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The Effects of Dichloromethylene Diphosphonate on Hypercalcemia and Other Parameters of the Humoral Hypercalcemia of Malignancy in the Rat Leydig Cell Tumor

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Summary. There is a high frequency of Leydig cell tumors associated with hypercalcemia in the aged Fischer 344 rat. We studied a transplantable tumor cell line (Rice D-6) which is associated with hypercalcemia, hypercalciuria, hypophosphatemia, renal phosphate wasting, increased urinary cyclic adenosine monophosphate (AMP) excretion, absence of bone metastases, increased osteoclastic bone resorption, and suppressed immunoreactive parathyroid hormone (iPTH) concentrations. We examined the ability of dichloromethylene diphosphonate (Cl_2MDP) to lower serum calcium and decrease the parameters of increased bone resorption. We used this drug also as a pharmacologic tool to determine the relationship of hypercalcemia and increased bone resorption to the abnormalities in renal tubular function associated with the humoral hypercalcemia of malignancy.

Daily administration of Cl_2MDP before development of hypercalcemia, in doses from 2.5–40 mg/kg body weight subcutaneously, delayed and suppressed both the hypercalcemia and hypercalciuria. There was an increase in bone mass and decrease in both osteoclast number and activity compared with bones from untreated tumor-bearing animals. The urinary hydroxyproline excretion in treated animals declined towards the normal range. There were no significant effects on serum phosphorus, urine phosphorus, or urine cyclic AMP excretion.

These data suggest that Cl_2MDP reverses the increased bone resorption that occurs in the humoral hypercalcemia of malignancy, and confirms that diphosphonates are effective agents in the prevention and treatment of increased bone resorption associated with malignant disease. They also suggest that renal phosphate wasting and increased urinary cyclic AMP excretion are not directly related to the hypercalcemia.

Key words: Dichloromethylene diphosphonate — Hypercalcemia — Rat tumor.

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It is probable that multiple mechanisms are responsible for the hypercalcemia associated with malignant disease [1]. One subset of patients with the hypercalcemia of malignancy has been shown to have a syndrome that comprises absence of bone metastasis, increased urinary cyclic AMP excretion, renal phosphate wasting, and decreased iPTH concentrations. This has been called the humoral hypercalcemia of malignancy [2–4]. Recently, we found that this syndrome occurs in a transplantable animal neoplasm, the rat Leydig cell tumor [5]. Animals carrying these tumors have increased bone resorption, hypercalcemia, increased urinary cyclic AMP excretion, decreased circulating iPTH concentration, and absence of bone metastasis.

The diphosphonates are synthetic analogues of pyrophosphate, a naturally occurring inhibitor of bone mineralization [6, 7]. Recently, there has been wide interest in the diphosphonates because of their potent inhibitory effects on bone resorption [7]. The diphosphonates have been shown to be effective in the treatment of Paget's disease of bone [8–10], and recently, several of the newer diphosphonates have been effective in the therapy of hypercalcemia of malignancy and the treatment of normocalcemic patients with malignant disease who have destructive bone lesions [11–13]. However, some of these studies are difficult to interpret because they have been performed on patients who

were simultaneously receiving antineoplastic therapy. In this study, we examined the effects of the diphosphonate Cl_2MDP on the serum calcium, bone histomorphometry, and the other parameters associated with the humoral hypercalcemia of malignancy in the rat Leydig cell tumor. In this model, the data is not obscured by the effects of simultaneous administration of antineoplastic treatments directed at the tumor. We found that Cl_2MDP is an effective inhibitor of bone resorption and hypercalcemia in these tumor-bearing animals. Inhibition of bone resorption by Cl_2MDP did not alter the other parameters of the humoral hypercalcemia of malignancy, namely renal phosphate wasting and increased urinary cyclic AMP excretion.

