Calcif Tissue Int (1990) 47:152–157

Calcified Tissue International

1,25 Dihydroxyvitamin D_3 Modifies Cyclosporine-Induced Bone Loss

Sol Epstein,[1] Mark Schlosberg,[1] Michael Fallon,[2] Steven Thomas,[1] Colin Movsowitz,[1] and Firhaad Ismail

[1]Albert Einstein Medical Center, and [2]University of Pennsylvania Medical School, Philadelphia, Pennsylvania, USA

Summary. We have previously shown that cyclosporin A (CsA) produces high bone remodeling with resorption exceeding formation and loss of bone volume in the rat. This may have important clinical implications where CsA is widely used in organ transplantation. 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a bone mineralizing hormone which also has immune modifying properties. Consequently, we studied the effect of combined CsA and $1,25(OH)_2D_3$ administration over 28 days in four groups of rats. Group A received vehicle (n = 10), group B CsA (15 mg/kg) (n = 10) alone, group C $1,25(OH)_2D_3$ plus CsA (n = 15), and group D $1,25(OH)_2D_3$ alone (20 ng/100 g) (n = 15). Rats were bled periodically at day 0, 7, 14, and 28 and Ca, parathyroid hormone (PTH), $1,25(OH)_2D$, osteocalcin (bone Gla-protein, BGP), BUN, and creatinine were measured. Rats were sacrificed on day 28 and bones were examined histomorphometrically. Compared to controls, CsA resulted in significant elevation of BGP and a transient increase in $1,25(OH)_2D$ with excess bone remodeling and loss of bone volume. $1,25(OH)_2D_3$ administration produced hypercalcemia, a significant rise in BGP, with suppression of PTH and increased osteoid volume. Combined therapy prevented the loss of bone volume probably due to increased osteoid tissue and enhanced osteoblast activity. Renal dysfunction, a side-affect of CsA, was not a factor. In conclusion, $1,25(OH)_2D_3$ combined with CsA restores bone volume which is accompanied by increases in serum calcium and BGP.

Send reprint requests to Sol Epstein, M.D., Albert Einstein Medical Center, Klein, 331, York and Tabor Roads, Philadelphia, PA 19141, USA.

Key words: 1,25 dihydroxyvitamin D_3 — Cyclosporine — Bone Gla-protein — Bone histomorphometry.

Cyclosporin A (CsA) is now a well-accepted form of antirejection therapy in patients receiving organ transplantation [1, 2]. It also has been used in the treatment of autoimmune diseases [3] and its administration will no doubt increase in the future as the indications for its use become broader. CsA, however, has been documented to produce such adverse effects as nephrotoxicity and hypertension [4]. Recently, we have demonstrated that enhanced bone remodeling with bone loss occurs in the rat administered CsA *in vivo* [5]. We postulated that this phenomenon is mediated via local factors and not through calciotropic hormones. Our results have been strengthened by reports of enhanced bone turnover and raised serum alkaline phosphatase and serum osteocalcin values in renal transplant patients receiving CsA [6, 7]. We have also observed that the deleterious effects of *in vivo* CsA on bone mineral metabolism is related to both dose and duration of administration with a tendency towards partial reversibility after withdrawal of the immunosuppressive agent [8]. Warren et al. [9], studied biomechanical properties and fracture healing in female rats after administering a lower dose of CsA over a shorter period of time. Although a reduction in biomechanical properties was observed between 12 and 16 weeks, the difference in dose, duration of administration, type of bone (i.e., predominantly cortical), and the lack of histomorphometric data do not allow for a direct comparison with our study. Based on the enhanced bone resorption associated with *in vivo* CsA administration, we have been actively engaged in attempting to prevent

or counteract these changes of rapid bone resorption using various pharmacological agents such as glucocorticoids and bisphosphonates (unpublished data). We feel that correction of cyclosporine-induced bone loss may have important clinical benefits regarding the long-term use of this agent. This article deals with the administration of $1,25-(OH)_2D_3$, a potent bone mineralizing hormone with immune modifying properties [10], given in combination with CsA in an attempt to overcome the bone-resorbing effects of CsA in immunosuppressive doses. The increasing evidence that $1,25(OH)_2D$ may regulate osteoclast function and impair interleukin 2 production, together with the data that indirectly suggest a role for local factors in the mediation of CsA-induced bone resorption, prompted us to study their combined effect on bone mineral metabolism.

