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Cyclosporin A Does Not Affect the Absolute Rate of Cortical Bone Resorption at the Organ Level in the Growing Rat

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Abstract. The weanling rat, an animal model of rapid bone turnover, was used to evaluate the effects of various doses of cyclosporin A (CsA) on various bones during different time periods. Sprague-Dawley male rats were extensively prelabeled with ^{3}H-tetracycline during 1–3 weeks of age. At 4 weeks of age, four groups of rats were given daily subcutaneous injections: vehicle or CsA—low dose (10 mg/kg), intermediary dose (20 mg/kg), or high dose (30 mg/kg) for 7, 14, or 28 days. Three different whole bones—the femur (low turnover), scapula (moderate turnover), and lumbar-6 vertebra (high turnover) were harvested intact at 4, 5, 6, and 8 weeks of age. The whole bones were assayed weekly for total dry defatted weight, calcium mass (formation), and loss of ^{3}H-tetracycline (bone resorption) following treatment with CsA. Serum CsA levels, calcium creatinine, and alkaline phosphatase were measured weekly. Significant decreases in serum calcium and alkaline phosphatase were observed at 1 and 2 weeks, and were normalized by 4 weeks of treatment. No significant changes in serum creatinine were noted. For all three doses of CsA, no effect was observed on the absolute rate of cortical bone resorption of three different, whole bones over three time periods. Body weight and bone formation in treated animals was significantly smaller in a dose- and time-related fashion compared with control animals at sacrifice. However, compared with the *initial* control animals, body weights and bone masses of the final treated animals were much larger, suggesting that the smaller bone masses were due to insufficient growth and slow gain in bone mass. Our isotopic data demonstrate that CsA has no effect on the basal rate of bone resorption and decreases rate of bone formation, as observed globally at the whole bone level. Bone measurements at the organ level may lead to different interpretations from those observed at the tissue level.

Key words: Cyclosporin — Bone mass — Resorption — Formation — Conservation.

The use of cyclosporin A (CsA) to prevent rejection of allograft tissue has stimulated the field of transplantation biology [1]. Despite these successes, the exact metabolic mechanisms of action are being gradually defined *in vitro* and *in vivo*. Recent studies [2] on CsA demonstrate that the *specific* suppression of T lymphocyte function occurs by inhibiting the activation of certain genes for the production of growth-inducing cytokines that may activate or inhibit bone metabolism. The inhibitory effect of CsA on the gene activation of cytokine production in T cells is *not* observed with B lymphocytes or stimulated accessory cells (human monocytes, monocytes in polyclonal systems, dendritic cells) that are required for T-cell responses [3]. Nor are stimulated macrophages, synovial fibroblasts, or a human fibroblast cell line directly inhibited by CsA [2, 4].

Studies *in vitro* have demonstrated that CsA specifically inhibits T lymphocyte-derived messengers such as interleukins (IL-1 alpha, IL-2, IL-3, IL-4), gamma-interferon, tumor necrosis factor-alpha, and GM-colony-stimulating factor [5, 6]. Some of these cellular messengers have been reported to influence bone resorption and formation in fetal bones *in vitro* [7]. The *in vitro* finding of a common regulatory pathway between the immune system and bone cells [7–9] have led to the hypothesis that CsA may also influence bone resorption and formation [10].

Studies *in vitro* [11] using fetal bones in tissue culture found that CsA inhibited the release of calcium from neonatal mouse calvaria in response to stimulation by calcemic hormones such as parathyroid hormone and vitamin D_3. When appropriate cells were treated coordinately with CsA, potential regulators such as cytokines (interleukin-1, IL-1) and prostaglandin E_2 (PGE_2), thrombin, and bacterial lipopolysaccharide [11] also inhibited calcium release. This inhibition was lost following removal of CsA from culture media. Earlier work by Stewart et al. [10] also found that CsA decreased the effect of PGE_2 and osteoclast activating factor (OAF) on calcium release in a dose-dependent, reversible manner [10, 12]. Neither group [10, 11] showed any effect of CsA on bone resorption in control cultures that were not previously stimulated by hormones or cytokines.