Methods

The Rice D-6 tumor was obtained initially from EG&G Mason Research Institute, Worcester, Massachusetts. The tumor was carried subcutaneously in male inbred Fischer rats of 180–250 g body weight, obtained from Charles River, Worcester, Massachusetts. They were fed standard Purina Rat Chow and had free access to water. Between 5 and 10 rats were used in each treatment group. The rats treated with Cl_2MDP received the drug by subcutaneous injection daily. In some experiments, treatment was started on the day of implantation of the tumor. In other experiments, the drug was administered after hypercalcemia was established.

Blood was obtained by cavernous sinus puncture and urine was obtained by housing the animals for 24-h periods in metabolic cages. The 24-h urine samples were preserved with 6 N HCl and frozen at −70°C until the time of sample determination. Serum and urine calciums were determined by atomic absorption spectrophotometry. Serum and urinary phosphates were determined by a modification of the Fiske and Subbarow method [14]. Creatinines were determined spectrophotometrically by the Jaffe method [15]. Urinary cyclic AMP was assayed according to the Steiner et al. method [16]. Urine hydroxyproline was assessed by the Nobels et al. method [17].

Some animals were sacrificed 19 days after the tumor was implanted and samples of the left proximal tibia and distal femur were collected for histomorphometric evaluation by standard techniques [18]. The tibial specimens were fixed in 10% neutral buffered formalin, decalcified in 14% EDTA, and embedded in paraffin or methyl methacrylate. Sections were then prepared, mounted on glass slides, and stained with hemotoxylin and eosin for evaluation of cellular changes. The femoral specimens were fixed in 70% ethanol and embedded in methyl methacrylate. Undecalcified sections then were prepared, stained with silver nitrate, and counter-stained with toluidine blue for evaluation of changes in the volume of metaphyseal mineralized tissue. The periosteal and endosteal surfaces lining the cortical bone just below the tibial epiphysis (and at the same site in each group of animals) were evaluated for change in the distribution of bone cells. This area was chosen because it is a site of active bone formation and resorption. In normal animals, the periosteal surface in this area has numerous osteoclasts, whereas the endosteal surface has numerous osteoblasts.

Quantitative measurements were made of the distribution of osteoblastic and osteoclastic cells at the endosteal surface with the aid of a reticle containing a 10 × 10 mm grid. The grid was oriented so that the horizontal lines intersected the endosteal surface on a plane perpendicular to the long axis of the bone. Two entire grid fields were examined at a magnification of 100 ×. The relative number of active osteoblasts was then determined by counting the number of grid lines that intersected these cells on the endosteal surface. The data were expressed as a percentage of the total surface measured. The relative number of active osteoclasts was determined by counting the total number of these cells within the two grid fields. Active osteoblasts were identified as cells lining the bone surface that had a plump columnar or spindle shape and eccentrically-located nucleus, a perinuclear clear zone corresponding to a Golgi apparatus, and basophilic cytoplasm. Active osteoclasts were identified as large multinucleated cells, adjacent to bone surfaces, having a foamy-appearing eosinophilic cytoplasm.

The volume of mineralized tissue present in the proximal metaphysis of the femur was measured to detect the effects of the drug treatments on bone resorption. The mineralized tissue in this area in normal animals is removed by resorption as long as bones grow in length. A preliminary examination indicated that this bone resorption was very active in rats bearing the Leydig cell tumor. Quantitative measurements were made of the percentage of the metaphysis occupied by mineralized tissue by interfacing a microscope with an image analyzer (Quantimet 720, Cambridge Instruments). Measurements were made at a magnification of 50×.

Results

Figure 1 shows the effects of Cl_2MDP on hypercalcemia and the other parameters of the humoral hypercalcemia of malignancy, namely, increased urine calcium, increased urine phosphorus, decreased serum phosphorus, increased urine hydroxyproline, and increased urine cyclic AMP excretion. In this study, the drug was administered to the animals from the time of tumor implantation. Animals treated with either 2.5 or 40 mg/kg body weight/day of Cl_2MDP had delayed onset of hypercalcemia and hypercalciuria. Although animals treated with 40 mg/kg body weight/day eventually became hypercalcemia, the increases in serum calcium were much less marked than in untreated tumor-bearing animals. Increases in urine hydroxyproline excretion also were less marked in treated animals. In contrast, treatment with either dose of the drug had no effect on serum phosphorus, urine phosphorus excretion, or urine cyclic AMP excretion. There was no significant change in creatinine clearance of either the treated or untreated animals (data not shown).