Materials and Methods

Fifty male Sprague Dawley rats approximately 400 g in weight were purchased from Harlan Sprague Dawley (Indianapolis, IN). All the animals were fed Agway RMH 3000 (calcium 0.97%. phosphorus 0.85%, and vitamin D_3 1045 IU/kg) and tap water *ad libitum*. The animals were randomly divided into four groups and received CsA (15 mg/kg bw) alone and in combination with $1,25(OH)_2D_3$ (20 ng/kg bw) according to the protocol described below.

CsA

CsA was obtained from Sandoz (East Hanover, NJ) and dissolved in olive oil. The olive oil-based vehicle in equivalent vol/wt served as the control. These agents were administered by daily gavage between 8:30 and 9:00 a.m. from day 0 to day 28.

$1,25(OH)_2D_3$

$1,25(OH)_2D_3$ was obtained from Hoffman la Roche (Nutley, NJ) and diluted in saline. Both $1,25(OH)_2D_3$ and saline in equivalent vol/wt were administered daily by subcutaneous injection from day 0 to 28.

Group A (n = 10) received vehicle (olive oil) by daily gavage and saline by s.c. injection for 4 weeks (n = 10). Group B (n = 10) received CsA by daily gavage and saline by s.c. injections for 4 weeks. Group C (n = 15) received CsA by daily gavage and 1,25 $(OH)_2D_3$ by s.c. injection for 4 weeks. Group D (n = 15) received $1,25(OH)_2D_3$ by s.c. injection and vehicle by daily gavage for weeks. All animals received two separate doses of tetracycline hydrochloride (Lederle Laboratories, Pearl River, NY) 1.5 mg/100 g bw by i.p. injection for measurement of dynamic bone histomorphometric parameters.

Blood Collection and Analysis

The animals were weighed and bled on days 0, 7, 14, 21, and 28 under Ketaset anesthesia, 15 mg/kg bw (Bristol Laboratories, Syracuse, NY). Tail vein blood was collected in heparinized capillary tubes for immediate determination of ionized calcium utilizing an ionized calcium channel analyzer (Radiometer Company, Copenhagen, Denmark). All other blood samples were obtained by orbital sinus puncture except when the animals were sacrificed by cardiac puncture on day 28. Serum samples were stores at −70° until assayed.

Serum BGP was measured by radioimmunoassay as previously described [5]. Antibody to rat BGP was raised in rabbits immunized by multiple-site intradermal injections of purified rat BGP. The antibody was used in a final dilution of 1:25,000. Purified rat BGP was used for the preparation of standards and ^{125}I-labeled tracer. An equilibrium assay was performed and antibody/BGP complexes were separated from free ^{125} I-labeled BGP by using goat anti-rabbit gamma globulin (Calbiochem, La-Jolla, CA); the lower limit of detection for the assay is 0.2 ng/ml. Intra- and interassay coefficients of variation were 9.9 and 8.36%, respectively. The validity of the BGP radioimmunoassay with stimulation of circulating levels by $1,25(OH)_2D$ and suppression by glucocorticoids has previously been published [11].

Serum immunoreactive parathyroid hormone (iPTH) was measured using a commercially available kit (Nichols Co, San Juan Capistrano, CA) with an antiserum to human PTH (1–34) that cross-reacts with rat PTH (1–34). The validity of this assay has been confirmed in our laboratory by showing low values for serum iPTH in parathyroidectomized and in $1,25(OH)_2D_3$-treated animals and elevated values after EDTA-induced hypocalcemia. The intra- and interassay coefficients of variation were 9.9 and 8.76%, respectively. The characteristics of this assay have also recently been published [12].

$1,25(OH)_2D$ was assayed using a commercially available INCSTAR kit (Stillwater, MN) which uses an extraction procedure followed by a radioreceptor-assay utilizing a thymus receptor that is specific for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. The interassay coefficient of variation was 8.9%.