Experimental data *in vivo* on the effects of oral CsA in the rat appear contradictory. Movsowitz et al. [13] concluded that there was increased bone resorption and formation in the proximal tibial metaphysis from treated young rats when evaluated by histomorphometric techniques (osteoclast number and tetracycline-labeled surface). It was concluded that oral CsA (7.5 and 15 mg/kg body weight) produced an overall osteopenic state in a dose- and time-dependant fashion [13] and that young rats (9 weeks old) lost much more trabecular bone than older rats 9 months old [14]. They also found that the metaphyseal osteopenia disappeared when CsA was discontinued [15]. In contrast, Orcel et al. [16] examined the effects of oral CsA (7 mg/kg body weight) on the seventh caudal vertebra of weanling rats using histomorphometric techniques as well as blood and urine biochemical markers. They found increased active formation surfaces after double tetracycline labeling and concluded that bone re-

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sorption was inhibited (decreased number of osteoclasts and decreased resorptive surfaces) [16]. The net effect of these two surfaces on the whole bone (as shown by bone ash and calcium content) was no effect of bone mass.

It is well known that in young and metabolic mature rats trabecular bone represents a small percentage (ca. 20%) of the total skeleton and cortical bone 80%. During growth of long bones, approximately 20% of the new mass is derived from linear endochondral bone formation and 80% from circumferential intramembranous bone formation. Thus, it may be difficult to extrapolate from data on trabecular bone changes to those at the organ level and vice versa. Whereas most previous experiments [13–16] have reflected *regional* trabecular bone in the growing metaphysis, our experimental design reflects mainly cortical bone at the organ level (whole bone). The variation in the previously reported results *in vivo* raised the following question: What are the *global* effects of CsA on rates of bone resorption and formation in whole bones when determined in a physiologic or pharmacologic setting *in vivo?*

The use of young, growing (weanling) rats provides a resilient animal model undergoing rapid bone turnover (formation and resorption) that is sensitive to external stimuli [17]. Long bones with low turnover (femur) and axial bones with high turnover (vertebral and scapula) were used [17] for quantifying rates of cortical bone resorption by measuring the weekly loss of ^{3}H-tetracycline from whole intact bones and weekly gain in bone mass. The purpose of this study was to determine the effect of three different doses of CsA (10, 20, 30 mg/kg body wt.) upon bone resorption and formation *in vivo* using a radioactive tetracycline method [18] in growing rats.

Methods and Materials

Animal Labeling and Preparation

Litters of male Sprague-Dawley rats with mothers were obtained on day 7 of life. On day 10 of life 54 pups were labeled by subcutaneous (s.c.) injections of ^{3}H-tetracycline every other day for 9 days (five injections) [18]. Each animal received a total of 180 μCi of ^{3}H-tetracycline. At day 24, all rats were weaned from their mothers and placed in one of nine groups (n = 6 per group). These rats were fed Purina rat chow (1% calcium, 0.65% phosphorus, and 4400 IU/kg vitamin) and water *ad libitum* throughout the experimental protocol. Control rat groups received an oil-based vehicle daily and were sacrificed at T = 0, 7, 14, and 28 days of the experiment (n = 6 per group). This was equivalent to 28, 35, 42, and 56 days of age. Control animals received daily an alcohol-olive oil vehicle s.c., and treated animals received daily s.c. injections of cyclosporin A (Sandoz, Basle, Switzerland) in an alcohol-oil base in one of the following regimes: 30 mg/kg body wt/day × 1 week; 10, 20, 30 mg/kg body weight/day × 2 weeks; or 20 mg/kg body wt/day × 4 weeks.

Animals were weighed twice weekly and monitored for abnormal appearance of coat, abnormal levels of activity, or respiratory distress. At the end of the appropriate treatment or control period, the animals were anesthetized using ether, and 3–5 ml of blood was obtained by direct cardiac puncture. The animals were weighed and sacrificed. The intact right femur, right scapula, and L_6 vertebra were harvested from each animal [19]. Each bone was cleaned of soft tissue, broken into several pieces, and defatted in $CHCl_3$: MeOH (2:1) solution [18]. The bones were then dried for 2 days in a desiccator over NaOH pellets.

Blood Analysis

All blood samples were analyzed in a Technicon SMA-24. Serum calcium, creatinine and total serum alkaline phosphatase levels, as well as heat-labile fractionated alkaline phosphatase were obtained for each animal. The heat-labile fraction of alkaline phosphatase, which represents the level of bone alkaline phosphatase, was used for comparison between control and experimental groups. Serum CsA levels for each animal were determined using high performance liquid chromatography [20].

