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Gut-mediated hypercalcemia in rabbits bearing VX_2 carcinoma: New mechanism for tumor-induced hypercalcemia

(prostaglandin E_2)

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ABSTRACT **The VX_2 carcinoma-bearing rabbit is an animal model for tumor-induced hypercalcemia, thought to be due to increased bone destruction effected by prostaglandin E_2. The present experiments suggest that the pathophysiology of the hypercalcemia differs from that previously proposed. Tumor was transplanted intramuscularly into 2.5- to 3-kg male New Zealand White rabbits, which were conditioned to a 1.5% calcium diet and treated with daily subcutaneous injections of dichloromethane diphosphonate (10 mg·kg^{-1}·day^{-1}), a potent inhibitor of bone resorption, or 0.9% NaCl (2 ml·kg^{-1}·day^{-1}). The diphosphonate had no significant effect on plasma Ca^{2+} in either group. After day 31, half the animals of each group were fed a calcium-free diet. This normalized the plasma Ca^{2+} in each VX_2-bearing rabbit within 3 to 4 days but had little effect in control rabbits. In a second series of experiments, VX_2-bearing rabbits maintained on standard rabbit chow were treated for 11 days with parenteral indomethacin (30–60 mg/day) or 0.9% NaCl. Although indomethacin normalized the markedly elevated urinary excretion of prostaglandin E_2, both treatment groups became severely hypercalcemic. Dietary calcium restriction promptly restored to normal the plasma Ca^{2+} concentration. In a third series of experiments, rabbits were fed standard rabbit chow and treated with oral indomethacin (40 mg/day) while control rabbits were pair fed an identical chow. Transplantation of VX_2 tumor into both groups caused hypercalcemia. We conclude that the hypercalcemia produced by this tumor strain is indomethacin resistant and dependent on an increase in gastrointestinal calcium absorption, not on skeletal calcium mobilization.**

Tumor-induced hypercalcemia is an aberration in calcium homeostasis caused by many different neoplasms (1). Hypercalcemia may be induced by direct bony invasion of tumor metastases. Some tumors, however, produce hypercalcemia without metastasizing to bone, presumably by secreting humoral substances such as parathyroid hormone, parathyroid hormone-like peptides, prostaglandins, osteoclast activitating factor, or vitamin D metabolites. Although the pathophysiology of the resultant hypercalcemia is poorly understood, tumor-induced hypercalcemia is thought to result primarily from bone resorption. This conclusion, however, is based primarily on *in vitro* evidence (1).

The VX_2 rabbit carcinoma produces fatal hypercalcemia by such a humoral mechanism, and the tumor secretes a substance, probably prostaglandin E_2 (PGE_2), that stimulates bone resorption *in vitro* (2–5). Both indomethacin and dichloromethane diphosphonate [$Cl_2C(PO_3H_2)_2$] reduce the bone calcium resorption produced by VX_2 rabbit carcinoma *in vitro* (4, 6) and the former is also effective *in vivo* (7). $Cl_2C(PO_3H_2)_2$ is a potent inhibitor of osteoclastic bone resorption *in vitro* and *in vivo* (8, 9). It has been used successfully to reduce the excessive bone resorption caused by Paget disease (10), multiple myeloma (11), and parathyroid hormone (12).

The original purpose of these experiments was to evaluate the efficacy of $Cl_2C(PO_3H_2)_2$ in controlling hypercalcemia in the VX_2 carcinoma-bearing rabbit *in vivo* but, as the work progressed it led to efforts to elucidate the mechanism of some unexpected aspects of the pathophysiology.