Histological Techniques

At the time of sacrifice, the right tibia was removed from each rat, dissected free of soft tissue, fixed in 70% ethanol, and embedded undecalcified in methyl methacrylate. Longitudinal 7 μm thick nondecalcified sections of proximal metaphyseal tibial bone taken from between one-third and one-half the thickness of each specimen were cut on a Jung Model K sledge microtome (American Scientific Instruments, Buffalo, NY), and were stained by a modification of the Masson trichrome technique. The tetracycline labels were measured on unstained sections viewed by epifluorescence. The following histological features were quantitated utilizing a semiautomated image-analysis system (Bio-Quant II Nashville, TN): (1) bone volume—the percentage of metaphyseal marrow space occupied by mineralized and unmineralized bone matrix, representing the bone mass; (2) osteoclast number (osteoclast-like cells)—number of multinucleated cells per millimeter of trabecular surface; (3) mineralizing surface—percentage of trabecular surface bearing dual fluorescent labels indicating the fraction of bone surfaces engaged in bone formation; (4) osteoid volume—the percentage of trabecular bone volume composed of unmineralized bone matrix, i.e., osteoid, an index of bone formation; (5) mineral apposition rate in micrometers per day, indicated by the mean distance between the midpoints of the double tetracycline labels at 30–40 randomly

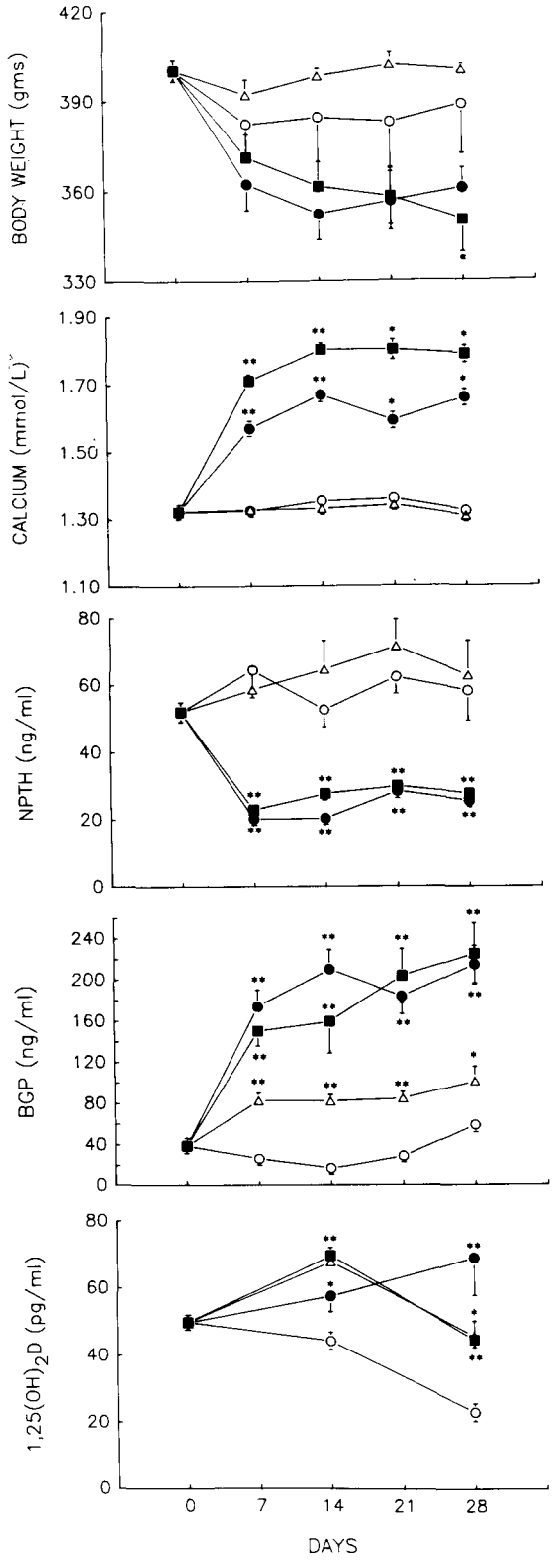

Fig. 1. The effects of CsA alone or in combination with $1,25(OH)_2D_3$ on body weight, serum calcium, PTH, osteocalcin (BGP), and $1,25(OH)_2D$ levels. Control (vehicle) (○); CsA (△); CsA + $1,25(OH)_2D_3$ (●); $1,25(OH)_2D_3$ (■). Values mean ± SEM. * $P < 0.05$ vs. control; ** $P < 0.005$ vs. control.

selected intercepts along the endosteal surface divided by the interdose duration.