Bone Analysis

Following desiccation, each bone in the experimental and control groups was weighed to 1.0 mg using a Mettler analytical balance to obtain its dry weight. Using the method previously described by Klein and Van Jackman [18], all pieces of each bone were placed in 5 or 10 ml of 0.5 N HCl solution for dissolving ^{3}H-tetracycline and calcium. Each bone was subjected to two or more extractions until the extract contained less than 5% of total tetracycline or calcium previously extracted from the bone. The supernatant fluid from each bone was then analyzed for ^{3}H-tetracycline and bone calcium [18, 19, 21]. An aliquot of the extract was counted in a liquid scintillation spectrometer (Mark II Searle, Chicago, IL), at least three times per bone. The total radioactivity per whole bone in the supernatant fluid represents the ^{3}H-tetracycline present at the end of the treatment or control period for each animal. Radioactivity present in zero time control animals represents the initial total radioactivity in whole bone (100%) prior to the control and treatment period. Radioactivity at the end of the T = 1, 2, or 4 weeks represents the amount of unresorbed bone remaining at the end of each time interval. Rate of bone resorption is presented as percentage of bone resorbed during 1, 2, or 4 weeks when compared with zero time controls. Bone calcium was analyzed by an automatic titrator, Model 4008 Calcette (Precision Systems, Sudbury, MA) using aminoethylether (EGTA) in the presence of calcium with a fluorometric endpoint [18, 21].

The present study quantifies cortical bone resorption in animals prelabeled with ^{3}H-tetracycline, using an organ technique (whole bone) that measures rates of cortical bone resorption. In actively growing animals, tetracycline has been shown to be rapidly and homogeneously taken up in newly formed mineralized bone which remains stable prior to resorption [18]. Tetracycline is rapidly excreted by the kidney after being released by bone resorption [21]. Intact bones are used for sequentially assaying bone mass and total loss of ^{3}H-tetracycline. Whole bone as the anatomic unit defines a reproducible unit of bone mass and circumvents the dilutional effect of growth upon isotope and chemical concentration [21]. In turn, whole bone permits a more absolute measurement of bone mass, formation, and resorption, and provides a quantitative model for *in vivo* testing of pharmaceuticals.

Data Analysis

Means and standard errors were computed in treatment and control groups for body weight and bone weight; calcium mass of whole bone; and content of ^{3}H-tetracycline, serum calcium, creatinine, total and heat-labile alkaline phosphatase, and CsA. A Student's *t*-test was used to determine statistical significance between treatment and control groups for all measurements of body weight, blood, and bone.

The radioactivity for treatment or control groups at 1, 2, and 4 weeks was compared with animals sacrificed at T = 0. Radioactivity lost for each bone represented bone resorption and was expressed as a percentage value of zero time according to the formula

$$\% = \frac{t_0 \text{ dpm} - t_x \text{ dpm}}{t_0 \text{ dpm}} \times 100$$

Means, standard errors, and statistical evaluation of bone resorption data were performed prior to conversion to the percentage form presented in Table 6.

Results

Animal Survival and Body Weights

All treated animals survived the experimental period. None

Table 1. Body weight (g)

CsA (mg/kg/day)	Duration of treatment 0 wk	1 wk	2 wk	4 wk
0	165 ± 2	240 ± 9	310 ± 6	460 ± 25
10	—	—	290 ± 12	—
20	—	—	270 ± 7[c]	350 ± 13[b]
30	—	210 ± 6[a]	250 ± 8[c]	—

Data are presented as means ± SEM when n = 6
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, statistical significance vs control animals

Table 2. Serum data for CsA level (ng/ml)

CsA (mg/kg/day)	Duration of treatment 0 wk	1 wk	2 wk	4 wk
0	0	0	0	0
10	—	600	700	—
20	—	—	1900	1400
30	—	2300	1950	2000

n = 2 per group

of these animals experienced the previously reported side effects of weight loss, hair loss, or decreased feeding behavior [22]. One control animal died at the end of the experimental period 2 days prior to planned sacrifice. At sacrifice, all animals underwent necropsy where abdomen, chest cavity, and brain were examined. No gross changes were noted.

