osteochondral allograft incorporation. Sprague-Dawley rats were randomly divided into six groups comprised of one control and five experimental groups. Each of the experimental groups received daily gavage administration of CsA dissolved in olive oil at one of the following dosages: 2.5, 5.0, 7.5, 10.0, and 15.0 mg/kg/day. The control group received plain vehicle. Each group received four weeks of treatment, with gavage five times per week. Rats were sacrificed on day 28 and tail vertebrae were removed, fixed in ethanol undecalcified, sectioned, and coverslipped in preparation for light and fluorescent microscopic analysis. A histomorphometric analysis of trabecular bone volume and bone formation rate was performed on the slides using an Osteoplan computer software system. Blood was collected periodically during the study and serum osteocalcin levels were analyzed by radioimmunoassay. Results revealed a statistically significant dose-dependent increase in trabecular bone volume in all except the highest dose group. There were no significant changes in the bone formation rate, as well as no statistically significant changes in serum osteocalcin levels within or between groups over time. Similarly, there were no alterations in creatinine clearance rates of animals in the highest dose group. In summary, the effects of cyclosporine in this rat model appear to be manifested by an increase in trabecular bone volume, unchanged bone formation rate supported by unchanged osteocalcin levels, and a resultant proportionate decrease in bone resorption rate. These findings are consistent with a low state of bone turnover and thus a less than optimal environment for allograft incorporation.

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Introduction

Cyclosporin A (cyclosporine, CsA) is an immunosuppressive agent with selective T helper cell suppression. This drug has been associated with improved survival of vascularized solid organ (kidney, liver, pancreas, lung, heart) and bone marrow transplantation, and may also prove useful in support of conventional or fresh, revascularized osteochondral allografts. In addition, there are at least five autoimmune diseases in which cyclosporine has been consistently efficacious in experimental studies. In spite of its potentially serious primary side effect of nephrotoxicity, the use of CsA in conjunction with azathioprine and prednisone has been associated with a reduced incidence of allograft rejection, acute bacterial and fungal infections, as well as reduced morbidity [12,24,51,67,70,74,82,84,89].

If cyclosporine is to play an important role in musculoskeletal transplantation, its effects on intact bone, fracture healing, and osteochondral allograft incorporation must be understood. As well, the skeletal effects of CsA therapy may have important implications for its use in non-osseous transplants. The complex interactions now being clarified between the immune system and bone cell populations further underscore the importance of defining cyclosporine's effects on bone.

The purpose of this study was to evaluate the dose dependent effects of cyclosporine at various dosages on the volume of trabecular bone, bone formation and bone resorption rates, and serum osteocalcin levels in a rat model.

Cyclosporine

Structure/Background Information

Cyclosporine is a neutral, lipophilic, cyclic endecapeptide with a molecular weight of 1203. All eleven amino acids have the L-configuration of natural amino acids except D-alanine in postion 8 and N-methylglycine in position 3, and the amino acids in positions 11, 1, 2, and 3 form a hydrophilic active immunosuppressive site (Figure 1) [2,50]. All natural and synthetic structural congeners with substitutions or deletions on the ring structure have less immunosuppressive activity than does CsA [97]. CsA is produced as a secondary metabolite from a fungal strain, *Tolypocladium inflatum* Gams, originally retrieved from soil specimens in the high plains area of southern Norway,

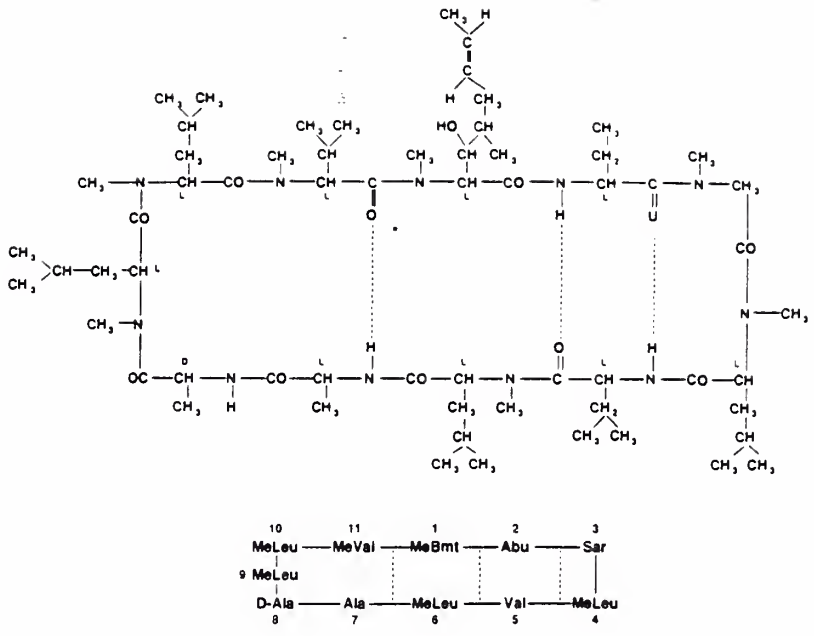


FIGURE 1: Atomic structure and alignment of amino acids of cyclosporine [from Kahan [50]]

Hardangervidda, by Sandoz scientists [81]. The number of solid organ transplant procedures performed annually has doubled since the introduction of CsA into clinical practice in December, 1983, [81] reflecting the enormous immunosuppressive effectiveness of this drug.

Pharmacokinetics

Cyclosporine is supplied as oral and intravenous preparations, stabilized with olive oil or castor oil vehicles, respectively. The relative bioequivalence of oral to intravenous doses is 1:3 [50]. There are threefold differences between individual patients in the average bioavailability of CsA (mean, 30 percent) and time to peak drug level (mean, 3.5-3.8 hours) as a result of slow, incomplete and variable gastrointestinal absorption [25,50]. Absorption is bile-dependent and adversely affected by biliary diversion, cholestyramine therapy, cholestasis, and slow gastric emptying; increased gastrointestinal mobility; steatorrhea; and reduced pancreatic exocrine secretion. The majority of CsA in the circulation is bound to lipoproteins of high, low, or very low density (34, 34, and 10 percent, respectively) and with chylomicrons [78]. Some drug circulates unbound but this "free" fraction does not correlate with the total blood level or with toxic side effects. Distribution is 33 to 47 percent in plasma, 4 to 9 percent in

lymphocytes, 5 to 12 percent in granulocytes, and 41 percent in erythrocytes [25]. The volume of distribution ranges from 4 to 8 liters per kilogram of body weight; the lack of correlation between volume of distribution and the presence of obesity suggests that factors other than the hydrophobicity of CsA are involved.

Elimination is primarily by metabolism, with a median half-life of 6.4 to 8.7 hours [50]. CsA is metabolized by the cytochrome P-450 hepatic enzyme system into 24 metabolites, 90 percent of which are excreted in bile, with about 6 percent excreted in urine [25,50]. Coadministration of drugs that interact with the cytochrome P-450 system may affect CsA metabolism. Cytochrome P-450 inhibitors known to increase CsA levels include ketoconazole, erythromycin, oral contraceptives, androgens, methylprednisolone, and calcium-channel antagonists; whether cimetidine and ranitidine inhibit CsA metabolism is controversial. Drugs known to induce the enzyme system and decrease cyclosporine levels include rifampin, phenobarbital, phenytoin, carbamazepine, valproate, and isoniazid. Children require more frequent and larger doses, as their clearance rate is 40 percent higher than that of adults. Drug clearance rates are slower in elderly patients and in patients with decreased levels of serum low-density lipoprotein triglyceride and cholesterol, and those with hepatic impairment. In the presence of elevated serum levels of bilirubin or alanine aminotransferase (ALT, SGPT), longer dosing intervals are necessary. In contrast, this change in administration pattern is not required with elevations of aspartate aminotransferase (AST,SGOT), lactate dehydrogenase, or alkaline phosphatase [100]. Because of the low level of urinary excretion, renal failure does not alter cyclosporine elimination.

Cyclosporine-Mediated Cellular Growth Regulation

Cyclosporine exerts varying effects on the growth of several cell types, and is currently known to inhibit helper and cytotoxic T cell activation and proliferation, possess antiparasitic and fungicidal activity, enhance IgE production and T-helper cell priming in antibody and in delayed hypersensitivity responses, reverse multidrug resistance to chemotherapeutic agents, and reduce or eliminate psoriatic skin lesions [81]. It is not currently known whether there exists a common molecular event that is controlled by CsA and responsible for these multiple actions. The most widely appreciated action of CsA has been its immunosuppressive action, and instead of suppressing the growth of all rapidly dividing lymphoid and myeloid cells as do cytotoxic drugs such as cyclophosphamide and azathioprine, cyclosporine exerts its primary effect in a more selective manner.

Immunosuppressive Actions

The most widely studied action of CsA is its ability to modulate the function of selective lymphocytic cell populations. The rejection of transplanted tissue are complex immune processes that involve the interaction and communication of several lymphocytic cell populations which, in turn, cause specific immunologic rejection patterns. The mixed lymphocyte reaction, in which cytotoxic T lymphocytes (CTLs) are generated and lymphokines are produced, is a widely used *in vitro* model of this process and is thus an informative system in which to study the cellular effects of CsA [81]. In particular, this model requires appropriate cell-cell collaboration resulting in the generation of cytotoxic T lymphocytes capable of recognizing and killing the specific target antigen, and it is thought that the CTL together with helper T cells and lymphokine release play a major role in graft rejection. The induction of CTLs requires several steps in addition to antigen recognition, including presentation of antigen by macrophages with subsequent production and release of interleukin-1 (IL-1), activation of precursor CTLs with acquisition of receptor for interleukin-2 (IL-2), activation of T helper lymphocytes with production and release of IL-2 (production of IL-2 is accentuated by IL-1), clonal amplification of activated CTLs by IL-2 which in turn directly attack donor tissue causing cellular rejection, and activation of suppressor T lymphocytes that modulate these responses [2,81]. The sites at which CsA exerts its inhibitory effect are thought to include the synthesis of IL-2 by T helper cells and to a lesser extent, the synthesis of IL-1 by macrophages/monocytes. CsA inhibition of IL-2 production suppresses the activation and subsequent proliferation of CTLs and T helper cell subsets of the T lymphocyte population, effects which are concentration-dependent [81]. In contradistinction, proliferation, in the presence of IL-2, of T cells that already express the IL-2 receptor on their plasma membrane, is resistant to CsA even at high concentrations. In addition, another population of T cells that is resistant to CsA inhibition is suppressor T cells. The inhibition of T helper cell and CTL activation and proliferation by CsA, while sparing inhibition of suppressor T cell growth, is thought to be the key cellular event by which CsA prevents rejection and graft-vs-host disease and suppresses autoimmune diseases [36].

An interesting and important observation is the Cd28 pathway of T cell activation and its CsA-resistant expression of the IL-2 gene [49]. This pathway is functional in helper T cells, but is not utilized by suppressor T cells which are resistant to CsA. That these *in vitro* studies were demonstrated in lymphocytes obtained from healthy young adults raises the question of whether such a pathway is operational in transplant

patients experiencing acute rejection episodes despite adequate blood concentrations of CsA.

The precise molecular mechanism of CsA's inhibition is not fully understood reflecting limited knowledge of the exact activation pathways in T lymphocytes. Evidence suggests that CsA is rapidly internalized through the plasma membrance by passive diffusion owing to its high hydrophobicity [2], and CsA-specific cell surface receptors have not been demonstrated. CsA does not affect the initial plasma membrane events, signal reception, transduction, and calcium influx (see Figure 2, Sites 1-4). Similarly, the alternate pathway which activates protein kinase C (Sites 5-6) is resistant to the drug. One theory of CsA's action implicates calcium-related cytoplasmic processes. Although CsA has only modest effects on calcium-channel permeability (Site 2) or intracellular calcium mobilization (Site 8), amino acids 2 and 3 of cyclosporine display the characteristic Type I-ß turn, which allows CsA to bind to calmodulin (Site 9) [50]. However, multiple inconsistencies limit the credibility of this theory that a receptor as ubiquitous as calmodulin can explain the T cell selectivity of CsA, including the fact that intracellular concentrations of CsA do not correlate with calmodulin content.

A second theory proposes that CsA inhibits the enzymatic generation of activation protein (or proteins) that mediates signal transduction from the cytoplasm to the nucleus (Site 10). The generation of the cytoplasmic activation signal that triggers DNA synthesis by resting lymphocyte nuclei is reduced by cyclosporine [50]. Cyclophilin, a cytoplasmic cyclosporine-binding protein, is one possible target of the cytoplasmic effect. This protein, a low molecular weight (17,737) basic protein binds CsA and its analogues in proportion to their immunosuppressive potency [35,50]. Cyclophilin has been shown to exhibit protein kinase activity and it has been proposed that this catalytic function may be involved in gene activation in T cells [35]. In addition, pig-kidney cyclophilin has sequence homology with peptidyl-prolyl-*cis-trans* isomerase [28]. This CsA-sensitive enzyme catalyzes cis-trans isomerization of proline imido peptide bonds, which may initiate folding of cytoplasmic proteins, thereby exposing DNA-binding domains [50]. Thus, CsA could block T cell activation at the level of protein kinase-mediated gene activation, as well as at the level of a proline isomerase-mediated inhibition of DNA binding.