Statistical Methods

Statistics were computed using the statistics package SPSS/PC plus (SPSS Inc, Chicago, IL) for all data until and including day 28. Overall significance was determined with *Multivariate analysis of variance* (MANOVA). Where significant differences occurred by MANOVA, a comparison was made between groups on different days using analysis of variance (ANOVA) with Scheffe's modification.

Results

Effects of CsA Alone or in Combination with $1,25(OH)_2D_3$

Weight. Weight loss occurred in the groups receiving either $1,25(OH)_2D_3$ alone (group D) or in combination with CsA (group C) and this weight loss persisted throughout the study period although it did not reach significance until day 28 in group D (Fig. 1).

Ionized Calcium. This rose significantly ($P < 0.005$) in groups C and D with the highest values occurring in group D; this increase was evident 1 week after administration of $1,25(OH)_2D_3$. It is of interest that serum calcium appeared to decrease significantly in group B ($P < 0.03$) at day 14, but at no other time point (Fig. 1).

N PTH. Significant decreases ($P < 0.005$) occurred in the same groups that had elevated ionized calcium values, i.e., groups C and D but no changes occurred in either the control (group A) or the cyclosporin A-treated animals (group B) (Fig. 1).

Serum Osteocalcin (BGP). In group B, BGP rose significantly with CsA administration ($P < 0.005$) by day 7 and remained elevated compared to controls thoughout the study period. The greatest increase in BGP occurred with the administration of $1,25(OH)_2D_3$ alone or $1,25(OH)_2D_3$in combination with CsA. This increase was evident by day 7 with the maximum values found at 28 days (Fig. 1).

$1,25(OH)_2D$. Serum values rose at day 14 in groups B, C, and D and thereafter declined at day 28 in groups A, C, and D. CsA independently increased serum $1,25(OH)_2D$ values at day 14 ($P < 0.05$ vs. group A) and day 28 ($P < 0.005$ vs. group A) (Fig. 1).

BUN and Creatinine. The groups that received CsA either singly or in combination had significant elevations in BUN and creatinine with the greatest increase occurring at day 14 in group C (Fig. 2).