All animals gained weight throughout the experimental period, though experimental animals gained less than their controls for all dosage regimens and all time intervals (Table 1). At 1 week, small but significant decreases in body weight occurred when the rats received 30 mg/kg body weight dose noted. By 4 weeks, body weights of experimental animals were significantly less than controls by greater than 100g.

Serum Data

Serum CsA levels were at or above therapeutic levels in all tested experimental animals (Table 2). These ranged from 700 to 2300 ng/ml and represented trough levels of CsA as none of the animals had received a dose of CsA for 24 hours prior to sacrifice. Serum CsA levels were proportional to the dose given with the animals receiving 10 mg/kg body weight per day, demonstrating the lowest serum levels 700 ng/ml), whereas animals receiving 30 mg/kg body weight per day had the highest serum levels (2300 ng/ml). Despite the administration of supra-therapeutic doses, none of our animals experienced renal failure. Creatinine levels were significantly higher in the 28-day treatment group (Table 3), but were not pathologically elevated.

Serum calcium and alkaline phosphatase levels showed more variability (Table 3). After 7 days of treatment, serum calcium decreased significantly (9.6 mg/dl ± 0.18 vs 10.1 ± 0.46 $P < 0.05$) for the highest dose (30 mg/kg body weight) when compared with controls. This trend continued at 14 days of treatment for all three doses when serum calcium decreased to its lowest level of 8.6 mg/dl ($P < 0.01$ when compared with controls). Heat-labile and total (not shown) serum alkaline phosphatase decreased progressively in control and experimental animals through the experimental protocol. At the highest treatment levels (30 mg/kg body weight), serum alkaline phosphatase was significantly lower than controls at 1 and 2 weeks of treatment (850 u vs 550 u, $P < 0.001$) and (780 u vs 580 u, $P < 0.01$).

Bone Weight

Differences between treated and control animals were noted in individual bone dry weights (see Table 4). All experimental bones weighed less than *final* controls for all treatment protocols but weighed more than *initial* controls at zero time. In the femur, marginal significance between controls and treated was noted only in 2-week (30 mg/kg) and 4-week (20 mg/kg) treated groups where experimental bones were only 91% and 79% of controls ($P < 0.05$ and $P < 0.01$, respectively). Scapulae showed significantly less total bone mass (86% and 83%) in treated animals compared with controls after 1 and 4 weeks of treatment. The L6 vertebral body showed significantly less total mass (83–71%) in all treatment groups after 2 and 4 weeks of treatment. When treated groups were compared with controls at zero time, none of the experimental bones were less in weight than the initial controls.

The ratios of bone weights to body weights for control and treated rats were compared at 1, 2, and 4 weeks. Significant increases in the ratios of treated rats (0.112 ± 0.0052, 0.121 ± 0.0066, 0.116 ± 0.0042; mean ± SD) compared with control rats (0.100 ± 0.0025, 0.107 ± 0.0035) were caused by larger decreases in body weights than in bone weights. The ratios were statistically significant at 20 and 30 mg CsA for 2 weeks ($P < 0.001$) and at 20 mg CsA for 4 weeks ($P < 0.01$).

Bone Calcium Mass

The calcium mass per whole bone (Table 5) paralleled the results found in the bone dry weight. The treated femur, scapula, and L_6 vertebral body all showed significantly less calcium mass after only 1 week of treatment. This difference remained after 2 weeks of treatment in all bones at the highest doses of cyclosporin. The scapula and L_6 vertebral body showed significantly less calcium mass at intermediate doses (20 mg/kg) than control bones whereas the L_6 vertebral body showed significantly less calcium at the lowest (10 mg/kg) cyclosporin dose. The differences persisted at 4 weeks as treatment groups had significantly less calcium mass in all bones, with the L_6 vertebral body most significantly affected ($P < 0.001$) (Table 5).

Bone Resorption

Absolute rate of bone resorption varied among the different control bones (Table 6). The femur showed the lowest basal rates of bone resorption with 41–42% resorption over the 4-week experimental course. The scapula and L_6 vertebra showed higher basal rates of resorption with 67–75% resorption by 4 weeks of treatment. For all doses and duration of treatment there was no significant difference in bone resorption between control and treated bones.