Histomorphometric evaluation indicated that there was a marked *increase* in osteoclastic bone resorption and a marked *decrease* in osteoblastic activity in the untreated tumor-bearing animals. Many active osteoclasts were observed on tra-

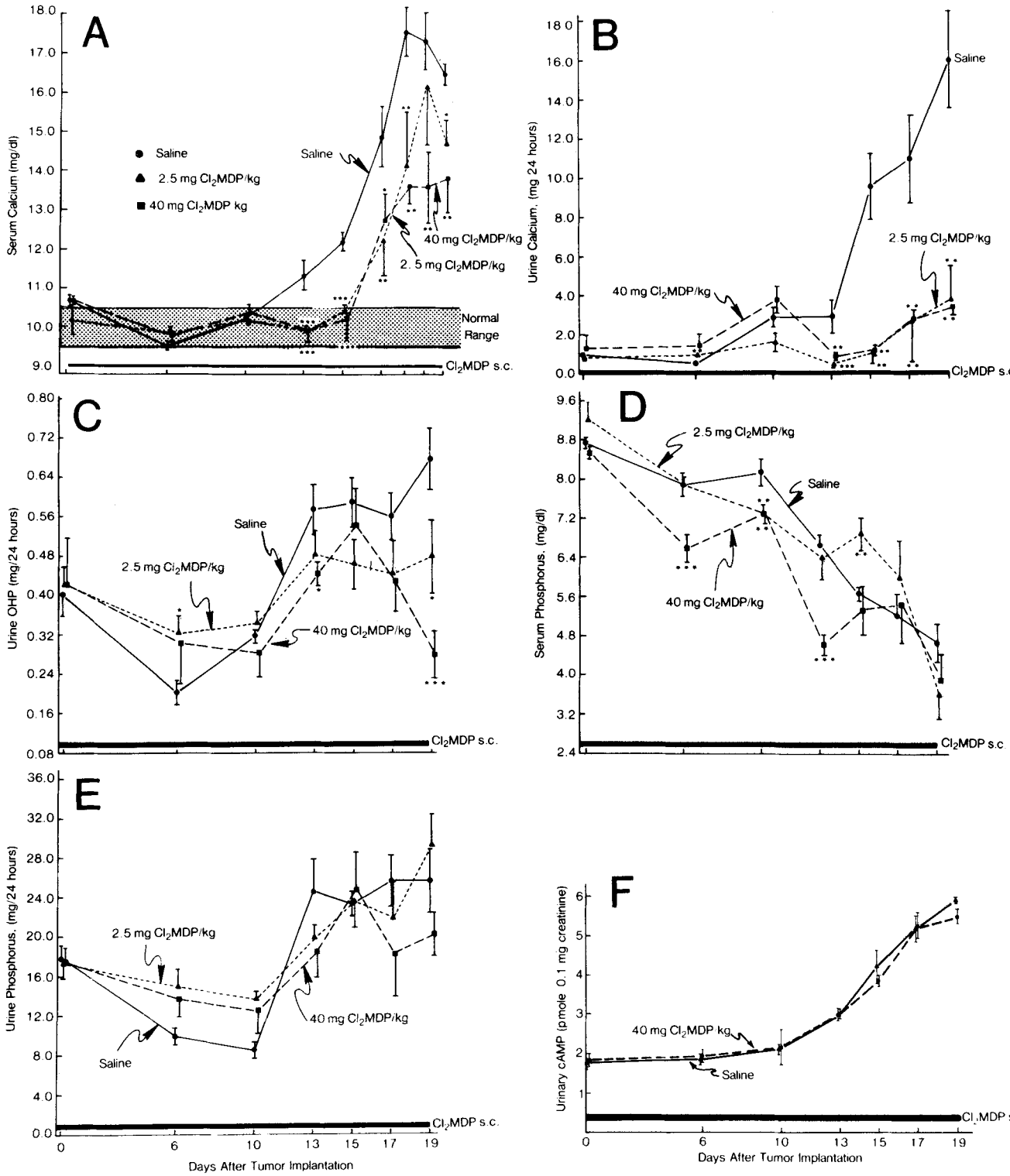

Fig. 1. Effects of Cl_2MDP administered subcutaneously to Fischer rats bearing Leydig cell tumors on **A** serum calcium, **B** urine calcium, **C** urine OHP, **D** serum phosphorus, **E** urine phosphorus, **F** urinary cAMP. The drug was given to the rats from the day of tumor implantation. Statistically significant at $P < 0.05$(*), $P < 0.01$(**), $P < 0.005$(***).