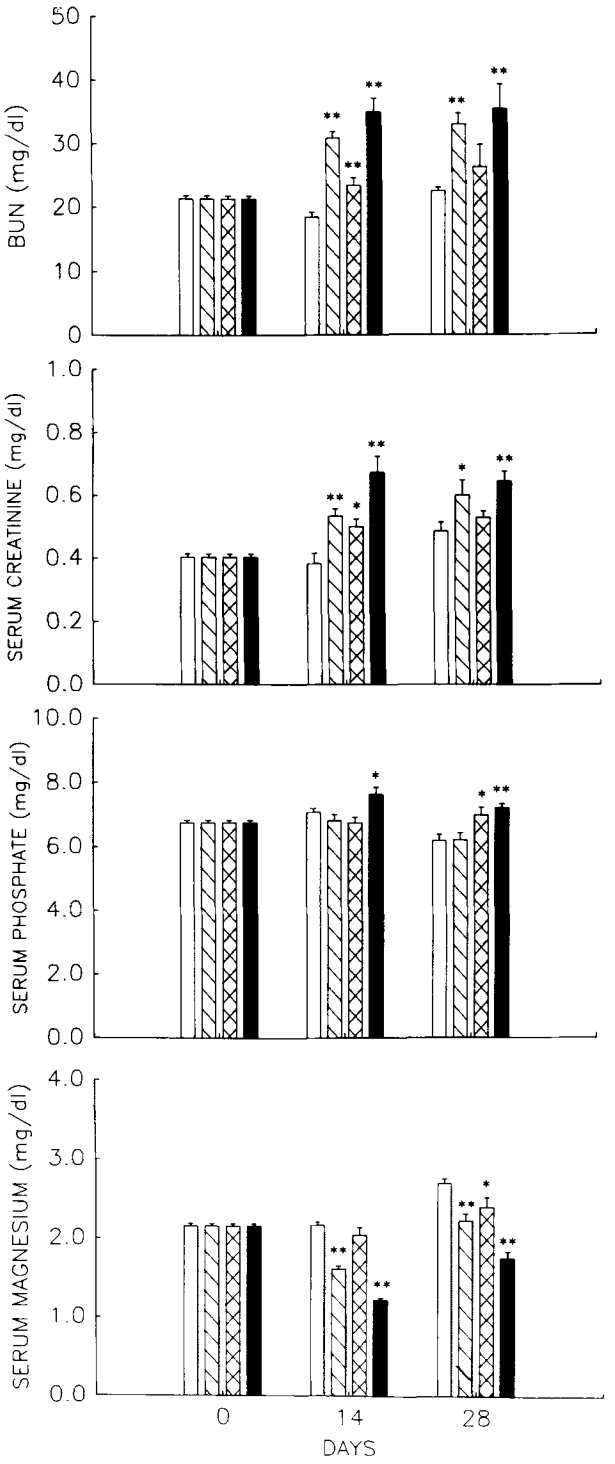

Fig. 2. The effects of CsA alone or in combination with $1,25(OH)_2D_3$ on BUN, creatinine, serum phosphate (PO_4), and magnesium levels. Control (□); CsA (▨); CsA + $1,25(OH)_2D_3$ (■); $1,25(OH)_2D_3$ (▩). Values mean ± SEM. * $P < 0.05$ vs. control; ** $P < 0.005$ vs. control.

Serum Phosphate (PO_4). Combined therapy (group C) appeared to increase serum PO_4 significantly at day 14 and day 28 ($P < 0.05$). This did not occur with CsA alone despite the greater increases in BUN and creatinine (Fig. 2).

Serum Magnesium (Mg). This decreased significantly in the CsA-treated animals (groups C and D) on day 14 and day 28 vs. control group (Fig. 2).

Bone Histomorphometry (Table 1)

Bone Volume. This was significantly diminished in the CsA-treated group (group B) ($P < 0.05$) and significantly increased in group D ($P < 0.005$). The combined therapy did not alter the percentage bone volume when compared to control animals.

Osteoclast-like Cells. At day 28, all groups had increased osteoclastic activity compared to controls with the greatest increase in group B. There was no difference in the effect of $1,25(OH)_2D_3$ with or without CsA on osteoclastic activity (group C vs. D) (Fig. 10).

Osteoid Volume. This was greatly increased in group D compared to all the other groups ($P < 0.005$) and this $1,25(OH)_2D_3$ effect also appeared in group C although not to a significant degree.

Mineral Apposition Rate. CsA significantly enhanced the mineral apposition rate (group B), and the addition of $1,25(OH)_2D_3$ appeared to decrease this effect (group C).

Mineralizing Surface. The results resembled the mineral apposition rate with CsA enhancing and $1,25(OH)_2D_3$ modifying this process, when compared to control animals ($P < 0.05$).

Discussion

We had previously described rapid bone remodeling with enhanced bone resorption produced by cyclosporine administration in the rat [5]. Because this adverse effect may have important clinical implications regarding human usage, we attempted to attenuate this manifestation by the administration of $1,25(OH)_2D_3$, a bone mineralizing hormone with significant immunomodulatory properties. The results of studies revealed that we improved some of the parameters of the bone remodeling process but may also have exaggerated others. $1,25(OH)_2D_3$ did not prevent the increase in osteoclast-like cells and

Table 1. Bone histomorphometry—day 28

	Group			
	A	B	C	D
Bone vol (%)	4.68 ± 0.41	2.31 ± 0.56[a]	4.35 ± 0.4	7.45 ± 0.68[b]
Osteoid vol (%)	0.8 ± 0.16	0.74 ± 0.2	1.43 ± 0.24	5.87 ± 0.77[b]
Osteoclast # (per mm)	0.67 ± 0.11	2.1 ± 0.22[b]	1.77 ± 0.21[b]	1.48 ± 0.2[b]
MAR (μm/day)	0.88 ± 0.13	2.23 ± 0.47[a]	1.25 ± 0.13	—
M.S. (%)	3.5 ± 0.72	11.53 ± 2.7[a]	6.85 ± 0.37[a]	—