METHODS

Sixty-five 2.5- to 3-kg male New Zealand White rabbits were conditioned to a 12-hr light/12-hr dark schedule and received ad lib water and modified Kitchovsky diet containing 1.5% calcium, 0.27% phosphorus.* Only animals who maintained or increased body weight and ate at least 60 g of food/day were used. After 1–3 weeks of equilibration, they were divided into four groups for treatment: (*i*) VX_2 tumor implant followed by $Cl_2C(PO_3H_2)_2$ (10 mg·kg^{-1}·day), (*ii*) VX_2 tumor implant followed by 0.9% NaCl (2 ml·kg^{-1}·day^{-1}), (*iii*) $Cl_2C(PO_3H_2)_2$, (*iv*) 0.9% NaCl. Tumor tissue for implant was harvested under sterile conditions from donor rabbits. The tumor was minced, passed through a tissue press and mixed with 0.9% NaCl (0.4 of tissue/ml of 0.9% NaCl), and 1 ml of the resulting suspension was injected into the hamstring muscles bilaterally. The $Cl_2C(PO_3H_2)_2$ was freshly dissolved in 0.9% NaCl (5 mg/ml) every 48 hr and stored in the dark. $Cl_2C(PO_3H_2)_2$ and 0.9% NaCl were administered in comparable volumes as single daily subcutaneous injections, starting the day after tumor transplant.

Heparinized blood was obtained serially from the ear anaerobically 3.5–7 hr after the light cycle began and 18–24 hr after the previous $Cl_2C(PO_3H_2)_2$ or 0.9% NaCl injection and placed immediately on ice, and the plasma was promptly analyzed for Ca^{2+} (Nova 2 analyzer, Nova Biomedical, Newton, MA) with a coefficient of variation of ± 1 to 2%, for total calcium by atomic absorption spectroscopy, and for phosphorus (13). After 31 days, half the rabbits in each group were switched to a calcium-free diet (ICN) otherwise identical to the 1.5% calcium diet. Additional blood samples were obtained over the next 4 days.

In a second series of experiments, 20 rabbits were maintained on standard rabbit chow (1.6% Ca, 0.45% P_i; Allied Mills, Chicago) for 1–3 weeks prior to VX_2 tumor transplantation as above. Serial blood samples were analyzed for plasma ionized calcium. Beginning 18 days after tumor transplant, half the rabbits received indomethacin while the other half received comparable injections of 0.9% NaCl. The indomethacin was administered

Abbreviations: $Cl_2C(PO_3H_2)_2$, dichloromethane diphosphonate; PGE_2, prostaglandin E_2.

* The Kitchovsky diet is a cholesterol-free rabbit diet made by ICN (Cleveland, OH) from pure constituents. The modified diet contained additional calcium salts.

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subcutaneously at increasing dosages: 7.5 mg every 6 hr for 4 days, 10 mg every 6 hr for 3.5 days, and 10 mg every 4 hr for 3.5 days. The indomethacin was prepared immediately prior to each injection as a 0.1% solution in 3 mM Na_2CO_3 as recommended by the manufacturer. Equal volumes of 0.9% NaCl were administered subcutaneously on the same schedule.

On day 30 after tumor transplant, while still receiving indomethacin or NaCl injections every 4 hr, the rabbits were lightly anesthetized with ketamine and acepromazine and catheterized urine samples were obtained for analysis of PGE_2 by radioimmunoassay (14), using an antibody provided by L. Levine, and creatinine (15). The indomethacin and NaCl treatments then ceased; half of each treatment group was then switched to the modified Kitchovsky calcium-free diet. The other half of each treatment group continued to receive the standard rabbit chow. Serial blood samples were obtained for the next 3–6 days and analyzed for Ca^{2+} and phosphorus.

In a third series of experiments, 15 rabbits were maintained on standard rabbit chow and food consumption was monitored. During the 2 weeks prior to tumor transplant, food consumption averaged 50–75 g/day. Several days prior to tumor transplant, rationed feeding was instituted, each animal receiving 50 g of food/day, gradually increasing to 75 g/day by 30 days after transplant. Forty milligrams of indomethacin was added to the daily food allotment of eight rabbits. The indomethacin was dissolved in 95% ethanol (1 g/50 ml) and sprayed onto a monolayer of food, which was then allowed to dry at room temperature. The remaining seven control rabbits had equal volumes of ethanol sprayed onto their daily food allotments. Unconsumed food was removed and measured daily. Serial blood samples were analyzed for Ca^{2+}. Twenty-eight days after transplant, the control group began indomethacin treatment and, after 7 days of such treatment, catheterized urine samples were obtained and analyzed for PGE_2 and creatinine.