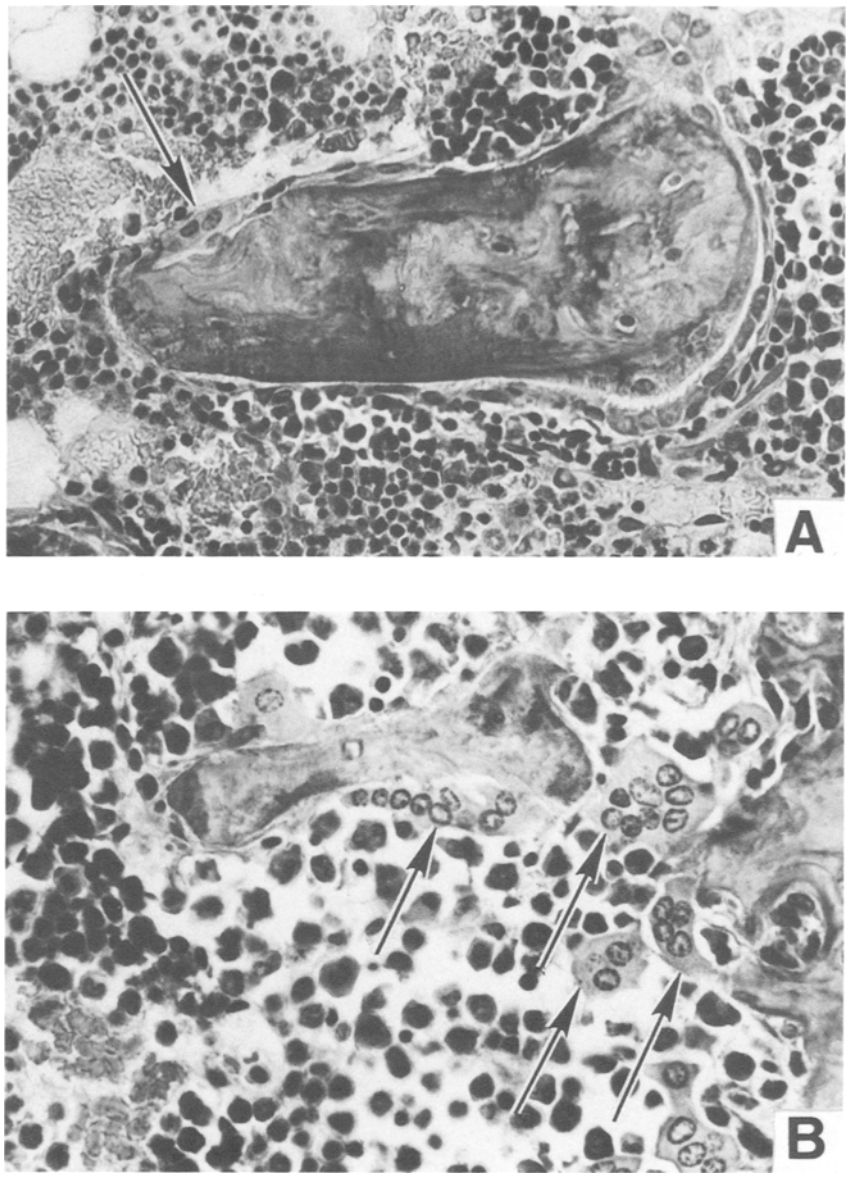

Fig. 2. Distribution of osteoclasts in the tibial metaphysis of normal and tumor-bearing rats. **A** Normal animal. Note the presence of a single "active" osteoclast on a trabecular bone surface (arrow). **B** Leydig cell tumor-bearing animal. Note that many more "active" osteoclasts are present (arrows), ×200.

Table 1. Effect of Cl_2MDP on bone cell distribution and metaphyseal mineral loss in the Leydig rat tumor

Treatment	Number of animals	Percent mineralized tissue (mean ± SE)	Number of active osteoclasts (mean ± SE)	Percent of endosteal surface with active osteoblasts (mean ± SE)
Nontreated control	5	30.4 ± 1.3[a]	8.0 ± 1[a]	100 ± 0[a]
Tumor-bearing control	4	12.4 ± 1.3	26.5 ± 3	0 ± 0
2.5 mg/kg/day Cl_2MDP	4	39.9 ± 1.6[a]	13.8 ± 1.3[a]	2.5 ± 2.5
40 mg/kg/day Cl_2MDP	3	46.9 ± 1.6[a,b]	1.7 ± 0.3[a,c]	10 ± 10

[a] Significantly different from tumor-bearing controls ($P < 0.05$)
[b] Significantly greater than the nontreated control ($P < 0.05$)
[c] Significantly less than the nontreated control ($P < 0.05$)

becular, endosteal, and periosteal bone surfaces (Fig. 2 and Table 1) and there was a marked loss of metaphyseal mineralized tissue (Fig. 3 and Table 1). Active osteoblasts, which are normally abundant on the endosteal and trabecular bone surfaces, often were replaced by osteoclasts or flat-shaped lining cells (Fig. 4 and Table 1).