A third potential site of action of CsA is the nucleus, as CsA selectively inhibits the capacity of nuclei isolated from human or rat lymphocytes, but not from liver, kidney, or tumor cells, to incorporate ^{3}H-labeled thymidine [50]. As well, the degree of impairment in nuclei isolated from the lymphocytes of different CsA-treated renal-transplant patients is inversely proportional to the intranuclear, but not serum, drug

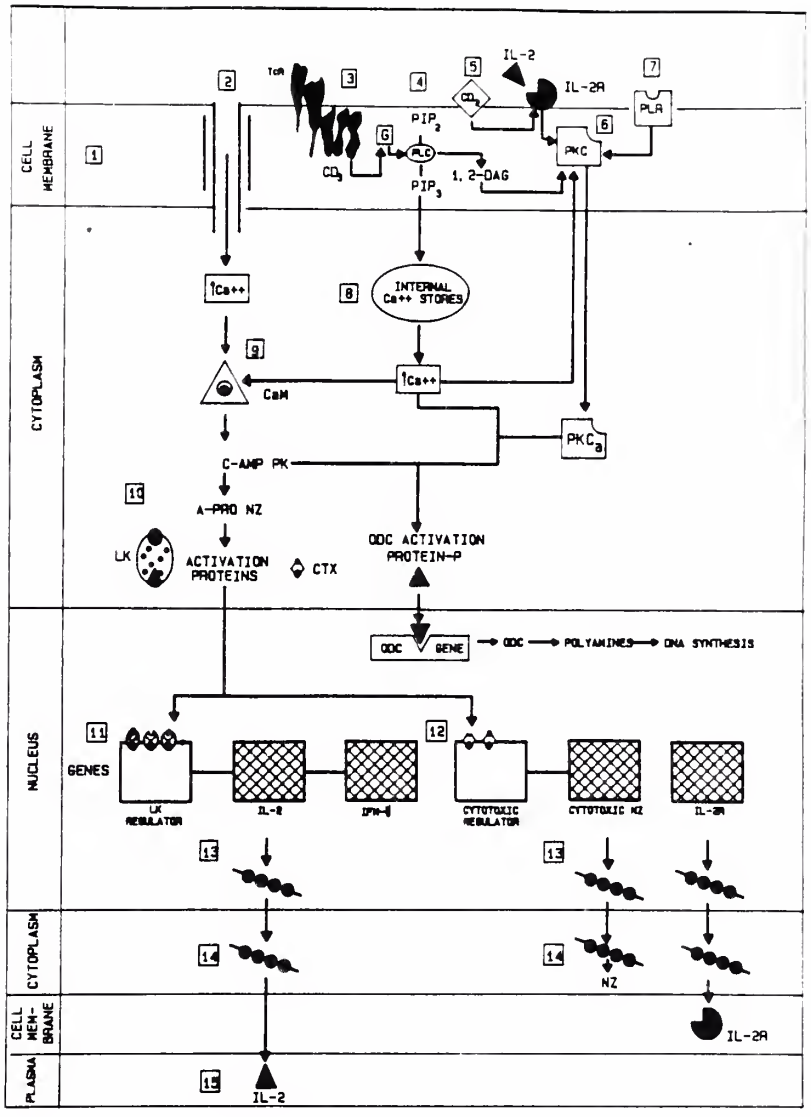


FIGURE 2: Cellular site of T-lymphocyte inhibition by cyclosporine [from Kahan [50]]

concentrations [21]. Thus, CsA potentially possesses inhibitory effects on transcriptional regulators (translocated cytoplasmic activation signals or independent intranuclear proteins), thus inhibiting the production of multiple lymphokines (Site 11), enzymes necessary for cytoaggression (Site 12), or both. At therapeutic concentrations, CsA disrupts lymphokine and proto-oncogene c-*myc* mRNA synthesis, but not that of interleukin-2 receptor mRNA (Site 14), and at high concentrations, CsA may have tissue-specific effects on mRNA transcription (Site 13). CsA has no effect on events that occur after gene activation; it does not inhibit constitutive production of

lymphokines by T cell clones, the stability or translation of lymphokine mRNA in the cytoplasm (Site 14), or the export of protein products (Site 15) [50].

Therapeutic Effects

In spite of its known toxicities, cyclosporine has drastically improved the clinical results of organ transplantation. It has significantly improved the initial survival of renal allografts, and has probably lengthened their long-term survival. CsA has substantially abrogated the impact of immunologic risk factors such as HLA mismatching and the absence of pretransplantation blood transfusions [50]. It has reduced morbidity, the incidence of allograft rejection, the period of initial hospitalization (from 6 to 3 weeks, or less), and the rate of readmission [67,82]. In addition, the absence of any myelotoxic effects permits faster alloengraftment [54].

It has been thought by numerous investigators that cyclosporine may have even greater utility in the treatment of autoimmune diseases, given their greater incidence and apparent high level of response. There are at least five autoimmune diseases which have been consistently responsive to CsA, although treatment remains investigational owing to its toxicity and relatively immediate relapses following discontinuation of therapy. In the most severe cases of psoriasis (defined as plaque-like psoriasis that covers at least 75 percent of the body and is unresponsive to other medications), CsA often produces a satisfactory if not complete remission. CsA offers a new approach to the treatment of the most severe cases of rheumatoid arthritis and related connective tissue disease, with responses comparable to those observed with other immunosuppressive agents. Most forms of autoimmune uveitis improve within a few weeks of treatment with CsA. In one three-year follow-up study, CsA was shown to be more effective than steroids or cytotoxic drugs in decreasing the inflammatory response and controlling the degree of vision loss from uveitis in Behcet's syndrome; however, it was less effective in improving systemic symptoms of Behcet's syndrome [25]. In Type I diabetes mellitus, remission from insulin dependence can be induced by treatment with CsA instituted within two months after the initiation of insulin therapy. In one study, insulin was not required for a full year in 50 percent of patients treated [25]. Minimal-change focal and segmental, membranous, and IgA nephropathies are all sensitive to CsA, although it is not clear whether the reduction in proteinuria reflects healing of the basement membrane or a decrease in the glomerular filtration rate [50]. There appears to be little efficacy of CsA in the treatment of myasthenia gravis, systemic lupus erythematosus, autoimmune thrombocytopenic purpura, and Crohn's disease.

Cyclosporine has an intrinsic antiparasitic effect in the rodent models of malaria, leishmaniasis, schistosomiasis, filiariasis, strongyloidiasis, and trichinosis [9]. There is some evidence suggesting an anti-toxoplasma activity of CsA, although no protective activity was observed in a rat model with *Pneumocystis carinii* infection. CsA's antiparasitic effects often seem to be host-mediated rather than directed against the parasite itself. It is not understood how the antiparasitic effects of CsA are mediated, and the clinical efficacy of CsA in parasitic infections remains unclear.

Recent work has revealed that CsA potentiates the effect of some cytostatic drugs *in vitro* and *in vivo*, in normal and tumor cells [25]. A clear potentiation of cytostatic drugs is seen in cells with acquired and pleiotropic drug resistance, or multi-drug resistance, compared with their sensitive counterparts. Thus, CsA may have a role as a resistance modifier in multi-drug resistant tumor. Unfortunately, the suggested explanations for this most attractive phenomenon are contradictory, and must await further investigations for a thorough evaluation of the therapeutic potential.

Extensive pharmacological studies on structure-activity relationships of cyclosporines began in the Sandoz laboratories in the mid-1970s with the goals of discovering a CsA derivative with: 1. much higher biological activity than CsA, 2. more selective spectra of activity than CsA, and 3. nonnephrotoxic effects while being equipotent to CsA. After much study, derivatives more potent than CsA itself and with acceptable side effects have not been found. (However, FK506, a chemically unrelated macrolide isolated from *Streptomyces tsukubaensis*, has been shown to have potent immunosuppressive activity at concentrations several hundredfold lower than CsA [34]. Studies of its clinical utility and side effects are ongoing.) Success has been quite limited in the search for a derivative with a narrow range of activity, with the most interesting profile being that of Val^2dihydro-CsA (DH-CsA-D). This derivative affects allograft rejection only weakly while suppressing a variety of cell-mediated responses similar to CsA. DH-CsA-D was selected for the treatment of autoimmune diseases because it proved remarkably active in an experimental autoimmune disease in the rat and Rhesus monkey [9]. Similarly, treatment with this analogue for a related disease in Lewis rats was found to be effective in preventing relapses after discontinuation of therapy, whereas a marked exacerbation of symptoms was noted after cessation of CsA therapy. Clinical efficacy of this derivative in a limited number of rheumatoid arthritis patients could not be conclusively established. Interestingly, DH-CsA-D had no obvious nephrotoxic effect, however due to other side effects, mainly hepatotoxicity and hypertension, clinical testing was abandoned [9]. Finally, the reduced nephrotoxicity of the best-known CsA derivative, Nva^2-CsA (CsA-G) initially raised hopes for a

nonnephrotoxic successor to CsA. In multiple *in vivo* tests, this derivative was usually found to be equipotent to CsA. Unfortunately, studies on toxicity have been rather confusing, yielding conflicting results in *in vivo* animal studies, most likely owing to the lack of standardized methods, the use of differing procedures, and strain and species variation [9]. The search for a less toxic alternative to CsA continues, including further evaluation of CsA-G in clinical pilot studies.

Therapeutic Monitoring and Dosage Regimens

The obvious goal in cyclosporine therapy is to achieve blood levels within a "therapeutic window" such that higher levels with resultant toxicity and lower levels with diminished graft rejection activity can be avoided. Because of the significant intra- and interpatient variability in the absorption and clearance of CsA, regular monitoring of blood (serum or plasma) levels is performed to guide dosage sufficient for the narrow therapeutic window. It is preferable to measure trough levels just prior to the next administration rather than peak concentrations, as the latter do not occur at uniform times after dosage and do not correlate with the occurrence of toxic complications [52]. In recipients of kidneys and bone marrow, trough serum levels above or below the therapeutic window correlate with episodes of graft rejection, nephrotoxicity, hepatoxicity, infection, and seizures. It is thought by several investigators that regular adjustment of CsA dosage to achieve and maintain trough levels within the narrow therapeutic range, rather than maintaining a fixed dose, may contribute to a further improvement of clinical outcome [50,81]. Assessment of pharmacologic variables prior to transplantation has also been used to predict the dosage of CsA likely to achieve post-transplantation target levels. CsA levels are measured either by high-performance liquid chromatography (HPLC) or radioimmunoassay (RIA). HPLC measures only CsA, whereas RIA measures the parent compound plus certain metabolites. RIA measurement is used in routine clinical monitoring, and HPLC is used for patients with abnormal liver function.

However, there are several problems with the monitoring of trough levels. Almost half of all renal transplant recipients have drug levels that are inconsistent with their renal status. In addition, the contribution of toxicity from CsA's metabolites remains an area of debate. As well, blood concentrations of CsA may prove to be a poor reflection of the true concentration at receptor sites, and thus may be relatively useless as a predictor of the adequacy of therapy.

The relationship between CsA concentration and graft rejection is clearly time dependent [54]. Based on the results of several studies comparing the clinical outcomes of transplant patients and trough blood concentrations of CsA, guidelines of recommended target ranges for CsA have been established (Tables 1,2).

TABLE 1: Recommended target ranges for cyclosporine concentrations (ng/ml) in whole blood [from Shaw [81]]

Transplant	First 3 to 6 months post-transplant	After 3 to 6 months post transplant
Kidney		
Living-related donor	150-250	80-125
Cadaver donor		
Serum creat >30 mg/L	80-120	80-125
Serum creat <30 mg/L	150-250	80-125
Liver		
Absence of toxicity	250-350	100-150
Presence of toxicity	80-120	100-150
Heart	350-450(day1-7) 250-350(day8-90) 150-250(day91-180)	100-150(>day180)

TABLE 2: Relationship between dose of cyclosporine and serum concentration as measured by HPLC and RIA after renal transplantation [from Keown et al. [54]]

Time after renal transplant	Cyclosporine dose, po (mg/kg/day)	HPLC (ng/ml)	RIA (ng/ml)
7 days	8.6 ± 1.0	63 ± 24	136 ± 30
14 days	14.6 ± 3.3	165 ± 39	209 ± 23
1 month	8.1 ± 2.4	67 ± 21	156 ± 20
2 months	7.6 ± 1.8	62 ± 21	99 ± 28
3 months	7.7 ± 2.2	40 ± 11	79 ± 17

In the first few weeks after transplantation, when the risk is greatest, rejection generally occurs when trough values are 170 ng per ml or less. A trough level around 200 ng per ml usually prevents rejection. With progressive host-graft adaptation, requirements gradually diminish such that by three months post-transplantion, trough levels of 50 to 125 ng per ml are usually sufficient to maintain quiescence. The initial intravenous doses of CsA in renal-transplant recipients, based on drug clearance

rates, have been calculated to achieve a steady-state concentration of 200 ng per milliliter, a level associated with a favorable balance between rejection episodes and nephrotoxic effects [50]. This correlates with a predicted intravenous dose of 3.8 mg per kilogram per day (mean), on the basis of the linear relationship between the intravenous dose and the steady-state concentration. The initial oral dose given to renal-transplant patients (generally 48 to 72 hours post-transplant) has been based on the linear relationship between the dose and the area under the serum concentration curve, but not on the trough value [50]. The oral dosage predicted to achieve an average serum concentration of 200 ng per ml displayed a bimodal pattern: in the majority of patients, the mean dose was 6.0 mg per kg per day (3.0 - 10.0), but nearly 30 percent of patients required 11 to 25 mg per kg per day. Another investigator suggests an initial intravenous dose of 4.0 mg per kg over 24 hours and an initial oral dose of 10.0 mg per kg in two divided doses, both of which most nearly approximate the 200 ng per ml target level in renal transplant patients. The persistent and significant variability in CsA levels despite pharmacokinetic studies and drug level monitoring highlights the challenge in achieving the "optimal" cyclosporine dosage.