Discussion

In contrast to previous investigations *in vivo*, our study demonstrates that CsA had little, if any, effect on cortical bone

Table 3. Effect of CsA dose and duration on serum calcium, alkaline phosphatase, and creatinine in the growing rat

Serum	CsA (mg/kg/day)	Duration of treatment 0 wk	1 wk	2 wk	4 wk
Calcium (mg/dl)	0	—	10.1 ± 0.2	9.9 ± 0.1	9.9 ± 0.1
	10	—	—	9.1 ± 0.3[a]	—
	20	—	—	9.2 ± 0.2[a]	9.9 ± 0.1
	30	—	9.6 ± 0.7[a]	8.6 ± 0.26[b]	—
Alkaline phosphatase (U/dl)	0	835 ± 74	850 ± 47	780 ± 44	580 ± 21
	10	—	—	870 ± 84	—
	20	—	—	720 ± 50	600 ± 82
	30	—	550 ± 35[c]	580 ± 28[b]	—
Creatinine (mg/dl)	0	0.48 ± 0.02	0.52 ± 0.04	0.70 ± 0.04	0.63 ± 0.03
	10	—	—	0.73 ± 0.06	—
	20	—	—	0.58 ± 0.03	0.72 ± 0.02[a]
	30	—	0.43 ± 0.02	0.68 ± 0.04	—

Data are presented as means ± SEM when n = 6
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, statistical significance vs control animals

Table 4. Effect of CsA dose and duration on bone dry weight (mg) in the growing rat

Bone	CsA (mg/kg/day)	Duration of treatment 0 wk	1 wk	2 wk	4 wk
Femur	0	167 ± 2	310 ± 14	320 ± 8.5	480 ± 14
	10	—	—	300 ± 5	—
	20	—	—	300 ± 14	390 ± 16[b]
	30	—	280 ± 19	290 ± 5.6[a]	—
Lumbar 6	0	61 ± 2	100 ± 4.5	140 ± 4	225 ± 7
	10	—	—	117 ± 5[a]	—
	20	—	—	114 ± 4[b]	160 ± 6[b]
	30	—	90 ± 3	117 ± 3[b]	—
Scapula	0	68 ± 4	100 ± 3	120 ± 4	180 ± 6
	10	—	—	110 ± 5	—
	20	—	—	110 ± 5	150 ± 8[a]
	30	—	86 ± 12[a]	120 ± 3	—

Data are presented as means (±SEM) when n = 6
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, statistical significance vs control animals

resorption despite the range of therapeutic and supratherapuetic dosages (10–30 mg/kg body weight). No effect was seen despite varying periods of CsA administration by s.c. injection in young animals undergoing rapid bone turnover due to modeling. Likewise, our study showed no significant increase or decrease in *basal* rates of bone resorption over the range of CsA dosages studied for the three different bones: femur, vertebrae, and scapula.

The CsA doses used for Sprague-Dawley rats were at the therapeutic level (10 mg/kg body weight, resulting in a serum value of 0.7 μg/ml), intermediary level (20 mg/kg body weight, and serum 1.6 μg/ml), and supratherapeutic level (30 mg/kg body weight, and serum 2.1 μg/ml). The specific, non specific, and toxic effects of CsA appear to occur at *similar* concentrations whether CsA is assayed on human or rodent blood or tissue fluid *in vivo*, or cells or organ media *in vitro* [23]. The *in vivo* doses of CsA in humans give rise to nonspecific side effects only in those fatty tissues [24] (kidney, pancreas, liver) that accumulate CsA to the greatest degree [25]. The concentration of CsA in bone and muscle was similar but only one-third of that in liver but three times higher than serum [25]. The most sensitive index of CsA side effects appears to be a decrease in renal function as seen by an increase in serum BUN and creatinine [26]. This renal side effect was seen only at the longest time of treatment (4 weeks) with CsA (20 mg/kg body weight) which showed a small increase in serum creatinine. Also, each animal was his own control as a means of improving the accuracy and sensitivity of the SMA-24 assay of creatinine.

None of our treated animals lost weight or exhibited hair loss or decreased feeding behavior as has been described in rats receiving comparably high doses of CsA (100 mg/kg body weight). Our young experimental animals showed significantly less body weight gain when compared with control animals throughout the treatment protocol, although there were no significant differences in the amount of food eaten between the control and experimental groups of rats.