In the Cl_2MDP-treated animals there was a marked, dose-dependent decrease in osteoclast number and in the loss of metaphyseal mineralized tissue (Table 1 and Fig. 3). In fact, in the 40 mg/kg group, osteoclast numbers were reduced and metaphyseal mineralized tissue values were increased over the values of the nontreated control animals (Table 1). This effect can be accounted for by the inhibitory effect that this compound can have on normal bone resorptive processes [1, 2]. Cl_2MDP treatment did not appear to have any effect on the reduction in osteoblastic activity caused by the tumor (Table 1).

Figure 5 shows the effects of treating tumor-bearing rats with Cl_2MDP after hypercalcemia was established. Administration of 2.5 mg/kg body weight of Cl_2MDP daily had minimal effects on the serum calcium, although serum calcium was significantly decreased by larger doses of the drug (Fig. 5). The effects on increased urine calcium excretion were even more marked (Fig. 5). Larger doses of the drug rapidly decreased the hypercalciuria, and hypercalciuria was even lowered by smaller doses of the drug (2.5 mg/kg body weight/day).

Discussion

Cl_2MDP effectively lowered the serum calcium in hypercalcemic animals. The drug was more effective in animals treated before they developed the tumor than in animals treated after the tumor had developed (Figs. 1 and 5). The fall in calcium occurred very rapidly with a near-maximal response produced within 24–48 h at the largest dose of the drug (Fig. 5). In animals treated with Cl_2MDP from the time of tumor implantation, the changes in the serum calcium were paralleled by simultaneous changes in the number of multinucleated osteoclasts seen at endosteal bone surfaces, and with changes in trabecular bone mass (Fig. 2, Table 1). The uri-