Toxic Effects

Cyclosporine has multiple toxic effects, yet nephrotoxicity is generally considered to be the most important. Some degree of renal impairment occurs in 25 percent of renal transplant recipients, 38 percent of cardiac transplant patients, and 37 percent of liver transplant patients [25]. CsA causes at least a 20 percent reduction in renal function in almost all patients. The clinical course of patients with CsA-induced renal impairment is generally benign [50]. In rat models, dose-response studies have shown that CsA nephrotoxicity is expressed acutely as a dose-dependent increase in renal vascular resistance, a decrease in renal blood flow, and a decrease in glomerular filtration rate (GFR) [81]. In humans, as in rats, vasoconstriction is the characteristic hemodynamic event manifested by increased renal vascular resistance, decreased renal blood flow, decreased GFR, decreased urine output, and increased BUN-creatinine ratio [54,81]. Patients receiving CsA for the treatment of autoimmune disease with presumed normal renal function have markedly reduced glomerular filtration and effective renal plasma flow rates during the first week of therapy. A characteristic syndrome of increased serum creatinine concentrations, body weight, and blood pressure appears within one month [71]. The mechanism of vasoconstriction is not entirely clear. One hypothesis is that CsA potentiates vasoconstrictor hormone-induced, transmembrane

calcium-ion influx, causing an exaggeration in the contractile responses in arteriolar smooth muscle and mesangial cells. An interesting pharmacologic-clinical assessment of this theory is reflected in the current therapeutic trials of calcium channel blockers as preventive therapy in CsA-induced renal damage. The most favored hypothesis suggests that CsA influences the balance of the vasodilator prostacyclin and its vasoconstrictor antagonist thromboxane A_2 in renal cortical tissue [50]. CsA consistently augments thromboxane A_2 synthesis, although its effects on prostacyclin are controversial. The augmented thromboxane A_2 synthesis produces renal vasoconstriction as well as proliferation of vascular smooth muscle cells into the intima with accumulation of cholesteryl esters in macrophages, converting them into foam cells. A single theory to link renal vasoconstriction with immunosuppressive drug effects hypothesizes that CsA inhibits gene transcription of critical humoral regulators of endothelial-cell or mesangial-cell production of local vasodilator substances necessary for normal vascular and glomerular interactions [50].

Clinically, nephrotoxicity can be divided into acute, subacute, and chronic courses. The acute form begins during the first 7 days of treatment, the subacute form most frequently between 7 and 60 days, and the chronic form as early as 30 days. Approximately 30 percent of transplant patients treated with CsA will become oligoanuric within the first week of initiation of therapy. When the baseline GFR declines by more than 25 percent, the resulting renal impairment is classified as drug-induced nephrotoxicity, since this degree of dysfunction may not be completely reversible after cessation of drug therapy. It is likely that certain factors predispose to CsA-induced renal damage, including coadministered nephrotoxic drugs such as catecholamines, nonsteroidal anti-inflammatory agents, radiocontrast agents, amphotericin B, aminoglycoside antiobiotics, and trimethoprim plus co-trimoxazole; endotoxin; renal ischemic injury; and pre-existing kidney damage in non-renal-transplant patients [50,81]. Clinicians often attempt to mitigate the early renal toxicity of CsA by administering it by continuous intravenous infusion. Avoidance of early exposure to CsA can be achieved by prophylactically administering polyclonal antilymphocyte serum to all kidney transplant recipients or to those with oligoanuria. Alternatively, initial therapy with azathioprine and prednisone reduces the risk of viral infection associated with antilymphoctye serum but increases the risk of acute rejection. Another option is to administer diltiazem immediately after operation which exerts cytoprotective effects and reduces intrarenal vasoconstrictive sensitivity to humoral stimuli, particularly angiotensin. However, diltiazem inhibits CsA metabolism, and

thus markedly reduced doses of CsA must be employed, which by itself may explain the diminished renal toxicity.

Subacute nephrotoxicity, a reversible injury, improves relatively promptly when the dose of CsA is reduced. Its clinical significance in renal transplantation occurs when it causes confusion between CsA nephrotoxicity and allograft rejection. Diagnosis is guided by several algorithms, with the putative diagnosis usually established according to the response of rejection episodes to corticosteroids and of toxic events to reduction of the CsA dose [50].

The course of chronic nephrotoxicity is variable, occuring in approximately 10-15 percent of patients treated with CsA [50,54]. In one group of cardiac transplant recipients receiving long-term treatment with CsA in doses of 10 to 17.5 mg per kg per day, the nephrotoxicity was found to be progressive. In contrast, renal-transplant patients have relatively stable reductions in renal function. A change from CsA to azathioprine results in a high risk of graft loss, suggesting ongoing, subclinical rejection. The most common approach is to reduce the dose of CsA to 2.5 mg per kg and to add azathioprine, although controlled studies are necessary to document the actual benefit of azathioprine in this setting [50].

Hepatotoxicity occurs in 4 to 7 percent of transplant patients [25] and is manifested by cholestasis with hyperbilirubinemia and elevation of serum levels of aminotransferases in renal-transplant patients, particulary alanine aminotransferase. Neurologic side effects occur in about 20 percent of recipients of kidney and liver transplants, resulting in symptoms of tremor, burning palmar and plantar paresthesias, headache, flushing, depression, confusion, and somnolence. Gingival hyperplasia is a relatively common side effect, as is hypertrichosis of the face, arms, shoulders, and back, developing in at least 50 percent of renal transplant patients treated with CsA. Frequent reports of anorexia, nausea, or vomiting have been associated with the type of oral preparation, and are now known to be corrected by an alternate method of administration. CsA has no direct toxic effects on the structure and function of gastrointestinal mucosa, and the frequency of the common complication of acute pancreatitis is unchanged in the presence of CsA. There is a reduced incidence of bacterial and fungal but not viral infections as compared to regimens lacking CsA and using steroids, and this has been ascribed to a reduced requirement for steroids. All immunosuppressive agents increase the risk of malignancy, and lymphoma and epithelial malignancies develop in 0.1-0.4 percent of patients treated with CsA. There is an increased incidence of thrombosis of the arterial and venous limbs of renal allografts as well as systemic veins, and this is likely due, in part, to CsA's effects on thromboxane

A_2 release. CsA occasionally produces hyperglycemia, thought not to reflect any change in insulin's response to glucose administration. Serum prolactin is increased and testosterone is decreased by CsA, causing gynecomastia in men, and impairing spermatogenesis or sperm maturation in rats [50].

The *In Vitro* Effects of Cyclosporine on Bone

It has been determined that certain components of the immune system play an important role in the regulation of normal bone remodeling. This school of thought has as its foundation the development of two inter-related observations: one, the controversial hematopoietic origin of the osteoclast; and two, the close association between inflammation and bone resorption as noted in rheumatoid arthritis and periodontal disease, and the identification of cytokines of immunocompetent cell origin capable of activating osteoclastic activity *in vitro*.

Ever since the discovery of the osteoclast in 1873 by Kolliker [57] and despite intensive investigation using light microscopy, tissue culture, electron microscopy, microcinematography, autoradiography, parabiosis, quail chick nuclear markers, giant lysosomal markers in beige mice, Y chromosomes, bone marrow cell culture, and monoclonal antibodies, the origin of the osteoclast remains controversial. It is now generally agreed that osteoclasts are derived from mononuclear cells that originate in the bone marrow or other hematopoietic tissues and migrate to bone via the circulation. However, the exact identity of the mononuclear progenitor cell is unclear. Experiments by Walker in 1975 [92-95] seemed to confirm the hematopoietic origin of the osteoclast, in which he showed that parabiotic union (vascular connection via a flap of skin) between a normal mouse and an irradiated osteopetrotic mouse would restore normal bone resorption in the affected animal [92]. (Osteopetrosis, also known as Albers-Schonberg disease, is a congenital skeletal disorder caused by a failure of normal resorptive mechanisms in bone tissue resulting from a decrease in osteoclast activity.) Furthermore, Walker showed that injection of normal spleen or bone marrow cell suspensions into irradiated osteopetrotic mutants cured the osteopetrosis [93], whereas injection of cell suspensions from osteopetrotic animals into irradiated normal mice induced the disease [95]. Kahn and Simmons [53] and later Jotereau and Le Douarin [48] used the quail-chick chimera to establish evidence pointing to the conversion of hematopoietic cells into osteoclasts. This system is based on the morphologic differences between the nuclei of the Japanese quail and the chick, and a chimera between a quail limb and bone

rudiments grown on the chorioallantoic membrance of the chick embryo produced a proportion of osteoclasts with nuclei of both species. Using fluoride-inhibitable nonspecific esterase (NSE) as a marker of the cells of the mononuclear phagocyte (MNP) system and tartrate-resistant acid phosphatase (TRAP) as a lysosomal marker for osteoclasts, Baron et al. [6] have shown that mature osteoclasts and their mononuclear precursors share the NSE as well as a number of morphologic features. They conclude that mononuclear precursors of the osteoclast are members of the MNP lineage and differentiate early to acquire the properties of the osteoclast. However, the hypothesis that osteoclasts originate from monocytes/macrophages has been weakened by several observations, including a lack of Fc and C3 receptors and other macrophage-specific antigens on osteoclasts [13,39,80], and electron microscope differences between these two differentiated cell populations [13-19,43,44]. Thus, although the osteoclast's origin remains contested, most concur that a mononuclear hematopoietic-derived precursor cell, possibly from the MNP lineage or from a separate stem cell lineage, migrate to bone via the blood where they fuse to form multinucleated mature osteoclasts with the capacity for bony resorption.

It has been known for some time that one of the manisfestations of an inflammatory response is bony resorption. Through histological studies, there is a clear association between the inflammatory infiltrate, composed of all types of leukocytes, and osteoclast-mediated bone resorption resulting in localized bone loss. It was proposed initially by Horton et al. [40] that an elaborated factor is responsible for the activation of osteoclasts and he termed this substance osteoclast activating factor (OAF). A lymphocytic source for OAF was shown in studies which disclosed that a radio-sensitive cell (lymphocyte) and not a radio-resistant cell (macrophage) was producing OAF in peripheral blood leukocyte cultures [41]. The requirement for both lymphocytes and macrophages to produce OAF has been clearly demonstrated in the mouse model studying spleen cell suspensions, in addition to the inability of B cells to be productive of OAF [5]. These studies confirm reports in humans of an interaction between lymphocytes and macrophages necessary for the production of this lymphokine with its target cell the osteoclast [20,41,101,102]. Biochemical studies have revealed that OAF is a protein in the 9 to 18 kD range [22,42,59,66].

To better understand the interactions of the immune system and bone turnover, a review of the events in sequential bone remodelling is helpful. A concept of primary importance in this process is the three levels of interaction: bone, bone marrow, and systemic components, the latter which include endocrine organs, other organs of the body, and the blood compartment. It is crucial to understand that both local and systemic

factors contribute to bone remodelling, although systemic factors affect all levels of bone remodeling [5]. The actions of systemic factors are exerted through the regulation of calcium concentrations and by way of other organs affecting remodelling (e.g., parathyroid gland, kidney), and local factors, as a result of their effects on bone marrow and directly at the level of the bone compartment.

It is generally agreed that the process of bone turnover comprises a balance between resorption and formation, and that resorption and formation are closely coupled in space and time. During bone remodelling, formation occurs only after resorption and at the same site [30]. Baron et al. [5] describe the process of bone remodelling, involving a series of several interactive events. The site at which bone activation normally occurs is an endosteal resting surface. Resting surfaces are the most prevalent surfaces in bone, representing 70-80% of the endosteal surface in humans. At this site can be identified the possible local generators of the initial event: bone matrix, bone extracellular fluid, endosteal lining cells, and osteocytes. Some alteration, such as conformational changes in matrix proteins or changes in fixed surface charge, may modify local cell configuration to initiate the process. The second event involves the transduction of the initial message from the bone matrix, bone extracellular fluid, or local bone cells, via the bone marrow, into a cascade of events resulting in local amplification of the initial event and local differentiation of osteoclast precursors. Lymphocytes and mononuclear phagocytes (MNP's), being acutely responsive, are the best candidates for cellular transducers, thought to act in concert by local lymphocytic proliferation with elaboration of OAF and other lymphokines. Stromal cells also influence the cellular milieu by releasing factors grouped under the term CSF (colony-stimulating factors) which regulate the local proliferation and differentiation of the mononuclear phagocyte and granulocyte lineages, in addition to constituting the origin of other bone remodelling cells: osteoblasts, osteocytes, and lining cells. The additive effects of the first and second events brings about the local formation of committed osteoclast precursors (OCPs), arising either from already committed hematopoietic stem cells or from the proliferation and differentiation of uncommitted hematopoietic stem cells within the mononuclear phagocyte lineage. OCPs, once formed, migrate to the remodelling site and attach to the bone surface, and it only when they are attached to the bone surface and perhaps even after initiating bone resorption that the mononuclear OCPs begin to fuse into multinucleated mature osteoclasts [90]. The resorption process is considered to be initiated once there is formation of the multinucleated osteoclast. During this process, fusion, recognition, and attachment are ongoing. Fusion is asynchronous until the number of osteoclast nuclei reaches a maximum, at which time

nuclei progressively "disappear." It is thought that the loss of nuclei is explained by intracellular digestion of nuclei (although evidence is lacking) or a fission mechanism whereby mononuclear "post-osteoclasts," perhaps MNP's, are dissociated. Evidence for fission exists *in vitro* and *in vivo*, although the fate of the cells after fission is not clear.