RESULTS

The plasma Ca^{2+} response to VX_2 tumor transplantation is shown in Fig. 1. Treatment with parenterally administered $Cl_2C(PO_3H_2)_2$ had no significant effect on the serum Ca^{2+} in the VX_2-bearing rabbits or in the controls. By 19 days after transplant, the VX_2-bearing rabbits were significantly hypercalcemic (mean difference from control, 0.384 mmol/liter, $P < 0.0001$). This difference increased to 0.828 mmol/liter by day 31 ($P < 0.0001$).

After day 31, each of the four groups of animals was divided into two subgroups matched for plasma Ca^{2+} concentration and weight. Plasma Ca^{2+} for the initial four groups was: group *i*, 2.517 ± 0.077 mmol/liter; group *ii*, 2.477 ± 0.077 mmol/liter; group *iii*, 1.691 ± 0.035 mmol/liter; group *iv*, 1.728 ± 0.029 mmol/liter (mean ± SEM). Half the subgroups were then switched to a calcium-free diet, while the remainder continued on the 1.5% calcium diet.

The VX_2-bearing animals switched to the calcium-free diet showed a dramatic decrease in plasma Ca^{2+} (mean decrease, 0.7 mmol/liter in 3 to 4 days; $P < 0.0001$ when compared with the value just before switching the diet). This eliminated the hypercalcemia and restored the Ca^{2+} level to the value before tumor transplantation. The VX_2-bearing rabbits maintained on the standard calcium diet showed little change in their Ca^{2+} values (Fig. 2). Half the animals switched to the calcium-free diet and half those maintained on the 1.5% calcium diet had continued treatment with $Cl_2C(PO_3H_2)_2$, but their Ca^{2+} concentrations were indistinguishable from the NaCl-treated counterparts on each diet, except that the plasma Ca^{2+} of the $Cl_2C(PO_3H_2)_2$-treated animals decreased somewhat more rapidly on the calcium-free diet (Fig. 2). The individual responses

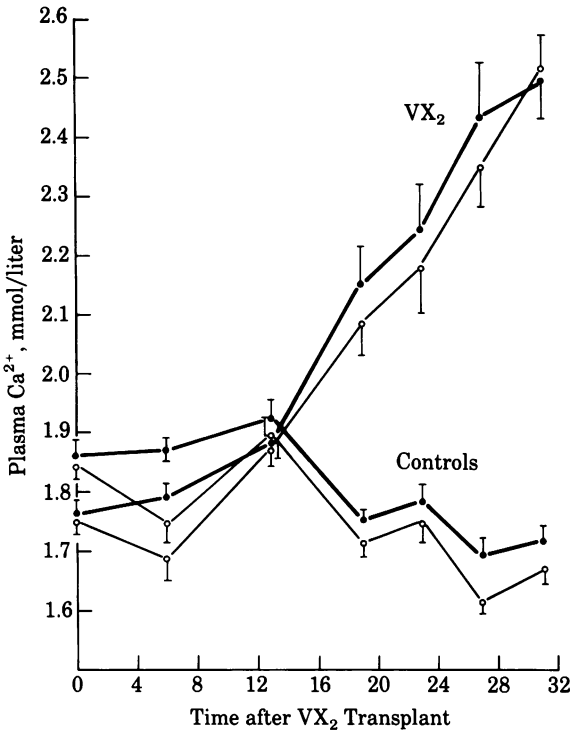

FIG. 1. Plasma Ca^{2+} response in control and VX_2 carcinoma-bearing rabbits with resistance of hypercalcemia due to treatment with $Cl_2C(PO_3H_2)_2$. Results represent mean ± SEM. Controls, $n = 13$ or 14; VX_2-bearing rabbits, $n = 18$ or 19. $P < 0.0001$ for VX_2 vs. controls on day 19 and day 31. ●——●, NaCl; ○——○, $Cl_2C(PO_3H_2)_2$ treated.

to changes in the diet in both saline-treated and $Cl_2C(PO_3H_2)_2$-treated VX_2-bearing rabbits are shown in Fig. 2 *B* and *C*. (The vertical scale in Fig. 2 *B* and *C* is compressed to one-half that in Fig. 2*A* to accomodate the marked changes produced by the diet switch.)