Our studies confirmed the decrease of serum calcium, previously reported [24, 27] for higher levels of CsA. This decrease of calcium could be a nonspecific effect due to an increased excretion by kidney and/or increased uptake by bone, and could be consistent with CsA inducing a change in calcium fluxes via an increase in cellular membrane permeability [26, 28]. The *in vitro* effect of CsA on cellular calcium fluxes is very cell- and dose dependent. At low levels (0.001–0.1 μg/ml), CsA specifically increases intracellular calcium in T-lymphocytes. But at intermediary levels (0.5–4 μg/ml), CsA nonspecifically increases intracellular calcium in many other cells tested: liver [29], kidney [30], pancreas [31], and vascular smooth muscle cells [32].

The significant decrease of serum alkaline phosphatase in the highest treatment groups over short treatment periods

Table 5. Effect of CsA dosage and duration of treatment on calcium mass (mg/bone) in the growing rat

Bone	CsA (mg/kg/day)	Duration of treatment 0 wk	1 wk	2 wk	4 wk
Femur	0	25 ± 0.4	58 ± 2	60 ± 1.6	102 ± 7.5
	10	—	—	57 ± 1.3	—
	20	—	—	55 ± 0.8[a]	79 ± 5[b]
	30	—	48 ± 2[b]	54 ± 1.4[a]	—
Lumbar 6	0	8.1 ± 0.2	17.9 ± 0.8	23.0 ± 0.6	41.3 ± 1.6
	10	—	—	18.7 ± 0.7[c]	—
	20	—	—	18.6 ± 0.4[c]	26.4 ± 1.7[c]
	30	—	14.5 ± 0.5[a]	18.1 ± 0.5[c]	—
Scapula	0	9.0 ± 0.3	17.8 ± 0.6	21.3 ± 0.7	34.6 ± 1.4
	10	—	—	20.6 ± 0.4	—
	20	—	—	19.3 ± 1	27.8 ± 1.6[b]
	30	—	15.0 ± 0.4[a]	18.6 ± 0.5[a]	—

Data are presented as means ± SEM when n = 6
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, statistical significance vs control animals

Table 6. Bone resorption[a]. Effect of CsA dosage and duration on rate of bone resportion in the growing rat

Bone	CsA (mg/kg/day)	Duration of treatment[b] 0 wk	1 wk	2 wk	4 wk
Femur	0	0	12 ± 2	33 ± 3	42 ± 5
	10	—	—	30 ± 3	—
	20	—	—	37 ± 3	41 ± 0.4
	30	—	15 ± 5	36 ± 4	—
Lumbar 6	0	0	25 ± 4	54 ± 3	74 ± 3
	10	—	—	60 ± 3	—
	20	—	—	58 ± 3	75 ± 1
	30	—	29 ± 3	58 ± 2	—
Scapula	0	0	20 ± 2	51 ± 3	71 ± 2
	10	—	—	50 ± 2.5	—
	20	—	—	52 ± 3	67 ± 1
	30	—	25 ± 4.6	50 ± 2.5	—

[a] As measured by the release of ^{3}H-tetracycline
[b] % Resorption compared with 0 time control for 0–1, 0–2, 0–4 weeks and no treatment for 1, 2, 4 weeks. Data are presented as means ± SEM when n = 6

(1–2 weeks) was consistent with a general decrease in body and bone growth. The decrease of serum alkaline phosphatase has been observed in earlier studies of Sprague-Dawley rats that were treated with CsA (25–100 mg/kg body weight/48 hours) [22, 33]. Higher doses of CsA (100 mg/kg body weight/24 hours) induced an increase in serum alkaline phosphatase [24]. *In vitro* studies by Okano et al. [34] demonstrated that high levels of CsA (10 μg/ml) decreased alkaline phosphatase activity and ^{3}H-thymidine uptake of a cloned osteoblastic cell line.

Significantly less mass of dry bone was seen in all three experimental bones when compared with corresponding controls. This appeared to be dependent on rate of growth of each bone as well as duration of treatment. These effects correlated well with a smaller bone calcium mass when compared with final controls. Experimental body weight, dry weight of whole bone, and total weight of bone calcium were compared with the initial and final control weights in order to distinguish between "relative" osteopenia due to insufficient growth, and "absolute" osteopenia due to a severe lack of growth and absolute loss of bone weight [35]. The smaller body and bone weights of treated rats (relative to final controls) were much larger relative to initial controls, thus indicating that CsA had an inhibitory effect on body growth [36] and bone formation. If loss of bone mass had occurred, the final experimental bone weights would have been smaller than the initial control weights.