The period following active resorption has been termed the reversal phase and is the transition zone between resorption and subsequent formation. At the end of resorption, mononuclear phagocytic cells appear in apposition to the bone surface. These cells arise either from chemotaxis and migration or osteoclast fission to form "post-osteoclasts," and may be involved in post-resorption scavenging and synthesis of future cement line components. These cells as well may be involved in the coupling phenomenon, although osteoclasts cannot be excluded from this role even though a period of a few days separates the phase of active resorption from formation. It may be significant that the osteoclast synthesizes and deposits a glucosamine-containing substance (lysosomal enzyme) at the bone surface [69] which may act as a chemotactic component attracting fibroblasts (possibly stromal cell components of the bone marrow) and macrophages. Thus, lysosomal enzymes and/or other components of the cement line may represent the local coupling factor responsible for recruiting osteoblasts. Once the surface of the previously resorbed bone is coated with cement line components, the post-osteoclastic MNP's are replaced by young osteoblasts, which form osteoid. After a delay of a few days, the osteoid undergoes mineralization. Osteocytes are formed when a proportion of osteoblasts are incorporated into the matrix, whereas other osteoblasts remain at the bone surface as flat lining cells characteristic of resting surfaces. The balance of activating and inhibiting local and systemic factors determines the length of the resting phase and thus the rate of bone turnover.

Because it has been suggested that immune cell derived regulators such as IL-1, IL-2, and OAF are involved in bone remodeling through calcemic hormones, studies have been undertaken to examine whether the immunosuppressive actions of cyclosporine would have an effect on the inhibition of bone resorption induced by calcemic hormones. Stewart et al. [85], responding to the knowledge that multiple factors stimulate bone resorption including parathyroid hormone (PTH), prostaglandin E_2, 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), and OAF, wondered if administration of CsA could suppress the resorption induced by these factors. Using the fetal rat limb bone culture system, and measuring the amount of ^{45}Ca released into the medium, they found that indeed CsA inhibited bone resorption in a dose-dependent fashion induced by each of the afore-mentioned substances, and found that the inhibition of resorption by CsA was in fact reversible upon removal of CsA. Interestingly, this inhibition of resorption was

observed with concentrations of CsA within the therapeutic range for human transplant patients. A related experiment by Klaushofer et al. [56],using the cultured neonatal mouse calvaria system, yielded similar results where CsA inhibited bone resorption induced by the same factors less OAF, as well as thrombin, IL-1, and bacterial lipopolysaccharide. A continuation of these studies by the same authors [87] examined the interaction between CsA's and calcitonin's inhibition of bone resorption, again in the neonatal mouse calvaria culture system. The results revealed that unlike CsA, calcitonin's inhibition of PTH stimulated bone resorption was not a lasting inhibition; rather, an "escape" from inhibition was observed after 24 hours of organ culture. Of note, pretreatment or coincubation with CsA allowed the "escape" phenomenon to be delayed to 48 hours.

The *In Vivo* Effects of Cyclosporine on Bone

Until recently, there have been few studies designed to examine the *in vivo* effects of CsA on bone homeostasis. The first documentation of osseous effects arose from incidental findings in CsA-treated renal transplant patients, where persistent elevations of alkaline phosphatase (aP) were observed [58]. In the European Multicentre Trial of CsA in renal transplantation, elevations of aP were attributed to hepatotoxicity [8], whereas Loertscher et al. were confident that the aP elevations were of an osseous origin, with suggestions of alterations in parathyroid hormone (PTH) as a contributory etiology.

The majority of *in vivo* studies of CsA's effects on bone have been conducted in the rat model by Epstein et al. [64,65,79]. This group performed histomorphometric analysis of rat tibiae, and has consistently found significant decreases in trabecular bone volume, and increases in bone formation rate and bone Gla protein (BGP, osteocalcin). A study comparing low dose CsA (7.5 mg/kg/day) with high dose CsA (15.0 mg/kg/day) on a short term (14 day) and long term (28 day) basis revealed significant decreases in bone volume in the high dose group on day 14 and in both the high and low dose groups on day 28. Osteoclast numbers (and thus bone resorption) were significantly increased in both low and high dose groups in the short and long term. Parameters reflecting bone formation rate including tetracycline-labeled surface and mineral apposition rate were significantly increased in the high dose group on days 14 and 28. In the low dose group, significant increases in tetracycline-labeled surface were seen on day 14, and increases in both parameters were present on day 28 [64]. In another protocol examining the

effects of withdrawal of CsA [79], animals were divided into three groups, the first group receiving vehicle (no CsA) until sacrifice of rats at day 14 or day 28, the second group receiving CsA (15.0 mg/kg/day) until sacrifice of rats at day 14 or day 28, and the third group receiving CsA (15.0 mg/kg/day) for 14 days followed by vehicle until sacrifice at day 28. In addition, some animals from each of the first two groups were studied until day 49. Results of this experiment were consistent with those of their previous studies in that there was a significant decrease in trabecular bone volume, even after 14 days of CsA administration (55% of control), and this diminished further after 28 days of CsA treatment (21% of control). In the third (withdrawal) group, continued diminution in bone volume was not observed after day 49 and, in fact, there was a suggestion of limited restoration, whereas in the second (CsA) group receiving CsA continuously, there was further decline in bone volume at day 49. As well, there were significant increases in bone formation rate, serum BGP levels, and osteoclast-like cells.

Another study by Epstein's group [65] examined the histomorphometry of rat tibiae in oophorectomized rats with and without the presence of CsA, recognizing that acute estrogen deficiency yields high turnover bone remodeling. Their results revealed that the group of animals receiving CsA had a significant decrease in trabecular bone volume and an increase in parameters of bone formation and bone resorption compared with control animals and oophorectomized animals without CsA.

Another group of investigators, Orcel et al., have studied the *in vivo* effects of CsA on caudal vertebrae of rats using histomorphometry [68]. Their protocol consisted of a 14 day course of CsA at an oral daily dose of 7 mg/kg, and they found a significant decrease in bone resorption as assessed by active resorption surface and number of tartrate-resistant acid phosphatase-labeled osteoclasts. In addition, bone formation evaluated by double-labeled surface and endosteal appositional rate was increased proportionately for the decrease in resorption, and thus the trabecular bone volume in treated animals was not significantly changed. Friedlaender et al. [29] using rats treated with a course of CsA at 7 mg/kg/day for 14 days with follow-up through 16 weeks, found that there was no significant change in trabecular bone volume. In addition, significant decreases in bone formation rate assessed by double-label were noted throughout the 16 week period of observation, whereas bone resorption parameters including the number of osteoclasts were reduced at 2 and 4 weeks but increased at 16 weeks. In related studies of CsA's effects on intact and fractured bone in the rat, Friedlaender's group found that a 14 day course of CsA at 7 mg/kg/day did not

significantly alter the biomechanical properties of fracture repair or intact bone turnover [96].

Changes in bone homeostasis in human patients treated with CsA have been studied by only a few investigators. This has been examined primarily in CsA-treated renal transplant patients. The potentially confounding factor in examining bone histomorphometry in renal transplant patients receiving CsA is the metabolic bone disease caused by renal osteodystrophy. This disease in manifested by osteoporotic signs and defects in bone formation and mineralization, and although it is thought that successful renal transplantation can mitigate this pathology, this point is not entirely clear [32,46]. Results of one review found abnormal (increased) numbers of osteoclasts and osteoblasts consistent with the "increasing repair hypothesis" [3]. In another study of nine patients [98], trabecular bone volume was normal but indices of osteoblast activity were increased with implicit increases in osteoclast activity and bone resorption.

With the introduction of cyclosporine into clinical practice, the prospect of using revascularized osteochondral allografts for segmental bone defects has been realistically entertained. Previous modalities of treatment in musculoskeletal reconstruction have included free vascularized bone autografts from donor sites of fibula, rib, and iliac crest. However, in instances of massive bone defects following tumor resection or trauma, autografts frequently are insufficient. Current treatment involves amputation, the use of synthetics, or reconstruction with large, preserved, non-vascularized allografts. It is known that fresh bone is strongly antigenic wheras cartilage is only weakly so and possibly immunopriveleged [27,31,38]. Experimentally, the intensity of response in animals relates directly to the genetic disparity between graft and recipient [99]. While hypothesized, this same circumstance has not yet been confirmed in humans, nor has a direct correlation between the presence of anti-HLA immune responses and bone allograft "success." Deep frozen osteochondral allografts have reduced antigenicity, and while associated with considerable clinical success, their use engenders a high incidence of serious complications [60,61,63,72,91]. Vascularized autografts of joints have been successfully transferred in recent years; however, clinical use is limited to the transfer of toe joints to the hand and of joints taken from amputated portions of extremities [26].

Despite the use of several drugs to suppress the immune response, vascularized allografts in animals have almost always been rejected within a few days. Now, with the advent of CsA, there has been dramatic improvement in the survival of a number of solid organ grafts, and there is evidence of success with osteochondral allografts, with two groups claiming long term survival of limb transfers in rats [37,55]. Several recent

studies in a number of animal models have assessed the survival of osteochondral allografts. In a study of revascularized rat knee allografts, Paskert et al. [73] examined the effects of continuous versus short-term (14 days) CsA (daily subcutaneous injection of 10 mg/kg) across either a weak or strong histocompatibility barrier. With a strong barrier, continuous CsA supported a successful graft, whereas short-term treatment, although without signs of rejection when followed 4 weeks, resulted in moderate rejection by 6 weeks. In the case of a weak immunogenetic barrier, both continuous and short-term CsA proved efficacious, without stigmata of rejection. In another investigation [45], rat hindlimb allografts were assessed in a similar way, with minor and major histocompatibility mismatch, receiving 16 days of subcutaneous CsA at a dose of 25 mg/kg/day. No evidence of allograft rejection was seen throughout the observation period of 16 weeks in the minor-mismatch group, whereas the major-mismatch group had a mean allograft survival time of 46.1 +/- 9.6 days.

Various immunosuppressive regimens, including cyclosporine, were compared by Rodrigo et al. [77] based upon their ability to inhibit the appearance of antibodies to donor strain lymphocytes in rat recipients of distal femoral devascularized osteochondral allografts using a lymphocytotoxicity assay. The protocol included varied doses and durations of CsA therapy: 25 mg/kg/day for 14 days, 25 mg/kg/day for 24 days, and 15 mg/kg/day for 28 days. A marked suppression of antibody response was observed (85, 100, and 85% reduction of antibody responses respectively after 6 weeks). These findings were superior to those of total lymphoid irradiation (30% positive antibody response), and courses of azathioprine/steroids or cyclophosphamide ($\geqslant$ 50%). Related experiments by the same authors consisted of 9 dogs divided into 3 autografts, 3 allograft controls, and 3 allografts treated with 25 mg/kg/day of CsA for one month. Despite the statistically insignificant number of animals, the 3 immunosuppressed allografts were significantly superior to the control allograft dogs in the measured (clinical, histologic, immunologic, biochemical, roentgenographic, and gross autopsy) rejection parameters.

In a similar study examining vascularized and non-revascularized dog knee joint allografts [26], the actual assessment of CsA (20 mg/kg/day) on the success of the transplantation was confounded by the coadministration of azathioprine. However, immunosuppression in vascularized allografts was successful for three of five animals (complications of failed vascular anastomosis and inadequate serum CsA levels resulted in the death of 2 animals), whereas both animals immunosuppressed for non-vascularized allografts had rapid rejection episodes.

Materials and Methods

Sprague-Dawley female rats weighing 200 ± 10 grams were randomly divided into six groups of twelve rats each, creating one control and five experimental groups. The animals were housed in AALAC approved animal housing facilities under normal circumstances and fed a diet of manufactured rat feed and water ad libitum. Cyclosporine (generously provided by Sandoz) was dissolved in pure olive oil and administered by gavage in 2 mg/ml solution, with the control group receiving plain vehicle. The five experimental groups each received the following dosage schedule of CsA: 2.5, 5.0, 7.5, 10.0, and 15.0 mg/kg/day. Each animal underwent four weeks of treatment, with daily gavage five times each week. Tail vein blood was collected under ether anesthesia on days 0, 3, 10, 17, and 24, and stored at -40 ^{o} C until tested. Using a metabolic cage, urine was collected on day 28 from animals in the 15.0 mg/kg/day dosage group and stored at -40 ^{o} C. Animals were weighed on days 0, 7, 14, 21, and 28. Calcein (DCAF, Merck) was given intraperitoneally at a dose of 30 mg/kg on days 13 and 27.

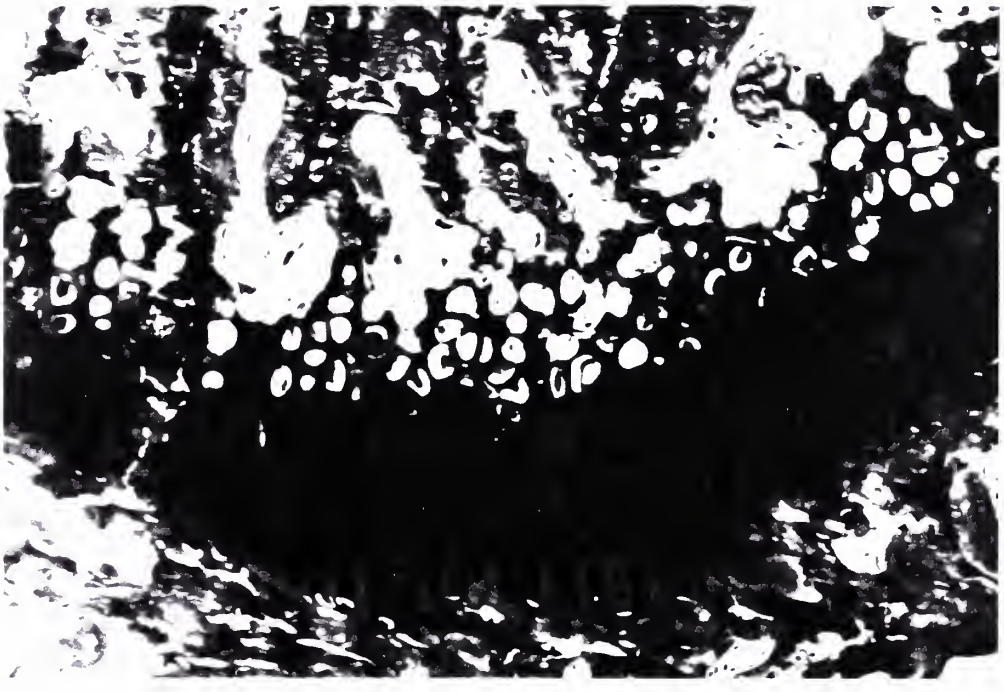
On day 28, animals were sacrificed and the third tail vertebral body was removed and fixed in 40% ethanol at 4 ^{o} C, and approximately 5 cc of blood retained from each animal. The tail vertebrae were dehydrated in a series of ethanol baths (70, 95, 100, and 100%) at 4 ^{o} C, cleared in xylene, and embedded in methylmethacrylate according to the technique by Baron et al. [4]. Vertebrae were sectioned on a Reichert-Jung microtome using a tungsten-carbide tipped blade into 4 micron and 12 micron sections. The 4 μ sections were deplastified and stained with toluidine blue 0 (Fisher) pH 3.7, cover-slipped with Permount (Fisher), and used for light microscope analysis. The 12 μ sections were left undeplastified and unstained, coverslipped with Uvinert, a UV inert glue, and analyzed with the fluorescent microscope. All slides were read on a Zeiss Osteoplan system using commercially available software (Osteoplan, program by H. Malluche) to quantitate trabecular bone volume and bone formation rate indices. Data were subjected to analysis of mean, standard deviation, and standard error, and t-test to determine significance.