The plasma phosphorus of the saline-treated rabbits initially was 5.6 ± 0.12 mg/dl (mean ± SEM). With the onset of hypercalcemia, the plasma phosphorus decreased and, on day 31 it was 4.6 ± 0.32 mg/dl ($P < 0.01$). After the switch to the calcium-free diet, the phosphorus also promptly normalized (6.05 ± 0.36 mg/dl; *P*, not significant for rabbits on calcium-free diet 2–4 days vs. initial values). The rabbits maintained on the 1.5% calcium diet continued to have a significantly lower plasma phosphorus level (4.31 ± 0.38 mg/dl; $P < 0.01$ for rabbits on 1.5% calcium diet at day 33–35 vs. initial values). The rabbits receiving $Cl_2C(PO_3H_2)_2$ showed the same phosphorus responses.

For comparative purposes, a similar dietary experiment was carried out in both groups of normal rabbits, which were divided into matched subgroups. The plasma Ca^{2+} decreased only 0.08 mmol/liter ($P < 0.05$) in normal animals switched to the calcium-free diet and was unaffected in normal rabbits maintained on the standard diet. Again the results were similar in the $Cl_2C(PO_3H_2)_2$-treated and NaCl-treated animals (−0.050 and −0.113 mmol/liter, respectively).

The total plasma calcium levels in the VX_2-bearing rabbits paralleled the plasma Ca^{2+} in all groups, although the magnitude of the changes was less marked. The total plasma calcium in all VX_2-bearing rabbits was initially 14.50 ± 0.17 mg/dl and increased to 16.75 ± 0.22 mg/dl by day 31 ($P < 0.001$). This represented a 16% increase in total calcium, as compared with the 47% increase in Ca^{2+} in the same animals. The calcium-free diet again eliminated the hypercalcemia and restored the total plasma calcium to the pretransplant values.