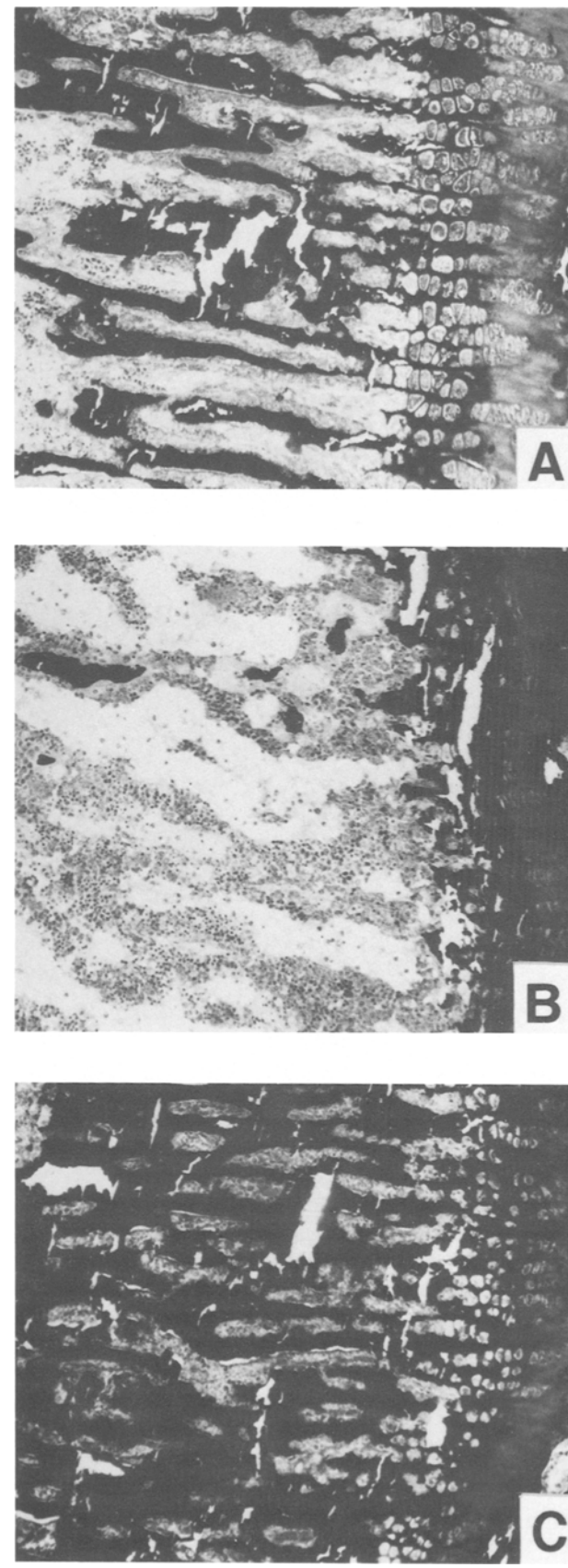

Fig. 3. Distribution of metaphyseal trabecular bone in normal, tumor-bearing, and tumor-bearing Cl_2MDP-treated rats. **A** Normal animal. Note the normal columnar distribution of trabecular bone (dark staining material). **B** Leydig cell tumor-bearing animal. Note that much of the trabecular bone is absent. **C** Tumor-bearing animal treated with Cl_2MDP at 40 mg/kg/day s.c. Note that there is much more trabecular bone present than in either the tumor-bearing or normal animals, ×200.