Previous experimental data on the effects of CsA on bone *in vivo* were based on regional histomorphometric analysis of growing tissue in tibial metaphyseal or vertebral bone [13–16]. This focal approach increases the possibility of sampling error as one region of the whole bone was examined at one time point. In addition, the proximal metaphysis of the tibia [13] and caudal vertebra [15] from growing rats are mainly involved in growth of new bone and its turnover. Also, the peak growth of vertebrae occurred later and over a longer period than the long bones [19]. In the presence of growth and turnover, morphometric analysis of osteoid scans or osteoclast number only indirectly reflect changes in bone formation and resorption. Under these conditions, it is difficult for histomorphometry to distinguish between osteopenia due to poor growth and that due to loss of preexisting bone.

The histomorphometric evidence for high turnover and loss of new bone mass observed in the trabecular bone of the tibial metaphysis [13–15] is not seen *globally* at the whole

bone level in three different types of bone (femur, vertebra, scapula), nor in the vertebral density and trabecular volume reported recently by del Pozo et al. [37, 38]. There are several reasons for these differences. The reported effects of CsA that are on endochondral bone growth and turnover in the metaphysis appear to be qualitatively and quantitatively different from that in intramembraneous bone growth and turnover beneath the periosteum. It is well known that during growth, the periosteum makes a much larger contribution (four- to fivefold) to the total mass of long bones than the metaphyses. Due to the efficient conservation of resorbed bone minerals under normal [39] and calcium-deficient conditions [35, 40], any loss of new bone in the metaphysis will result in a redistribution of resorbed minerals [41] to new bone in the periosteum.

Our major reason for obtaining data from whole bones was to circumvent the problem of (1) structural and metabolic heterogeneity that exists at the tissue level and (2) the conservation of bone minerals that exist at the tissue and systemic levels [35, 39–41]. Structurally, the long bones are more heterogenous than the axial bones (vertebra, scapula). This heterogeneity is greater in rats, mice, and rabbits where the resorption rates rapidly decline during growth [17, 21, 42], and thus, lead to greater tissue heterogeneity than in growing chicks, dogs [42], and man [43] with continuously high resorption rates. Metabolic heterogeneity also exists among different bones in rats [17, 21], where whole long bones demonstrate lower rates of bone resorption (loss of ^{3}H-tetracycline) than whole vertebrae and scapula, thus, the bone mass of long bones is more resistant to loss.

CsA studies *in vitro* with fetal calvaria and long bones also have shown that CsA did not effect the basal rate of ^{45}Ca release (bone resorption) at therapeutic doses of 0.005–0.1 μg/ml [44] and 0.01–0.1 μg/ml [10, 11]. At what is considered to be supratherapeutic [23] doses of CsA (10 and 100 μg/ml), the basal rate of bone resorption was inhibited only 10–20% [45] compared with a much larger inhibition (70–80%) of stimulated bone resorption [10, 11]. However, the basal rate of ^{45}Ca release *in vitro* needs to be interpreted cautiously because the majority of the ^{45}Ca released into the medium appears to be nonosteoclastic physiochemical exchange of ^{45}Ca [46]. The basal data of bone resorption, whether obtained *in vitro* or *in vivo,* were consistent in demonstrating no effect of CsA on bone resorption at therapeutic or somewhat higher doses. Most likely our basal rate of bone resorption *in vivo* is occurring under conditions of normally low endocrine stimulation and minimal immune stimulation. However, the basal data on young bones *in vivo* cannot be directly compared with the effect of CsA on fetal bones *in vitro* that were stimulated hormonally or with cytokines.

The absence of an effect of CsA on the basal rate of bone resorption *in vivo* would suggest that in the normal, unstimulated, or low-stimulated state for cytokines or calcemic hormones, the inhibition of the immune system (T lymphocytes) may not have an important regulatory effect on bone resorption. This does not exclude regulatory control by a stimulated immune or endocrine system.

In conclusion, we found that CsA had no significant effect on basal rates of whole bone resorption *in vivo* at pharmacologic to suprapharmacologic doses. In contrast, significant decreases in serum alkaline phosphatase, lower dry weights, and lower total calcium mass per whole bone demonstrated that CsA has a dose-dependent, depressive effect on bone formation. At the organ level, osteopenia appears to be due to impaired growth rather than an increase in bone resorption and loss of mass.

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