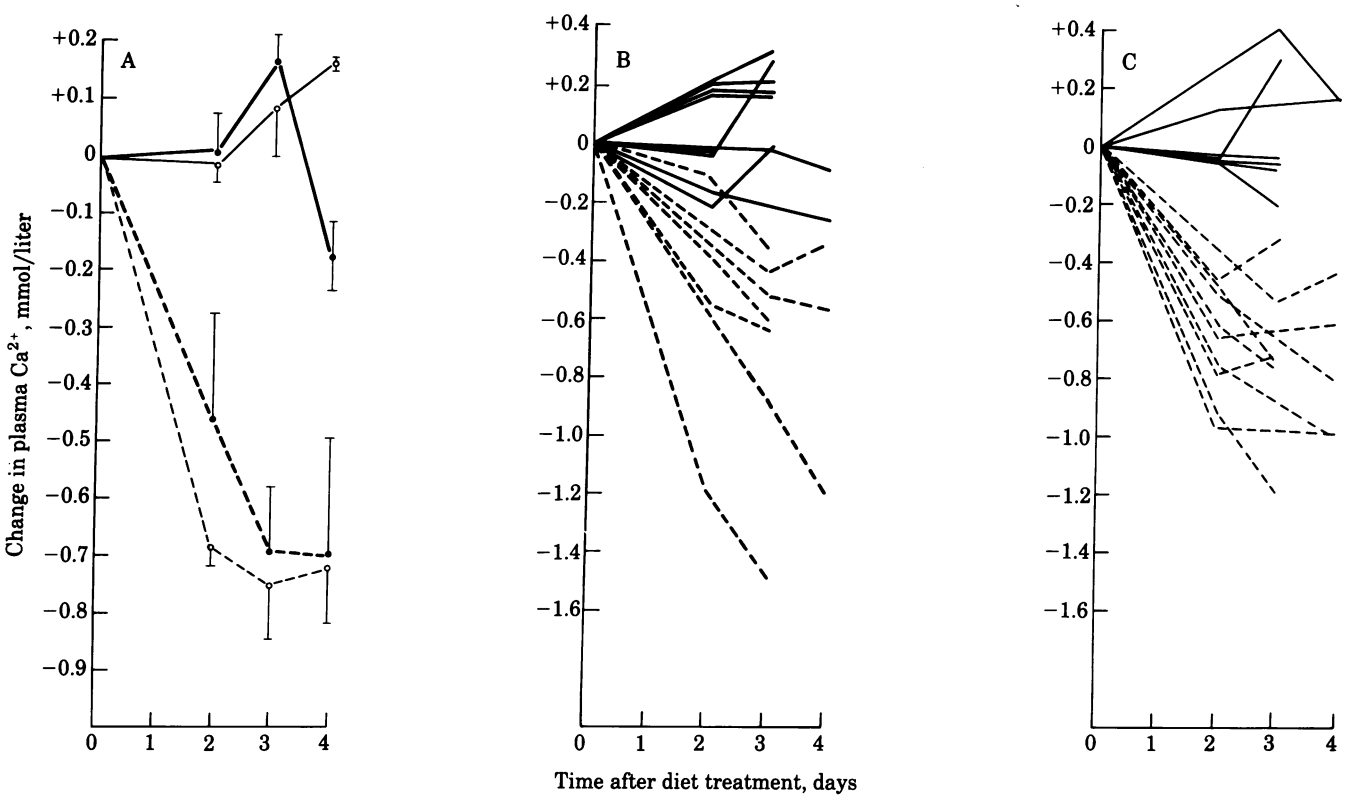

FIG. 2. (*A*) Diet dependence of hypercalcemia in VX_2 carcinoma-bearing rabbits. Results represent mean ± SEM for saline/high-calcium diet (●——●; $n = 7$ at days 2 and 3 and 2 at day 4), $Cl_2C(PO_3H_2)_2$/high-calcium diet (○——○; $n = 6$ at days 2 and 3 and 2 at day 4), saline/calcium-free diet (●- - -●; $n = 7$ or 8 at days 2 and 3 and 3 at day 4), $Cl_2C(PO_3H_2)_2$/calcium-free diet (○- - -○; $n = 7$–9 at days 2 and 3 and 5 at day 4). $P < 0.0001$ at days 3 and 4 for all animals on calcium-free diet vs. prediet treatment values. (*B*) Individual plasma Ca^{2+} response to diet treatment in VX_2-bearing rabbits receiving daily saline injections. Results represent change from plasma Ca^{2+} before diet treatment. (*C*) Individual plasma Ca^{2+} response to diet treatment in VX_2-bearing rabbits receiving daily $Cl_2C(PO_3H_2)_2$ injections. Results represent change from plasma Ca^{2+} before diet treatment. (*B* and *C*) —, High-calcium diet continued; ---, calcium-free diet started.

The plasma Ca^{2+} response of rabbits maintained on the standard rabbit chow in the second series of experiments is shown in Fig. 3. The plasma Ca^{2+} averaged 2.57 mmol/liter by 17 days after tumor transplant and 2.89 mmol/liter by 19 days after transplant, both being significantly elevated from initial values ($P < 0.00001$). Parenteral indomethacin treatment failed to affect the hypercalcemia, which actually increased by 0.483 mmol/liter (from 2.788 to 3.271), which paralleled the increase in plasma Ca^{2+} in the rabbits receiving parenteral NaCl injections.