Once thawed, tail vein serum was analyzed by radioimmunoassay to quantitate serum osteocalcin (BGP) levels. These data were subjected to analysis of mean, standard deviation, and standard error, with t-test and analysis of variance to assess significance.

Photographs 1-4 represent the microscope images of both light and fluorescent

slides, showing both trabecular bone (photos 1-2) and fluorescent single and double label (photos 3-4)

PHOTOGRAPH 1: Light microscope slide of trabecular bone



PHOTOGRAPH 2: Light microscope slide of growth plate

PHOTOGRAPH 3: Fluorescent microscope slide - double label

PHOTOGRAPH 4: Fluorescent microscope slide - single label

Results

The following represent data collected from light microscopy, fluorescent microscopy, and osteocalcin radioimmunoassay.

Light Microscopy

The light microscopic analysis consisted of assessing the following parameters from the sections of rat tail vertebrae: trabecular bone volume, surface volume, and the diameter of trabecular bone. Table 3 contains the values for these parameters for each of the animals in the control and five experimental groups. Table 4 compares the experimental values with control values, using a student's t-test for assessment of significance. Figure 3 represents a comparison of trabecular bone volume among the six groups.

There is a dose-dependent increase in trabecular bone volume, with a maximum reached at the 7.5 mg/kg/day dose, and a decrease in this parameter toward control with higher doses. There is a similar increase in trabecular surface volume with a maximum value also at the 7.5 mg/kg/day dose, and analogous decrease toward control value. The diameter of trabecular bone, as with trabecular bone volume and surface volume, is greater than control value for each of the experimental values. However, the peak value occurred at the 5.0 mg/kg/day dose, with a similar diminution of magnitude toward control value as the highest dose of 15.0 mg/kg/day is approached.

Fluorescent Microscopy

The following parameters were measured by fluorescent microscopy: mean distance between double label (MD-d), fraction of trabecular surface exhibiting double label (LAB-TS-d), fraction of trabecular surface exhibiting single label (LAB-TS-s), appositional rate per day (AR/D), bone formation rate: tissue level-surface referent (BFR ts), bone formation rate: tissue level-volume referent (BFR tv), and bone formation rate: cell level-surface referent (BFR cs). Table 5 contains the values for each of these parameters with mean, standard deviation, and standard error for each group. It should be noted that there are fewer observations than in the light microscopy data, since several slides had insufficient fluorescent label for accurate interpretation.

Table 6 is a comparison of experimental versus control values for each of the fluorescent microscopy parameters, including a t-test analysis for significance. There were no statistically significant differences between experimental groups and the control group for any of the values with the exception of increases in MD-d, AR/D, and BFR cs in the 10.0 mg/kg/day dose group. Figures 4-6 represent intragroup comparisons of the three measurements of bone formation rate.

Osteocalcin (Bone Gla Protein, BGP) Radioimmunoassay

Serum osteocalcin levels were assessed by radioimmunoassay (RIA) from tail vein blood. Table 7 lists the osteocalcin values averaged per group compared to that of control values, including mean, standard deviation, and standard error, with analysis of variance to assess significance. Figure 7 represents the osteocalcin levels over time for each group.

Table 8 represents creatinine clearance rates as assessed for animals in the 15.0 mg/kg/day dosage group compared to those values obtained from control animals.

There are clearly no statistically significant intra- or intergroup differences over time in osteocalcin levels. In addition, creatinine clearance rates of those in the highest dose CsA group are not reduced as compared to those of control animals.

TABLE 3: Light Microscope Data

GROUP	Animal	Bone Volume	Surface Volume	Diam Trab Bone
control	116	221	5372	210
	133	209	4217	252
	145	158	4123	195
	207	177	5380	167
	216	134	4698	146
	222	212	5396	200
	238	239	6986	174
	307	219	5432	206
	316	221	6131	183
	325	256	5200	251
	335	237	5864	206
2.5 mg/kg	404	244	6062	205
	417	256	6301	207
	435	265	5812	232
	448	285	5236	277
	504	263	6303	213
	516	213	4443	244
	525	248	5537	228
	535	246	5883	213
	604	281	6786	211
	616	265	5584	242
	6252	221	4981	226
	637	232	5854	201
5.0 mg/kg	705	241	4769	257
	714	252	4325	296
	727	228	6139	189
	737	202	5446	189
	805	294	6109	245
	817	282	6069	236
	838	219	4328	258
	907	286	7431	196
	912	317	8016	201
	928	261	6348	209
	935	260	6249	212
7.5 mg/kg	1005	259	6506	203
	1016	266	6571	206
	1025	274	5876	238
	1038	229	5136	227
	1107	289	6221	237
	1118	243	5653	219
	1125	193	5882	167
	1134	353	6992	257
	1208	265	6161	219
	1217	229	5678	205
	1222	325	8449	195
	1234	264	5271	255
10.0 mg/kg	1307	173	4231	209
	1316	240	6164	198
	1324	292	7235	205
	1335	210	5525	194
	1417	267	6182	220
	1424	244	5265	236
	1433	220	4479	250
	1504	269	6063	226
	1516	227	6507	177
	1525	255	6273	207
	1533	263	7464	179
15.0 mg/kg	1608	221	6481	173
	1614	203	5023	206
	1625	189	4930	195
	1637	247	5650	222
	1707	212	5684	189
	1716	186	4806	197
	1723	314	5664	282
	1734	243	6042	205
	1807	225	6034	190
	1817	198	5740	176
	1828	272	5900	234
	1832	197	5492	183

TABLE 4: Light Microscope Statistics

		Bone Volume	Surface Volume	Diam Trab Bone
cont vs. 2.5	cont value, avg	207.5	5345.4	199.1
	expt value, avg	251.6	5731.8	224.9
	t-value	3.52	1.27	2.27
	D.F.	21	21	21
	p < 0.05	yes	no	yes
	% change	21.3	7.2	13
cont vs. 5.0	cont value, avg	207.5	5345.4	199.1
	expt value, avg	258.4	5929.9	226.2
	t-value	3.33	1.35	1.89
	D.F.	20	20	20
	p < 0.05	yes	no	yes
	% change	24.5	10.9	13.6
cont vs. 7.5	cont value, avg	207.5	5345.4	199.1
	expt value, avg	265.8	6199.7	219
	t-value	3.48	2.38	1.64
	D.F.	21	21	21
	p < 0.05	yes	yes	no
	% change	28.1	16	10
cont vs. 10.0	cont value, avg	207.5	5345.4	199.1
	expt value, avg	241.8	5944.4	209.2
	t-value	2.3	1.52	0.85
	D.F.	20	20	20
	p < 0.05	yes	no	no
	% change	34.3	11.2	5.1
cont vs. 15.0	cont value, avg	207.5	5345.4	199.1
	expt value, avg	225.6	5620.5	204.3
	t-value	1.15	0.98	0.4
	D.F.	21	21	21
	p < 0.05	no	no	no
	% change	8.7	5.2	2.6

FIGURE 3: Bone Volume, Control and Experimental Groups

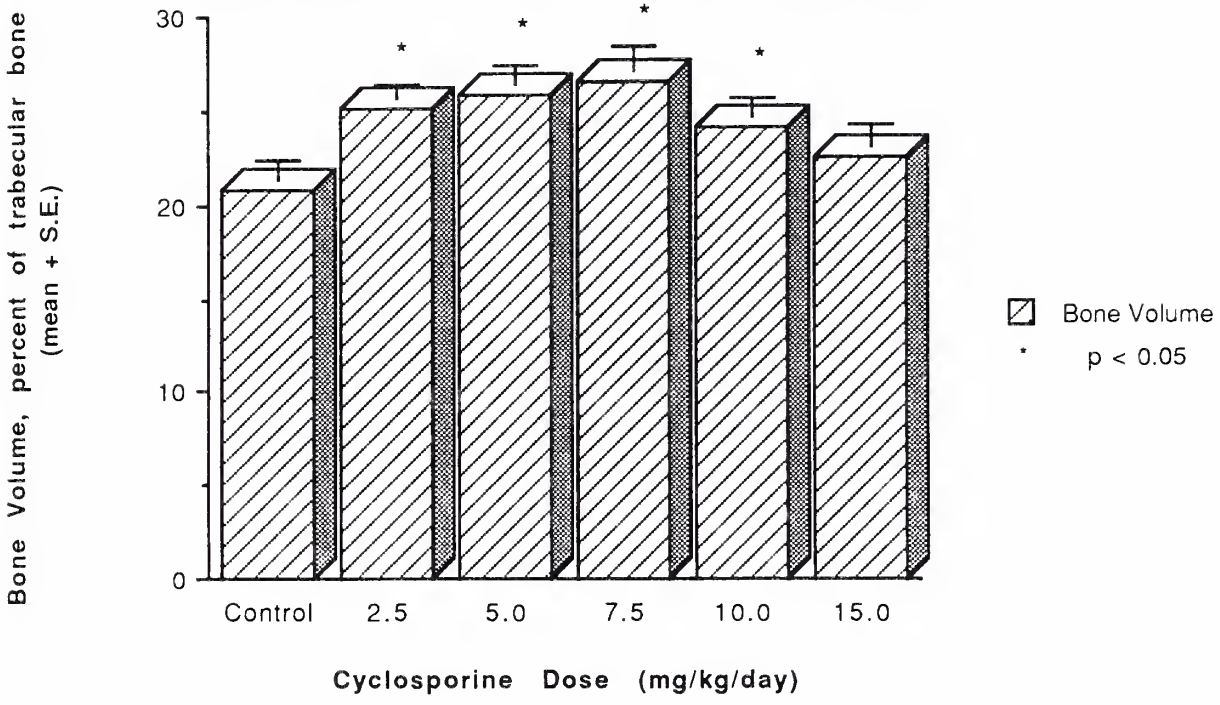


TABLE 5: Fluorescent Microscope Data

Group	Number	MD-d	LAB-TS-d	LAB-TS-s	AR/D	BFR ts	BFR tv	BFR cs
Controls	116	4.99	0.047	0.049	0.26	0.004	0.108	0.095
	133	6.332	0.178	0.16	0.33	0.021	0.432	0.12
	145	5.606	0.045	0.055	0.292	0.005	0.124	0.107
	207	6.955	0.05	0.081	0.363	0.007	0.2	0.132
	216	6.334	0.139	0.115	0.33	0.017	0.583	0.121
	222	6.989	0.117	0.133	0.364	0.016	0.394	0.133
	238	7.066	0.109	0.127	0.368	0.015	0.426	0.134
	307	6.397	0.099	0.061	0.334	0.012	0.299	0.122
	316	7.647	0.06	0.102	0.399	0.009	0.244	0.146
	325	7.585	0.095	0.126	0.395	0.014	0.277	0.144
	mean	**6.59**	**0.0939**	**0.101**	**0.344**	**0.012**	**0.309**	**0.125**
	S.D.	**0.838**	**0.0442**	**0.0377**	**0.0437**	**0.0056**	**0.15**	**0.0159**
	S.E.	**0.265**	**0.014**	**0.0119**	**0.0138**	**0.0018**	**0.0475**	**0.005**
2.5 mg/kg	404	8.731	0.104	0.072	0.455	0.017	0.428	0.166
	417	5.049	0.047	0.079	0.263	0.005	0.112	0.096
	448	7.221	0.052	0.042	0.377	0.007	0.13	0.137
	504	6.126	0.123	0.087	0.319	0.014	0.342	0.117
	516	7.603	0.126	0.111	0.396	0.018	0.378	0.145
	525	9.976	0.091	0.076	0.52	0.017	0.385	0.19
	616	8.304	0.111	0.091	0.433	0.017	0.367	0.158
	6252	8.052	0.196	0.088	0.42	0.03	0.676	0.153
	637	6.543	0.036	0.153	0.341	0.004	0.112	0.125
	mean	**7.512**	**0.0984**	**0.0888**	**0.392**	**0.0143**	**0.326**	**0.143**
	S.D.	**1.479**	**0.0498**	**0.0303**	**0.0772**	**0.0081**	**0.184**	**0.0281**
	S.E.	**0.493**	**0.0166**	**0.0101**	**0.0257**	**0.0027**	**0.0614**	**0.0094**
5.0 mg/kg	727	6.268	0.071	0.068	0.327	0.009	0.229	0.119
	737	5.793	0.078	0.091	0.302	0.009	0.231	0.11
	838	6.112	0.13	0.135	0.319	0.015	0.298	0.116
	mean	**6.058**	**0.093**	**0.098**	**0.316**	**0.011**	**0.253**	**0.115**
	S.D.	**0.242**	**0.0322**	**0.034**	**0.0128**	**0.0035**	**0.0393**	**0.0046**
	S.E.	**0.14**	**0.0186**	**0.0197**	**0.0074**	**0.002**	**0.0227**	**0.0027**
7.5 mg/kg	1005	6.136	0.063	0.057	0.32	0.007	0.184	0.117
	1025	6.257	0.13	0.124	0.326	0.015	0.33	0.119
	1038	7.352	0.061	0.041	0.383	0.009	0.191	0.14
	1107	7.709	0.053	0.041	0.402	0.008	0.167	0.147
	mean	**6.864**	**0.0768**	**0.0658**	**0.358**	**0.0098**	**0.218**	**0.131**
	S.D.	**0.785**	**0.0358**	**0.0396**	**0.0409**	**0.0036**	**0.0753**	**0.015**
	S.E.	**0.393**	**0.0179**	**0.0198**	**0.0205**	**0.0018**	**0.0377**	**0.0075**