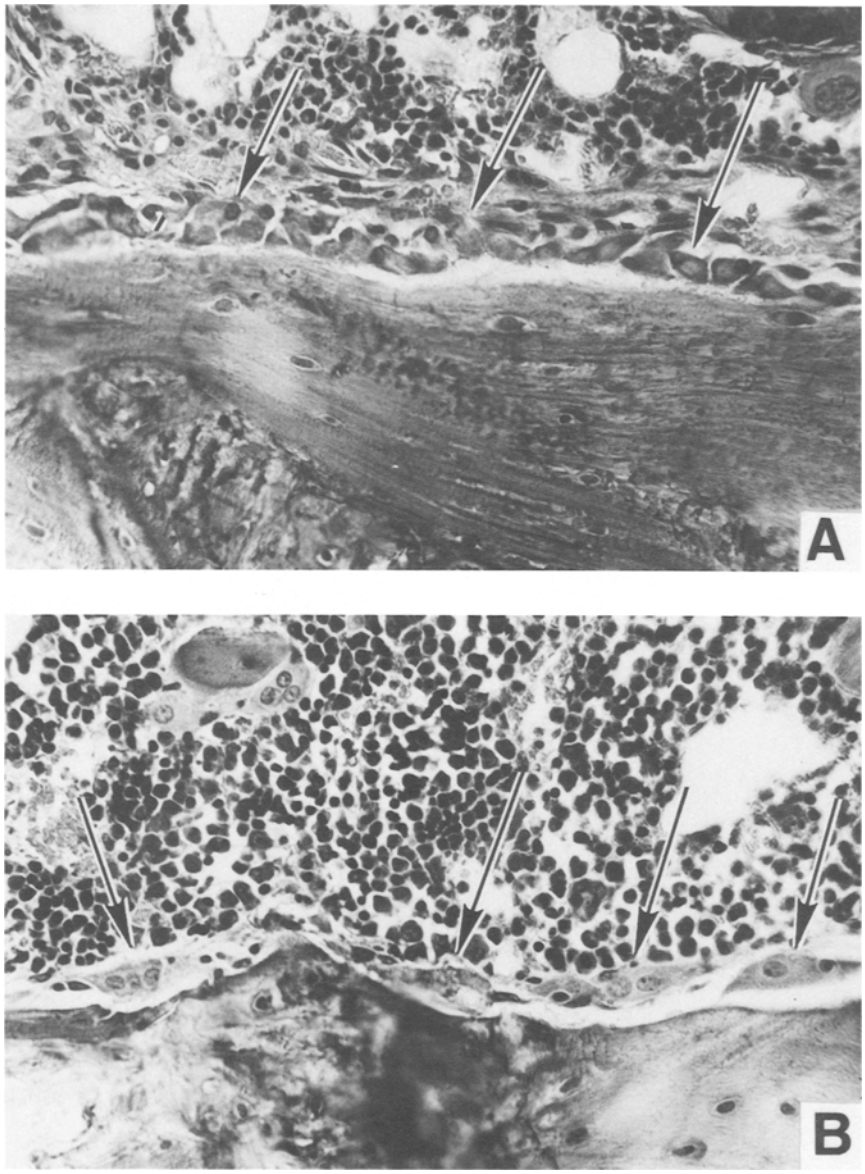

Fig. 4. Distribution of osteoblasts in the tibial metaphysis of normal and tumor-bearing animals. **A** Normal animal. Note the presence of many "active" osteoblasts on the endosteal bone surface (arrows). **B** Leydig cell tumor-bearing animal. Note the absence of osteoblasts; instead there is are numerous osteoclasts lining the endosteal surface, ×200.

nary hydroxyproline, which is an index of increased bone resorption, also decreased with treatment, indicating that the drug lowered the serum calcium by inhibition of bone resorption (Fig. 1). Although it is conceivable that Cl_2MDP may have produced these effects due to tumor cell cytotoxicity, this seems unlikely because there was no difference in tumor size in animals treated from the day of tumor implantation (Fig. 1), and Cl_2MDP had no effect on other nonbone-related features of the tumor, such as the renal tubular abnormalities.

The relationship between hypercalcemia and the other parameters associated with the humoral hypercalcemia of malignancy that are due to alterations in renal tubular function, namely, renal phosphate wasting and increased urine cyclic AMP excretion, is not known. In patients with breast cancer, it has been suggested that hypercalcemia causes phosphaturia directly [19]. The data produced in this study indicate that hypercalcemia and increased bone resorption are not related directly to renal phosphate wasting, hypophosphatemia, and increased urine cyclic AMP excretion, since the latter persisted in animals in whom Cl_2MDP inhibited bone resorption and lowered the serum calcium (Figs. 1 and 2, Table 1). It seems more likely that the humoral factor responsible for the hypercalcemia also has an independent effect on the renal tubules, similar to that of PTH. When the primary tumor was excised from a hypercalcemic animal, there was an