The urinary PGE_2 excretion on day 30, expressed as ng/mg of creatinine, was dramatically lower in the indomethacin-treated rabbits (2.047 ± 0.759 ng/mg of creatinine) than in the saline-treated rabbits (11.087 ± 4.187 ng/mg of creatinine; $P < 0.01$). Urinary PGE_2 excretion in six normal rabbits on the same diet was 2.594 ± 0.869 ng/mg of creatinine (VX_2-bearing saline-treated rabbits vs. controls, $P < 0.05$; VX_2-bearing indomethacin-treated rabbits vs. controls, P not significant).

Although the hypercalcemia was unaffected by indomethacin treatment, subsequent dietary calcium restriction resulted in a prompt return of the plasma Ca^{2+} to pretreatment values (Fig. 3). After 11 days of treatment with indomethacin, the persistent hypercalcemia was associated with a decrease in plasma phosphorus (4.9 ± 0.14 mg/dl) compared with initial values (6.6 ± 0.18 mg/dl; $P < 0.0001$). The normalization of Ca^{2+} to pretreatment values after dietary restriction of calcium was also associated with normalization of the plasma phosphorus values (6.2 ± 0.02 mg/dl) as seen in the first series of experiments.

Since parenteral indomethacin was ineffective in reversing the hypercalcemia resulting from VX_2 tumor implant, a third series of experiments was carried out to see whether early continued treatment with oral indomethacin would prevent the hypercalcemia. Prior to beginning oral indomethacin treatment, the food consumption of all animals was monitored daily. Based on these measurements pair feeding of 50 g/day was instituted when treatment began. The unconsumed food was also measured daily. Once treatment began, rabbits receiving oral indomethacin showed an immediate reduced food consumption to an amount that averaged 77% of that for ethanol-treated rabbits. Twenty-eight days after tumor transplant, the rabbits receiving ethanol treatment were switched to oral indomethacin treatment. Their food consumption also decreased immediately on changing treatment, this decrease averaging 35% for the 2 days before and after treatment change. Postmortem examination showed gastritis and, in cases of sudden death, gastric perforation in the indomethacin-treated rabbits.

By the 28th day after tumor transplant, the plasma Ca^{2+} had increased by 0.617 ± 0.15 mmol/liter in the six surviving rabbits receiving oral indomethacin treatment ($P < 0.01$), while the plasma Ca^{2+} had increased by 1.33 ± 0.23 mmol/liter in the eight rabbits receiving vehicle. Thereafter, these control rabbits received treatment with oral indomethacin, despite which their mean plasma Ca^{2+} increased from the values on the 28th day by an additional 0.391 mmol/liter after seven days of treatment ($n = 7$) and 0.101 mmol/liter ($n = 4$) after 11 days of treatment. The hypercalcemia persisted despite subnormal urinary PGE_2 excretion after 7 days of oral indomethacin treatment [0.329 ± 0.083 ng/mg of creatinine ($n = 5$) for VX_2-bearing oral indomethacin-treated rabbits vs. normal rabbits; $P < 0.01$].