TABLE 5: Fluorescent Microscope Data

Group	Number	MD-d	LAB-TS-d	LAB-TS-s	AR/D	BFR ts	BFR tv	BFR cs
10.0 mg/kg	1316	8.779	0.032	0.04	0.458	0.005	0.139	0.167
	1324	6.703	0.083	0.034	0.35	0.011	0.263	0.128
	1417	6.475	0.07	0.056	0.338	0.009	0.199	0.123
	1504	7.543	0.129	0.103	0.393	0.018	0.416	0.144
	1516	6.396	0.037	0.103	0.334	0.004	0.128	0.122
	1525	8.948	0.142	0.084	0.467	0.024	0.593	0.17
	1533	8.8	0.133	0.083	0.459	0.022	0.632	0.167
	mean	**7.663**	**0.0894**	**0.0719**	**0.4**	**0.0133**	**0.339**	**0.146**
	S.D.	**1.165**	**0.046**	**0.0268**	**0.0607**	**0.0081**	**0.211**	**0.022**
	S.E.	**0.44**	**0.0174**	**0.0108**	**0.0229**	**0.0031**	**0.0796**	**0.0083**
15.0 mg/kg	1614	6.298	0.09	0.063	0.328	0.011	0.266	0.12
	1625	6.325	0.074	0.076	0.33	0.009	0.232	0.12
	1637	8.095	0.099	0.064	0.422	0.015	0.35	0.154
	1707	6.795	0.07	0.083	0.354	0.009	0.242	0.129
	1723	6.954	0.076	0.068	0.363	0.01	0.181	0.132
	1734	6.018	0.07	0.125	0.314	0.008	0.2	0.115
	1807	8.153	0.105	0.067	0.425	0.016	0.437	0.155
	mean	**6.948**	**0.0834**	**0.078**	**0.362**	**0.0111**	**0.273**	**0.132**
	S.D.	**0.863**	**0.0145**	**0.0219**	**0.0449**	**0.0031**	**0.0907**	**0.0163**
	S.E.	**0.326**	**0.00547**	**0.00828**	**0.017**	**0.0012**	**0.0343**	**0.0062**

Index:
MD-d: Mean distance between double labels
LAB-TS-d: Fraction of trabecular surface exhibiting double labels
LAB-TS-s: Fraction of trabecular surface exhibiting single labels
AR/D: Appositional rate per day
BFR ts: Bone formation rate, tissue level - surface referent
BFR tv: Bone formation rate, tissue level - volume referent
BFR cs: Bone formation rate, cell level - surface referent

TABLE 6: Fluorescent Microscope Statistics

		MD-d	LAB-TS-d	LAB-TS-s	AR/D	BFR ts	BFR tv	BFR cs
cont vs. 2.5	cont value, avg	6.59	0.0939	0.101	0.344	0.012	0.309	0.125
	expt value, avg	7.512	0.0984	0.0888	0.392	0.0143	0.326	0.143
	t-value	1.69	0.21	0.77	1.69	0.74	0.22	1.71
	D.F.	17	17	17	17	17	17	17
	p < 0.05	no	no	no	no	no	no	no
cont vs. 5.0	cont value, avg	6.59	0.0939	0.101	0.344	0.012	0.309	0.125
	expt value, avg	6.058	0.093	0.098	0.316	0.011	0.253	0.115
	t-value	1.06	0.032	0.12	1.05	0.29	0.62	1.09
	D.F.	11	11	11	11	11	11	11
	p < 0.05	no	no	no	no	no	no	no
cont vs. 7.5	cont value, avg	6.59	0.0939	0.101	0.344	0.012	0.309	0.125
	expt value, avg	6.864	0.0768	0.0658	0.358	0.00975	0.218	0.131
	t-value	0.56	0.69	1.56	0.56	0.74	1.13	0.58
	D.F.	12	12	12	12	12	12	12
	p < 0.05	no	no	no	no	no	no	no
cont vs. 10.0	cont value, avg	6.59	0.0939	0.101	0.344	0.012	0.309	0.125
	expt value, avg	7.663	0.0894	0.0719	0.4	0.0133	0.339	0.146
	t-value	2.22	0.2	1.71	2.23	0.39	0.34	2.23
	D.F.	15	15	15	15	15	15	15
	p < 0.05	yes	no	no	yes	no	no	yes
cont vs. 15.0	cont value, avg	6.59	0.0939	0.101	0.344	0.012	0.309	0.125
	expt value, avg	6.948	0.0834	0.078	0.362	0.0111	0.273	0.132
	t-value	0.86	0.6	1.44	0.86	0.36	0.57	0.85
	D.F.	15	15	15	15	15	15	15
	p < 0.05	no	no	no	no	no	no	no

Index:

MD-d: Mean distance between double labels
LAB-TS-d: Fraction of trabecular surface exhibiting double labels
LAB-TS-s: Fraction of trabecular surface exhibiting single labels
AR/D: Appositional rate per day
BFR ts: Bone formation rate, tissue level - surface referent
BFR tv: Bone formation rate, tissue level - volume referent
BFR cs: Bone formation rate, cell level - surface referent