Group A = control, group B = CsA treatment, group C = CsA + $1,25(OH)_2D_3$ treatment, group D = $1,25(OH)_2D_3$ treatment
Osteoclast # = number of osteoclast-like cells; MAR = mineral apposition rate; M.S. = mineralizing surface; — = unmeasurable. All values mean ± SEM
[a] $P < 0.05$ vs. group A, [b] $P < 0.005$ vs. group A

hence the potential for resorption, but it did attenuate it. The loss of percentage bone volume, which is a prominent feature of CsA therapy and presumably poses a very serious hazard in the long term, was prevented by concomitant $1,25(OH)_2D_3$ administration. The bone volume, however, was not as great as in the animals receiving $1,25(OH)_2D_3$ alone, suggesting that CsA may have had an inhibitory effect on the action of $1,25(OH)_2D_3$ [13]. Alternately, the loss of percentage bone volume may have been too great or the duration of administration of $1,25(OH)_2D_3$ too short to allow further increase in the percentage bone volume. The increase in the percentage osteoid volume by $1,25(OH)_2D_3$ (group D), which far exceeded the other groups, did appear to be blunted by concomitant CsA administration (group C).

Unfortunately, the other parameters of bone formation and accretion such as tetracycline labeling and mineral appositional rate could not be evaluated with regard to $1,25(OH)_2D_3$ or combined therapy. CsA administration on its own did not result in an increased osteoid volume as we had previously observed [5]. However, the rats in the present study were at least a month older than our previous report [5]. We have observed in our laboratory that with aging there is lower bone remodeling and serum BGP [14]. The low BGP, osteoid, and baseline bone volume reflect a lower rate of bone remodeling in the animals in this study which may compromise the effect of cyclosporine. It is unlikely that the time of day of CsA administration [15] may account for the difference in osteoid volume between our present and previous studies, as the time of administration was always consistent in the mornings.

Overall, the effect of combined $1,25(OH)_2D_3$ and CsA administration appears to be maintenance of percentage bone volume without significant alterations in the other parameters of bone resorption. The lack of significant effect on bone resorption occurred despite suppression of PTH and elevated ionized calciums levels and this failure to suppress resorption may also be a "toxic" manifestation of high dose $1,25(OH)_2D_3$.

It is also of interest that combined therapy enhanced the BGP production by osteoblasts to a greater degree than CsA alone. This may be an advantage of combined therapy and may account for the maintenance of the percentage bone volume in group C. The addition of $1,25(OH)_2D_3$ to CsA despite the potential advantages mentioned above may be offset by the greater increases in BUN, creatinine, and phosphate than that observed with CsA administration alone. The elevation in calcium and the weight loss are also disadvantages. These adverse effects of $1,25(OH)_2D_3$ are probably dose related, but we chose this schedule based on our previous results with glucocorticoid-induced bone loss in the rat [11]. Further experiments with smaller doses of $1,25(OH)_2D_3$ need to be conducted over a longer period of time to determine if the adverse effects of CsA therapy could be offset without the development of these potentially harmful manifestations. The biochemical alterations seen in this study with CsA therapy alone has been previously noted by us [5, 8], viz, increase in serum BUN, creatinine, as well as $1,25(OH)_2D_3$ with an accompanying decrease in serum magnesium. Another reason for using $1,25(OH)_2D_3$ was that in view of the biological actions of CsA on the immune system [16] and the newly described roles of local factors in bone resorption [17, 18], we felt that $1,25(OH)_2D_3$ may well influence bone resorption.

Growth factors or products of the lymphokine cytokine system such as interleukin-1 [19] were not measured, so we cannot conclusively state their role in bone resorption in this present study. However, the histomorphometric analysis did not show significant effects of $1,25(OH)_2D$ on CsA-induced changes in bone resoprtion.

In conclusion, the addition of large doses of $1,25(OH)_2D_3$ to CsA therapy maintains bone vol-

ume but does not correct all the histomorphometric abnormalities and is associated with significant increases in serum calcium, BUN, and creatinine. Further studies with smaller doses of $1,25(OH)_2D_3$ or with newer vitamin D analogs [20] that are reputed to produce less hypercalcemia ought to better define the value of this combination therapy.

Acknowledgments. We wish to thank Sandoz Pharmaceuticals and the Albert Einstein Society for their support and the secretarial assistance of Ms. Selina Plummer.

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Received April 3, 1989, and in revised form June 19, 1989.