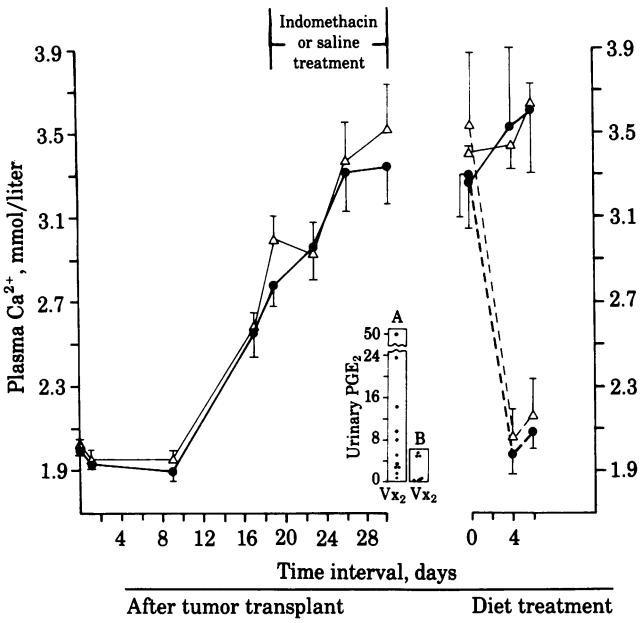

FIG. 3. (*Left*) Plasma Ca^{2+} response in VX_2 carcinoma-bearing rabbits with resistance to parenterally administered indomethacin. Results represent mean ± SEM. ●, Indomethacin treated (n = 10); △, saline treated (n = 10). (*Middle*) Individual urinary PGE_2 excretion on day 30 (ng/mg of creatinine). (*A*) Saline treated (n = 11); (*B*) indomethacin treated (n = 9). (*Right*) Plasma Ca^{2+} response in same rabbits after diet treatment. Results represent mean ± SEM. (●——●, Standard chow, previously indomethacin treated (n = 3 or 4); △——△, standard chow, previously saline treated (n = 5); ●- - -●, calcium-free diet, previously indomethacin treated (n = 4); △- - -△, calcium-free diet, previously saline treated (n = 5).

DISCUSSION

In initial experiments with VX_2 carcinoma in rabbits, we uncovered a surprising result. Hypercalcemia in tumor-bearing rabbits could be demonstrated only if the animals were fed a diet high in calcium. This finding seemed inconsistent with previous views that the hypercalcemia was due principally to increased bone destruction; hence, the experimental design was altered to the protocol summarized in this report.

We conclude that the VX_2 carcinoma in the rabbit produces hypercalcemia consistently within 2 to 3 weeks but only if the animals ingest ample calcium in their diet. The prompt return of the plasma ionized and total calcium to initial values following removal of dietary calcium shows that the primary source of the excess blood calcium is the gut, conflicting with previous reports that hypercalcemia is due to increased bone resorption (4, 16, 17). Failure of parenterally administered $Cl_2C(PO_3H_2)_2$ to prevent or inhibit the hypercalcemia is strong evidence against an osseous source of the hypercalcemia because this drug effectively inhibits bone resorption (8) and, as we and others have shown, given parenterally at 10 $mg \cdot kg^{-1} \cdot day^{-1}$, very effectively controls bone resorption in rats and rabbits and hypercalcemia of bone origin in rats (6, 12), whereas dietary restriction of calcium alone does not (12).

Bone may play, of course, a passive role in maintaining the hypercalcemic state. At any steady-state calcium level, there is an equilibrium between calcium fluxes into and out of bone, the latter being due to bone resorption and fluxes of calcium from bone mediated by the osteoblast–osteocyte lining-cell syncytium. With hypercalcemia (here of gut origin), there is an increased flux of calcium into bone (18). If the calcium efflux from bone were increased in parallel, the bone could at least play a passive role in maintaining the elevated plasma calcium. However, calcium kinetic studies have not been reported in rabbits bearing this tumor.

The following observations have suggested to others that the VX_2 carcinoma produces prostaglandin-mediated hypercalcemia by stimulating bone resorption. (*i*) The VX_2 carcinoma secretes prostaglandins of the E series *in vivo* and *in vitro*, and plasma levels of PGE_2 are elevated in rabbits bearing the VX_2 carcinoma. (*ii*) Tissue extracts and tissue culture media of this tumor exhibit a bone-resorbing activity that, in culture media, is decreased by culturing in the presence of indomethacin. (*iii*) Synthetic PGE_2 also stimulated bone resorption *in vitro*. (*iv*) Bone turnover is increased in rabbits bearing the VX_2 tumors.

The recent discovery, however, that PGE_2 stimulates renal 25-hydroxyvitamin D-1-hydroxylase activity emphasizes the need for caution in extrapolating *in vitro* data to a more complex *in vivo* model (19). In fact, the *in vivo* evidence that prostaglandins mediate the hypercalcemia of the VX_2 tumor is tenuous. (*i*) The ability of intravenous PGE_2 infusions to produce hypercalcemia *in vivo* is disputed (20–22). (*ii*) Indomethacin treatment only occasionally reverses the hypercalcemia of patients with tumor-induced hypercalcemia and evidence of excessive PGE_2 secretion (23). (*iii*) The addition of indomethacin to the diet of VX_2-bearing rabbits only variably reduces the hypercalcemia (3, 4) according to the published literature, and we cannot confirm any consistent reduction of plasma calcium in VX_2-bearing rabbits receiving indomethacin doses equal to or greater than those reported in the literature as long as consumption of food with a generous calcium content remains normal.