FIGURE 4: Bone Formation Rate, tissue level - surface referent

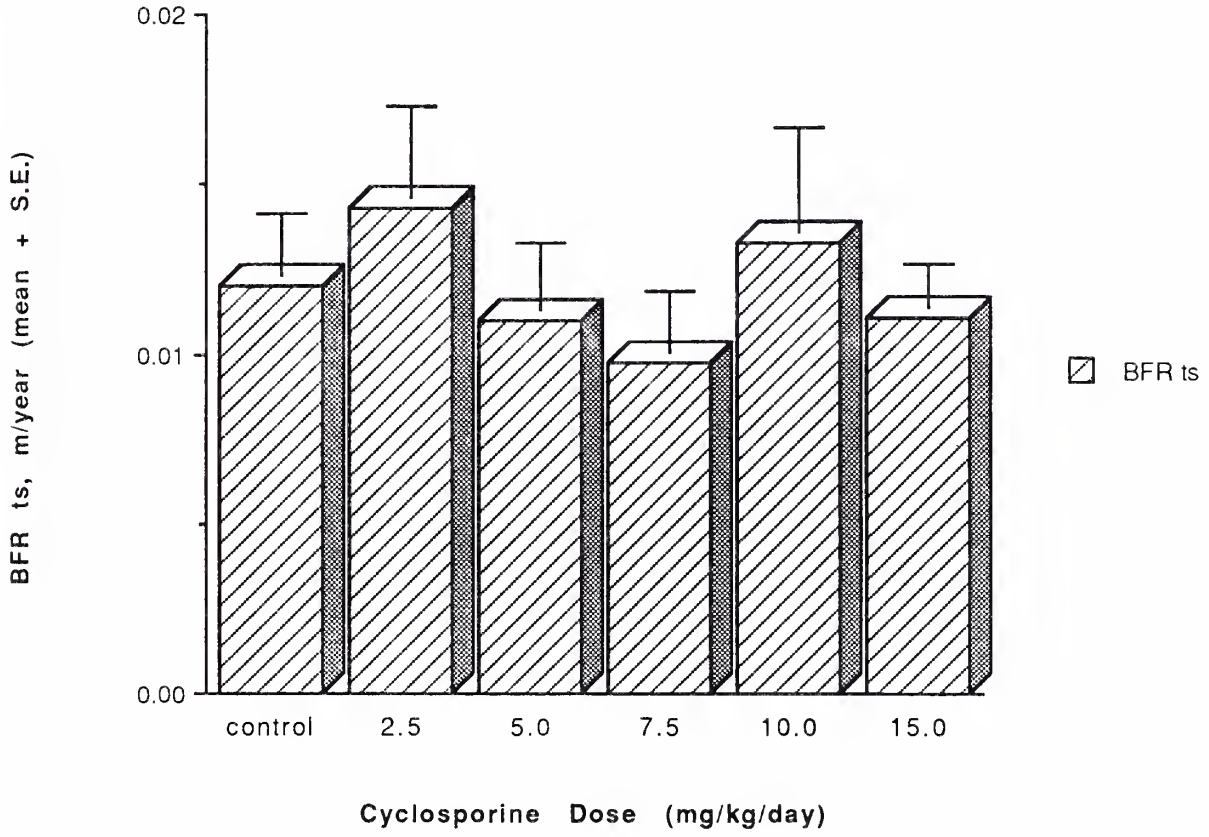


FIGURE 5: Bone Formation Rate, tissue level - volume referent

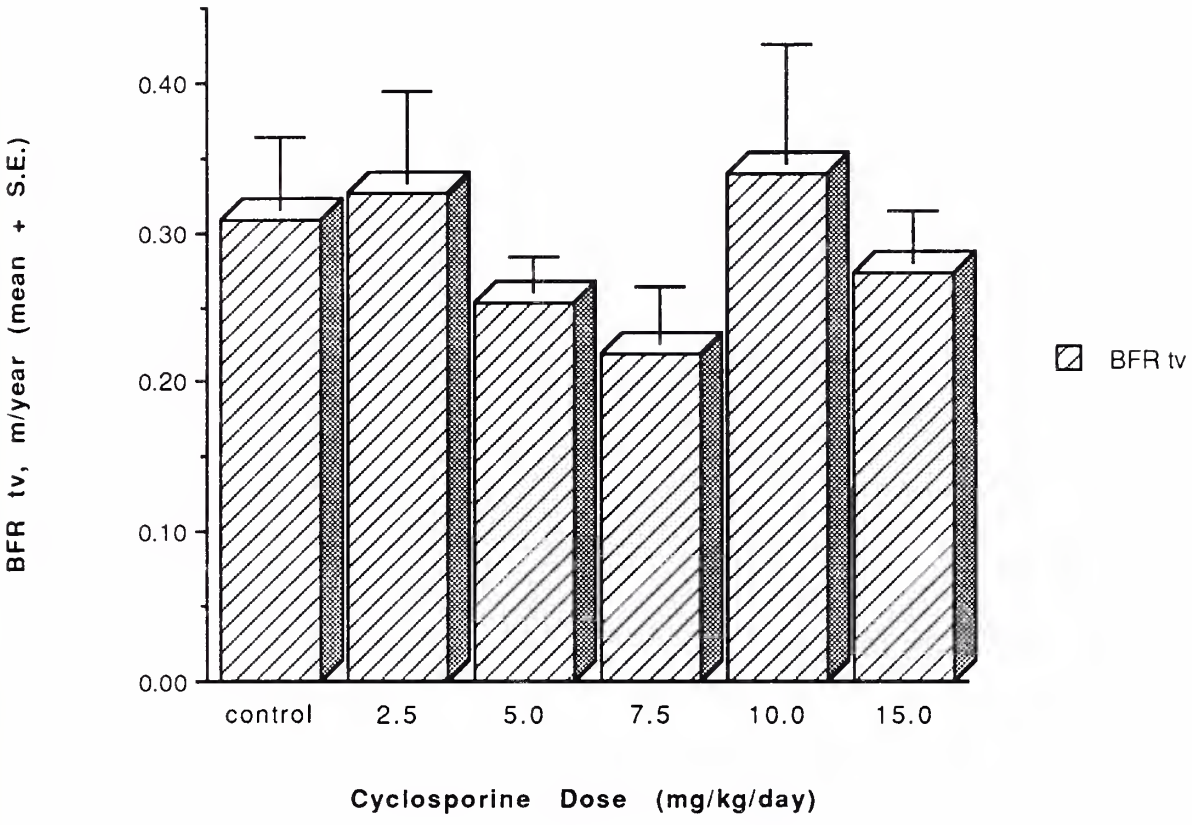


FIGURE 6: Bone Formation Rate, cell level - surface referent

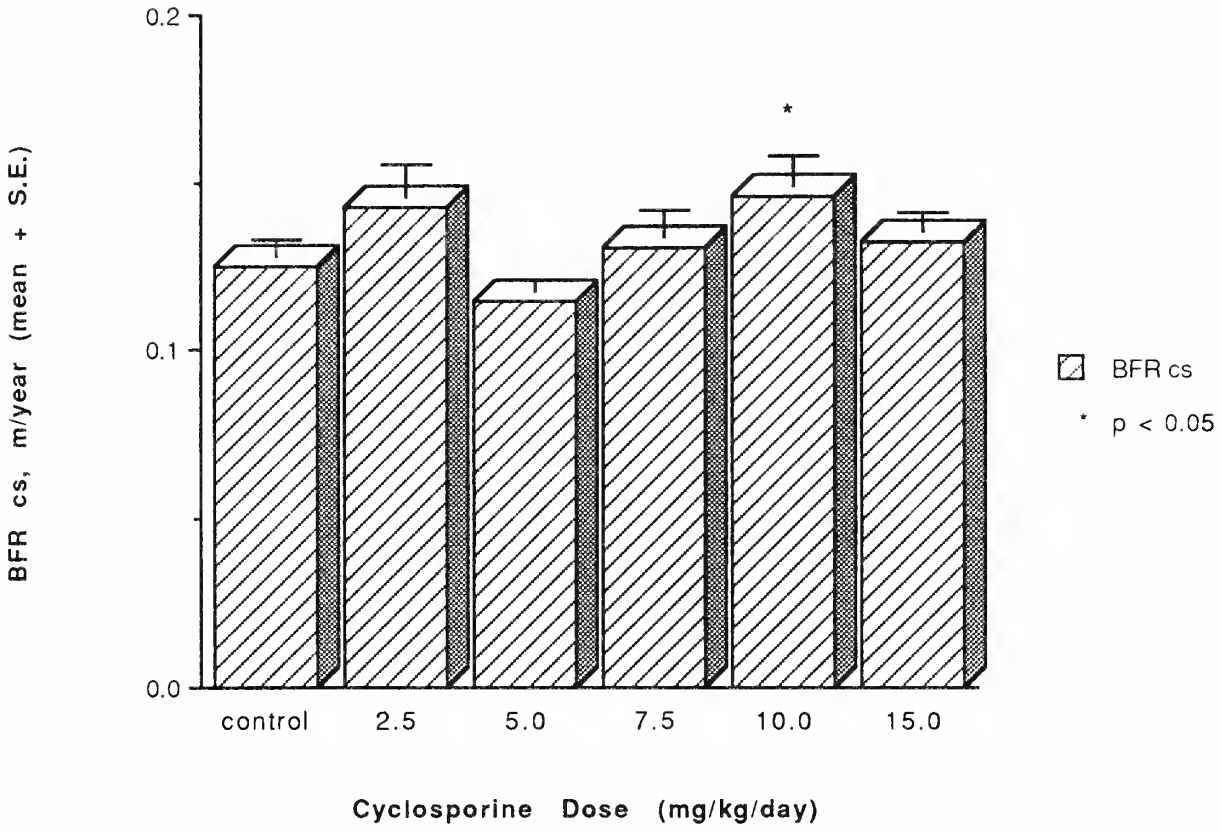


TABLE 7: Osteocalcin Data

Group	Animal	Osteocalcin level, ng/ml:	Day 0	Day 3	Day 10	Day 17	Day 24
Control	1-1		65.6	77.9	61.2	65.6	96.9
	1-3		69.7	61.2	69.7	63.8	73.8
	1-4		73.8	65.6	65.6	63.6	77.9
	2-0		68.9	71.4	68.9	135.3	81.6
	2-1		67.7	61.5	69.7	67.7	77.9
	2-2		69.7	63.6	63.6	77.9	56.1
	2-3		61.5	71.4	73.8	63.6	51
	3-0		76.5	82	66.3	77.9	69.7
	3-1		82	82	68.9	63.8	68.9
	3-2		82	102.5	63.8	82	71.4
	3-3		61.2	69.7	61.2	73.8	61.5
		Mean	**70.78**	**73.53**	**66.61**	**75.91**	**71.52**
		S.D.	**7.17**	**12.18**	**3.97**	**20.85**	**12.66**
		S.E.	**2.16**	**3.67**	**1.2**	**6.29**	**3.82**
2.5 mg/kg/day	4-0		56.1	89.3	61.2	61.2	66.3
	4-1		34.7	56.1	40.8	117.3	63.8
	4-3		38.8	51	61.2	102	53.6
	4-4		29.6	40.8	68.9	71.4	56.1
	5-0		76.5	53.6	61.2	102	68.9
	5-1		76.5	61.2	89.3	66.3	61.2
	5-2		76.5	81.6	89.3	107.1	132.6
	5-3		71.4	50	76.5	61.2	51
	6-0		68.9	56.1	66.3	-	68.9
	6-1		102	56.1	86.7	89.3	56.1
	6-2		86.7	71.4	96.9	76.5	81.6
	6-3		66.3	53.6	66.3	147.9	53.6
		Mean	**65.33**	**60.07**	**72.05**	**91.11**	**67.81**
		S.D.	**21.81**	**13.93**	**16.1**	**27.23**	**22.18**
		S.E.	**6.29**	**4.02**	**4.65**	**8.21**	**6.4**
5.0 mg/kg/day	7-0		68.9	68.9	86.7	43.9	66.3
	7-1		71.4	66.3	76.5	66.3	68.7
	7-2		68.9	68.9	81.6	81.6	66.3
	7-3		71.4	81.6	81.6	68.9	76.5
	8-0		71.4	76.5	102	81.6	81.6
	8-1		63.8	56.1	68.7	53.6	68.9
	8-3		68.9	107.1	102	102	127.5
	9-0		68.9	96.9	68.7	102	71.4
	9-1		96.9	96.9	117.3	96.9	127.5
	9-2		71.4	86.7	102	96.9	127.5
	9-3		71.4	63.8	68.7	66.3	71.4
		Mean	**72.12**	**79.06**	**86.69**	**78.18**	**86.69**
		S.D.	**8.53**	**16.2**	**16.63**	**20.02**	**26.58**
		S.E.	**2.57**	**4.88**	**5.01**	**6.04**	**8.01**

TABLE 7: Osteocalcin Data

Group	Animal	Osteocalcin level, ng/ml:	Day 0	Day 3	Day 10	Day 17	Day 24
7.5 mg/kg/day	10-0		61.2	61.2	61.2	81.6	81.6
	10-1		89.3	76.5	63.8	68.9	53.6
	10-2		71.4	96.9	107.1	81.6	53.6
	10-3		81.6	76.5	102	68.9	51
	11-0		43.4	63.8	63.8	66.3	63.8
	11-1		63.8	76.5	76.5	56.1	47.9
	11-2		43.4	107.1	86.7	71.4	47.9
	11-3		61.2	56.1	76.5	66.3	53.6
	12-0		86.7	86.7	68.9	53.6	47.9
	12-1		56.1	89.3	86.7	71.4	53.6
	12-2		107.1	53.6	76.5	81.6	45.9
	12-3		76.5	68.9	68.9	63.8	50
		Mean	**70.14**	**76.09**	**78.22**	**69.29**	**54.2**
		S.D.	**19.09**	**16.55**	**14.87**	**9.2**	**9.82**
		S.E.	**5.51**	**4.78**	**4.29**	**2.66**	**2.83**
10.0 mg/kg/day	13-0		79.1	79.1	74	68.9	79.1
	13-1		71.4	76.5	79.1	89.3	66.3
	13-2		74	104.6	66.3	84.2	66.3
	13-3		76.5	84.2	68.9	74	-
	14-1		81.6	89.3	117.3	76.5	68.9
	14-2		84.2	76.5	68.9	102	84.2
	14-3		79.1	76.5	96.9	66.3	89.3
	15-0		84.2	76.5	89.3	66.3	61.2
	15-1		74	81.6	68.9	76.5	66.3
	15-2		102	81.6	79.1	79.1	76.5
	15-3		66.3	84.2	61.2	71.4	102
		Mean	**79.31**	**82.78**	**79.08**	**77.68**	**76.01**
		S.D.	**9.3**	**8.35**	**16.44**	**10.78**	**12.83**
		S.E.	**2.8**	**2.52**	**4.96**	**3.25**	**4.06**
15.0 mg/kg/day	16-0		68.9	66.3	79.1	74	66.3
	16-1		79.1	74	132.6	140.3	89.3
	16-2		71.4	76.5	79.1	81.6	68.9
	16-3		68.9	71.4	74	66.3	61.2
	17-0		68.9	104.6	81.6	66.3	63.8
	17-1		79.1	81.6	112.2	74	74
	17-2		74	84.2	79.1	76.5	76.5
	17-3		84.2	74	79.1	63.8	66.3
	18-0		76.5	76.5	61.2	79.1	66.3
	18-1		68.9	76.5	71.4	76.5	79.1
	18-2		58.7	71.4	71.4	61.2	58.7
	18-3		74	68.9	81.6	76.5	68.9
		Mean	**72.72**	**77.16**	**83.53**	**78.01**	**69.94**
		S.D.	**6.66**	**9.98**	**19.54**	**20.65**	**8.53**
		S.E.	**1.92**	**2.88**	**5.64**	**5.96**	**2.46**

FIGURE 7: Osteocalcin (OC) Levels, Days 0-24

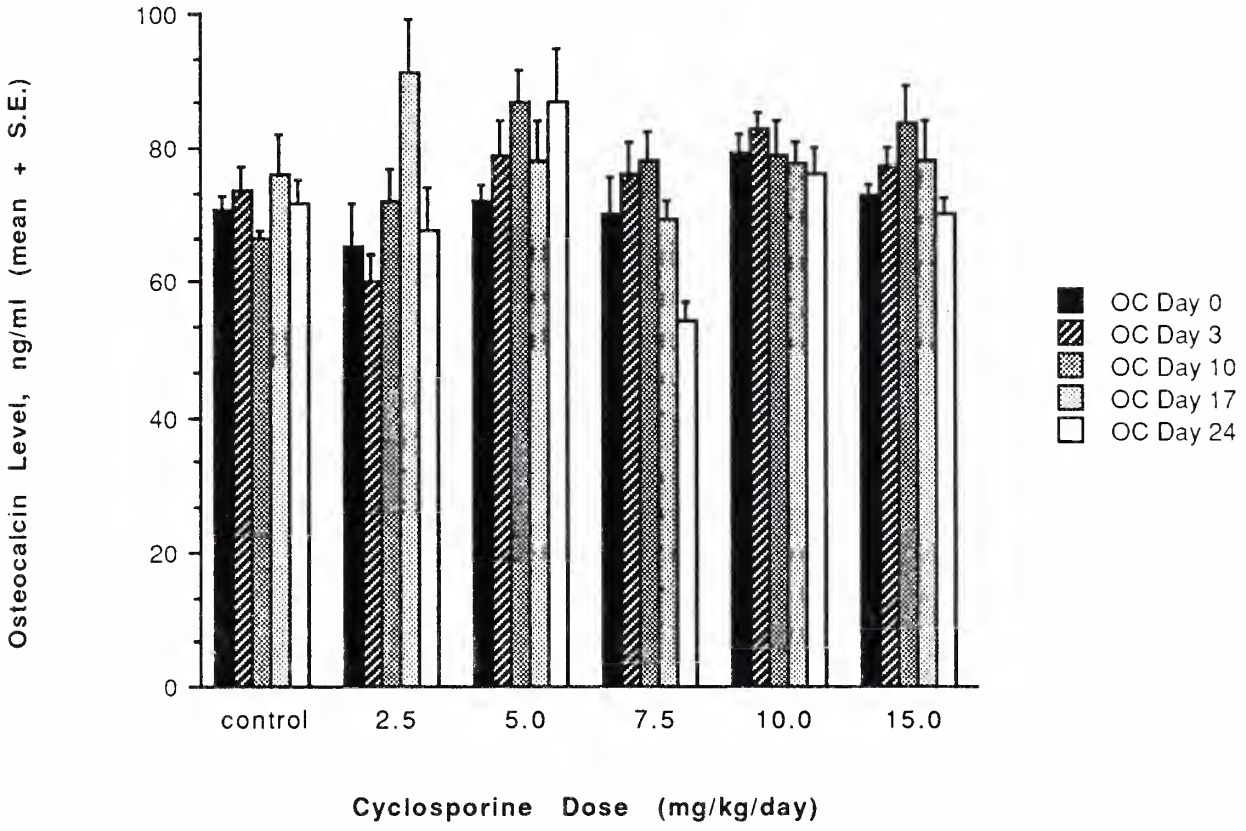


TABLE 8: Creatinine Clearance - 15.0 mg/kg/day dose vs. control

	Creat clearance (15.0 mg/kg/day)	Creat clearance (control)
	38.4	15.6
	17.5	12.8
	49.5	21.3
	10.7	16.1
	22	15.2
	12.9	25
	18.4	27.9
	11.8	14.8
	18.8	13.9
	33	21.3
	31.7	12.3
	33.6	
Mean	24.86	17.84
S.D.	12.19	5.22

Discussion

The objective of this project was to evaluate the effects of cyclosporine on intact bone homeostasis, as reflected in the trabecular bone volume and bone formation rate of tail vertebrae in the rat model. Five doses of CsA, ranging from 2.5 mg/kg/day to 15.0 mg/kg/day, were employed, reflecting the range of dosage used in a variety of clinical regimens for transplant rejection prophylaxis and the treatment of autoimmune disease. Because of similarities between the rat and human with respect to the physiology of bone turnover, this rodent system has frequently been used as a model for evaluation of skeletal effects of therapeutic agents.

This project is of interest for two major reasons. First, it is important to understand the osseous effects of cyclosporine since solid organ transplant patients are committed to a life-long course of treatment with this medication. It is valuable to demonstrate any alterations in bone homeostasis caused by this immunosuppressive drug. Second, based upon the success of CsA in solid organ transplantation, there is optimism about the success of vascularized bone allografts and perhaps enhanced behavior of conventional non-revascularized osteochondral allografts currently used in multiple clinical settings. Consequently, it is crucial to examine the systemic effects of this immunosuppressive agent on intact bone as well as any deleterious effects CsA may have on osteochondral or composite tissue allografts.