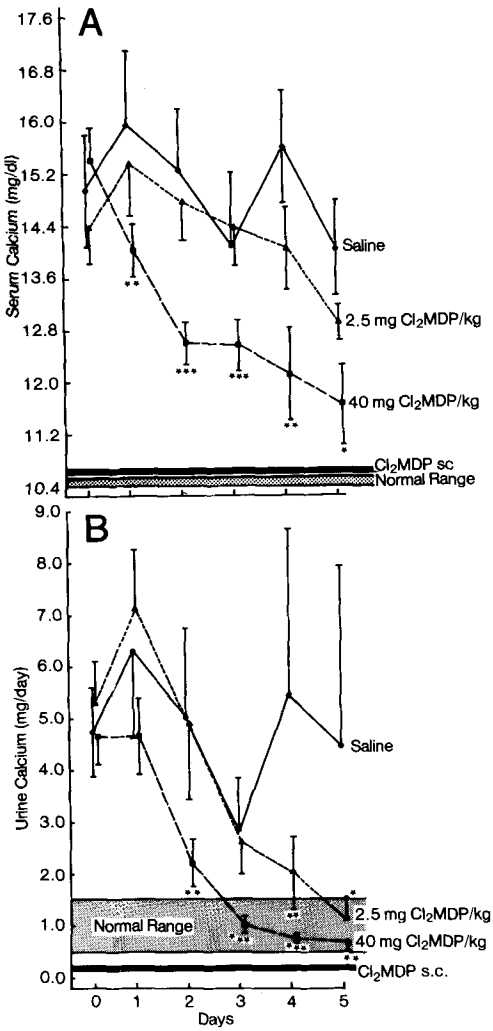

Fig. 5. Effects of Cl_2MDP administered to hypercalcemic tumor-bearing rats. At the time Cl_2MDP therapy was commended, the rats had been carrying the tumors for 12 days. Statistically significant at $P < 0.05$(*), $P < 0.01$(**), $P < 0.005$(***).

immediate fall in the serum calcium parallel with a fall in urine cyclic AMP excretion [5], which is consistent with this hypothesis.

The diphosphonates are efficacious compounds for the treatment of increased bone resorption associated with malignant disease. Three diphosphonates have been studied clinically: EHDP (ethane-1-hydroxy-1,1-diphosphonate), Cl_2MDP, and APD (amino-hydroxy propane diphosphonate) [8–11]. Although their mode of inhibiting bone resorption at the cellular and molecular level is still unclear, it has been shown both *in vitro* and *in vivo* that they inhibit osteoclastic bone resorption [7]. This has led to their clinical use in treating patients with disorders associated with increased bone resorption, such as Paget's disease [8–10] and the hypercalcemia of malignancy [11–13, 20]. Their effects when used orally are not as striking as when used parenterally, possibly because the absorption of the diphosphonates from the gastrointestinal tract ranges from about 2–7% of an oral dose [21]. Peak blood concentrations occur about 1½ h after administration and the drugs are excreted primarily from the kidney.

The agents that have received the most attention in the treatment of increased bone resorption associated with malignant disease are APD and Cl_2MPD. APD was used in a series of patients with myeloma and with breast cancer and was shown to lower the serum calcium and the urinary calcium in most of them [9]. Cl_2MDP has been used both orally and intravenously in patients with myeloma, in patients with breast cancer, and recently in patients with hyperparathyroidism and parathyroid carcinoma [12, 13, 20, 22, 23]. It is very effective when given either orally or intravenously. Myeloma studies were performed in patients treated for 3 months in a double-blind crossover study [12]. This was necessary because these patients were being treated simultaneously with cytotoxic drugs which were effective in therapy of multiple myeloma. Preliminary studies of EHDP indicate that it may be very effective in treating hypercalcemia when administered intravenously [24].

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