Urinary PGE_2 excretion is generally considered to reflect renal PGE_2 production (24, 25). PGE_2 is primarily metabolized in the lung and only small quantities are cleared intact by the kidney (25). It is surprising that the urinary PGE_2 excretion of VX_2-bearing rabbits is so high. This may reflect either increased renal production of PGE_2, inadequate pulmonary and renal clearance of tumor-produced PGE_2 due to extremely high blood PGE_2 levels, or the effect of extremely high levels of urinary PGE_2-metabolites that crossreact with our antibody in the radioimmunoassay (L. Levine, personal communication). Indomethacin is known to act systemically, blocking PGE_2 synthesis regardless of the site of production. The marked decrease in urinary PGE_2 excretion to normal in our indomethacin-treated rabbits implied that an effective systemic dose of indomethacin was achieved. Despite this, the plasma Ca^{2+} continued to increase. The half-life of parenteral indomethacin is 2 hr (26). Considering the dosage and frequency of injections and the marked reduction of urinary PGE_2 excretion, it seems unlikely that insufficient drug was used.

In the third series of experiments, the addition of indomethacin to the diet produced gastritis, resulting in decreased food consumption and occasional gastric perforation. Nevertheless, significant hypercalcemia was seen in all rabbits surviving >3 weeks, and three of seven showed marked hypercalcemia. The dose of oral indomethacin used in this experiment was that reported by Voelkel *et al.* (4) to reverse, delay, or prevent hypercalcemia in VX_2-tumor-bearing rabbits. Nonetheless subsequent oral indomethacin treatment for 11 days of the seven rabbits with established hypercalcemia 28 days after tumor transplant effected no response. Serious doubt is therefore raised as to a primary role for prostaglandin in mediating the hypercalcemia in this animal model. PGE_2 may merely be a tumor marker as suggested by Caro *et al.* (27). Our tumor was obtained indirectly from the Mason Research Institute, which traces the lineage back to Kidd and Rous (28). Nonetheless, it is possible that our strain of VX_2 tumor is different from that used by other investigators (4). Independent of such consid-

eration, however, these data establish a new mechanism for tumor-induced hypercalcemia in animals.

In a recent series of experiments, we inoculated *nude* mice subcutaneously with VX_2 carcinoma cells, with subsequent growth of large subcutaneous tumors. These animals developed severe hypercalcemia when fed diets containing 1.2% calcium; the calcium level reverted to normal despite continued tumor growth when diets were switched to low-calcium content. These results (*i*) suggest that the tumor-elaborated hypercalcemic factor is not limited in action to rabbits, (*ii*) confirm the dietary calcium dependence of the hypercalcemia due to tumor, and (*iii*) may prove useful in efforts to isolate the active principal in the tumor and further characterize its biological actions. Additional work will be necessary to determine whether vitamin D or its metabolites or peptides related to parathyroid hormone are involved in the gut-mediated hypercalcemia produced by VX_2 carcinoma or whether some other factor or factors differing from known hormones mediate the inappropriately high intestinal calcium absorption in these animals.

Of further interest is the possible relevance of these studies to the hypercalcemia frequently seen in humans with cancer. Intestinal hyperabsorption of dietary calcium is important in the pathogenesis of hypercalcemia in patients with sarcoidosis and vitamin D intoxication, but the hypercalcemia of cancer patients has generally been attributed to excessive calcium fluxes from bone. The present studies suggest that the role of intestinal hyperabsorption of calcium should be investigated more fully in cancer patients, particularly patients with humorally mediated hypercalcemia that is not reversed by indomethacin treatment.

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