In review, the results of this study revealed a dose-dependent increase in trabecular bone volume peaking at the intermediate dose of 7.5 mg/kg/day with no significant changes in bone formation rate as measured by double fluorescent label and no significant changes over time or dose in serum osteocalcin levels as assessed by radioimmunoassay. The significance of these data centers on the three following interrelated and normally linked parameters: trabecular bone volume, bone formation rate, and bone resorption rate. The net result of rates of formation and resorption dictate the bone volume, with increased bone formation and decreased bone resorption producing increased bone volume, and vice versa. Likewise, the speed of bone turnover is dependent on the bone formation rate and bone resorption rate. In this study, the bone volume is statistically significantly increased over control value at all doses except for 15 mg/kg/day, and bone formation rate is unchanged as compared to control values at all doses. Therefore, the bone resorption rate must be decreased in proportion to the

increases observed in trabecular bone volume. Similarly, with decreases in bone resorption rate, and no change in bone formation rate, bone turnover is also reduced.

The process of bone remodelling has been described as a system of multiple interactions between bone, bone marrow, and systemic components. It is well known that this process involves a close coupling between resorption and formation, and that it is possible that lymphocytes and mononuclear phagocytes represent the cellular transducer responsible for the amplification of bone resorption activity at the cellular level. The lymphokine osteoclast activating factor (OAF), elaborated by T helper lymphocytes, is thought to be intimately involved in the initiation and amplification of the bone resorption process. In addition, interleukin-2 (IL-2) elaboration by T helper cells and interleukin-1 (IL-1) production by mononuclear phagocytes likely play a routine role in bone remodelling mediated through the calcemic hormones [5]. The mechanisms by which the calcemic hormones (parathyroid hormone [PTH], prostaglandins, and 1,25 vitamin D_3 metabolites) induce bone resorption are not entirely clear. It is possible that immune cell factors such as IL-1, IL-2, and OAF increase the sensitivity of target bone cells to the resorbing actions of PTH, $1,25(OH)_2D_3$, and prostaglandins [23]. In addition, PTH affects the proliferation of bone marrow hematopoietic stem cells [74], mononuclear leukocytes have high affinity PTH receptors [75], lymphocytes have functional receptors for calcitonin [62], and interactions between lymphocytes-macrophages and $1,25(OH)_2D_3$ have been reported [1,88].

Cyclosporine is thought to exert its inhibitory effects on resorption by reducing synthesis of IL-2 by T helper cells and IL-1 by mononuclear phagocytes [81]. The inhibition of IL-2 production in turn suppresses the activation and proliferation of cytotoxic T lymphocytes and the feedback enhancement of additional T helper cell populations. Thus, the elaboration of other lymphokines, including OAF, will be diminished by CsA in therapeutic concentrations. It is tempting to speculate that CsA-induced inhibition of OAF, IL-1, and IL-2 contributes to the inhibition of osteoclast and thus bone resorption activity. The observation that two non-immunosuppressive analogues of CsA, cyclosporine H and cyclosporine F, do not inhibit bone resorption *in vitro* lends support to the hypothesis of lymphocytic involvement in bone homeostasis [85]. However, the fact that CsA inhibits bone resorption induced by a broad range of stimuli lends support to a more nonspecific action of CsA.

A brief review of calcium and bone metabolism is helpful. An important function of bone is its storage of calcium, magnesium, phosphorus, sodium, and other ions, and its contribution to calcium homeostasis. The hormonal and ionic interactions in calcium homeostasis involve a close interrelation between parathyroid hormone (PTH),

calcitonin (CT), and vitamin D, and the transport of calcium and phosphate by renal, intestinal, and bone cells. PTH acts directly on bone and kidney and indirectly on intestine through its effect on the synthesis of 1,25-dihydroxyvitamin D to increase serum calcium, and in turn, the concentration of PTH is closely regulated by a feedback mechanism dependent on the concentration of ionized serum calcium. Calcitonin is a physiologic antagonist to PTH and is a potent hypocalcemic, hypophosphatemic hormone, which acts to reduce bone resorption and increase renal calcium clearance. With adequate exposure to sunlight, vitamin D is actually a hormone. When skin is exposed to sunlight, 7-dehydrocholesterol is transformed into vitamin D_3. In the liver, vitamin D_3 is metabolized to 25-hydroxyvitamin D [25(OH)D] by vitamin D-25-hydroxylase, and this is loosely regulated by product feedback mechanism. In the kidney, 25(OH)D is metabolized by a number of enzymes, with 25(OH)D-a-hydroxylase producing 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], the only known important metabolite of vitamin D. Metabolic influences operate on this hydroxylase to closely regulate the circulating concentration of $1,25(OH)_2D$. Under physiologic conditions, $1,25(OH)_2D$ is believed to be synergistic with PTH in bone resorption. When serum calcium falls below normal, secretion of PTH is enhanced, resulting in increased production of $1,25(OH)_2D$.

Thus, there is a complex interaction between ionic and hormonal factors which exists to regulate serum calcium concentration, and this regulatory mechanism is highly dependent on bone. In certain clinical settings, the assessment of mineral metabolism is often complicated. Disturbances in mineral metabolism caused by renal failure can produce secondary hyperparathyroidism, where excessive production of PTH occurs because of partial resistance to the metabolic actions of the hormone. In progressive kidney disease, retention of phosphate because of reduced renal capacity to excrete phosphate and reduced concentrations of $1,25(OH)_2D$ lead to hypocalcemia which in turn leads to reduced skeletal responsiveness to PTH and the development of secondary hyperparathyroidism. Since calcium deposition in bone is critically dependent on the availability of phosphate, the retention of phosphate in renal disease facilitates calcium entry into bone and thus contributes to the hypocalcemia and elevations of plasma PTH. The bone disease in patients with secondary hyperparathyroidism is termed renal osteodystrophy, and concomitant osteomalacia (vitamin D deficiency) and osteitis fibrosa cystica (excessive PTH action) may also be seen. The significance of bone disease, therefore, in patients treated with cyclosporine is clear: the osseous effects of CsA may be obfuscated by chronic renal failure which necessitated kidney tranplantation, or concurrent renal disease in patients receiving CsA for other reasons. For the purposes

of this study, then, knowledge of the state of mineral metabolism is helpful in interpreting the histomorphometric analysis of bone. Calcium, phosphate, vitamin D and PTH levels are not reported in this study, but in animals with no preexisting renal disease and with no changes in serum creatinine or creatinine clearances, it is safe to assume that there were no renal-induced alterations in bone and mineral metabolism. It can also be assumed, then, that any alterations in bone turnover as evaluated in this study were effected by cyclosporine.

A simple explanation of the *in vitro* findings would suggest that there was inhibition of osteoclastic activity with resultant increases in bone volume, and without compensatory increases in osteoblastic activity, perhaps mediated through alterations in humoral factors (cytokines). There are, however, no *in vitro* studies reflecting CsA's effects on bone formation rate. In applying these findings to the phenomenon of coupling between osteoclastic bone resorption and osteoblastic bone formation, it would follow that CsA would effect a decrease in the bone formation rate, yielding a state of low bone turnover. It is possible, however, that CsA interferes with this coupled mechanism which may explain the discrepancy in some of the *in vivo* results. It is not fully clear whether an assessment of bone remodelling *in vivo* is entirely valid since there may be disturbances in this coupled homeostatic mechanism.

Another explanation of the *in vitro* data is a direct toxic effect of CsA on osteoclasts. Epstein et al. suggest that this is unlikely because if true, one would also expect evidence of nephrotoxicity, which was not observed in their study or the present study. However, it is possible that in the absence of nephrotoxicity, osteoclasts exhibit a lower tolerance to the toxic effects of CsA. Studies of this nature have not been found in the literature.

It is interesting to note that hypomagnesemia and decreased bone magnesium levels have been observed during CsA therapy [7]. This is of importance because the bone histological changes described with Mg deficiency are reductions in both bone resorption and formation [46]. Although the present findings reveal only a decrease in bone resorption, it is interesting to consider the potential contribution of hypomagnesemia to these results. However, presuming the hypomagnesemic effects of CsA are mediated through effects on the kidneys, and considering the absence of changes in renal function assessed at the highest dose of CsA, it is unlikely that this could contribute to the findings of this study.

The results of this *in vivo* study differ from the few other similar studies reported to date. Epstein et al. [63,64,78] found decreased trabecular bone volume, increased bone formation rate, and increased serum osteocalcin levels. Orcel et al. [68]

discovered no change in trabecular bone volume and synchronous increases in bone formation and resorption rates. Friedlaender et al. [29] found no change in trabecular bone volume, decreased bone formation rate, and reduced bone resorption rate. Thus it is clear that the current findings of increased trabecular bone volume, decreased bone resorption rate, and unchanged bone formation rate represent yet another finding in the reported effects of CsA on rat bone histomorphometry.

The osteopenia and increased bone formation rate observed by Epstein et al., consistent with a high bone turnover state, is found in the absence of changes in ionized calcium, phosphate, or PTH levels. This, in combination with unchanged creatinine levels, suggests that the effects of CsA are mediated at a cellular level via cytokines. These observations contrast with those *in vitro*, and these authors suggest that this may be explained by the fact that the whole animal is more representative of the multiple interactions between CsA and the cytokine system. Furthermore, they contend, their findings compare favorably to the bone histology from human renal transplant patients receiving CsA [3], as well as with the evidence of increased alkaline phosphatase of osseous origin reflecting increased bone turnover [58]. In addition, Wilmink et al. [98] provide evidence of increased bone turnover with no changes in trabecular bone volume in CsA-treated renal transplant patients. The issue of renal osteodystrophy must be discussed in conjunction with these findings. After successful renal transplantation, patients may recover from metabolic bone disease; however, defects in bone formation, mineralization, and signs of osteoporosis may persist and even get worse [32,46]. Thus, in these clinical examples of increased bone alkaline phosphatase and parameters of bone turnover, it is difficult to interpret these findings as resulting from an effect of CsA, persistence of metabolic bone disease, or a physiologic response to recovering osteodystrophy.

In contrast, Orcel et al. have shown decreases in bone resorption which are supported by the *in vitro* findings, yet they observed increases in bone formation rate yielding a net absence of change in trabecular bone volume, again suggesting the hypothesis of a disrupted coupling phenomenon. They further suggest that the inhibitory effects of IL-1 on osteocalcin production by osteoblasts shown *in vitro* to be antagonized by CsA [82] may explain their findings of increased bone formation. These investigators found no evidence of renal toxicity or any modification of vitamin D metabolites, and thus a toxic effect of CsA to explain decreased osteoclast activity is unlikely.

The findings of Friedlaender et al. to date have shown no changes in trabecular bone volume, decreases in bone formation rate, and proportionate decreases in bone resorption rate. Thus, they observed a low bone turnover state which persisted 16

weeks after the initiation of the two week course of CsA. However, coupling between resorption and formation appears to be preserved. Thus, it remains uncertain whether the inhibition of resorption by CsA observed *in vitro* by Stewart et al. and *in vivo* by Friedlaender et al. is explained by an effect of CsA or is complicated by a nonphysiologic uncoupling of bone resorption and formation.

The current findings of decreased bone resorption and no change in bone formation are consistent with the *in vitro* findings. As well, they support a disorganization in this coupling phenomenon. It remains unclear why there is such variability between investigators in the determination of the histomorphometric effects of cyclosporine in the rat. The present findings are consistent with a low bone turnover state as are those of Friedlaender et al. In contrast, Orcel et al. found essentially control values of bone turnover, whereas the results of Epstein's group support a high bone turnover state. In an application of these findings to an experimental design of osteochondral allograft survival, it would seem that the latter results would be optimal. A state of high bone turnover would intuitively support a more vigorous phase of bone remodelling and likely improve the success of the allograft. In regards to technique, there are minor variations in the dose and duration of therapy with CsA, however in each protocol presented here, anywhere from 7.0 to 15.0 mg/kg/day of CsA for 14 to 28 days has been used. This is not drastically different from the range of CsA of 2.5 to 15.0 mg/kg/day for 28 days used in this study. Epstein et al. have mentioned a difference in technique as perhaps contributing to the different results. Their group performed histomorphometry on rat tibiae and not on rat tail vertebrae as was done in this study. Thus the issue of CsA differentially affecting weight-bearing versus non-weight-bearing bone is to be considered. One would conjecture that the degree of bone remodelling would be higher in bone exposed to the stress of weight, although how CsA would enter into this equation and either support or interfere with the preexisting histomorphometry is unclear. The age of the animals used differs among these studies. Epstein's group employed 250 gram, 300 gram, and 340 gram rats. Orcel et al. used weaning rats, Friedlaender et al. used 200-250 gram animals, and the current study employed 200 gram rats. It is possible that the bone remodelling sequence differs at different stages in the maturing rat. Thus, the varied results could represent a reflection of a differential effect of CsA on the bone remodelling sequence dependent on the age of the animal. The results of this study and those of Friedlaender et al. and Orcel et al. are the most similar, and have been derived from younger animals. In contrast, Epstein's findings, while entirely consistent in several studies, were derived from older, more mature animals.

The major shortcomings of this experiment involve the inadequate numbers of fluorescent slides used to assess values for bone formation rate. Several of the slides had such poor fluorescent label that analysis of them for double label would yield erroneous and non-reproducible results. This investigator is satisfied that the reading of the few well-labeled fluorescent slides is representative of actual findings, yet it remains unknown whether more appropriate numbers of slides would alter the bone formation rate results. Despite the relative paucity of fluorescent slides for analysis, the consistent absence of changes in osteocalcin levels in all groups over time is supportive of these results.

Future studies are necessary to confirm or challenge these results. They should involve different durations of therapy with CsA and a comparison of histomorphometry between weight-bearing and non-weight-bearing bones. Bone histomorphometric studies of cyclosporine therapy in a rat model of renal failure and in rats immunosuppressed after renal transplantation would be most informative.

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