Effects of Calcium and Magnesium Acetates on the Carcinogenicity of Cadmium Chloride in Wistar Rats

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ABSTRACT

The effects of calcium and magnesium acetates on the formation of injection site and testicular tumors in male Wistar rats over 2 years following s.c. injections of cadmium chloride (CdCl₂) were determined. The rats (25/group) received a single s.c. dose of CdCl₂ (0.02 or 0.04 mmol/kg; 0.9% NaCl solutions). Calcium and magnesium acetates were administered as 3% dietary supplements for 2 weeks prior to and 2 weeks after the CdCl₂ injection, or as three daily s.c. injections (0.16 mmol calcium acetate per kg, 4 mmol magnesium acetate per kg; 0.9% NaCl solutions) at the same site as CdCl₂ on the day before, the day of, and the day after CdCl₂ dosing. Control groups were given 0.9% NaCl solution instead of CdCl₂ plus s.c. or dietary calcium and magnesium acetates. In rats given injections of CdCl₂ alone, the final tumor yields were 33 and 34% of rats at risk at the injection site (mostly fibrosarcomas) and 86 and 85% of rats at risk in the testes (mostly interstitial cell tumors), respectively, for the low- and high-CdCl₂ doses. In control rats, the corresponding tumor yields were 0% at the site of 0.9% NaCl solution injection and 30% in the testes. Dietary calcium and magnesium acetates or s.c. calcium acetate did not affect significantly the tumor yields and latent periods. Simultaneous injections of magnesium acetate at the same site completely prevented the development of injection site tumors for both CdCl₂ doses but had no effect on the final yields of testicular tumors. CdCl₂ injection also caused significant elevation of incidence of the pancreatic islet cell tumors (8.5 versus 2.2%) regardless of any other experimental treatment. These results provide further evidence that the divalent carcinogenic metals may exert their activity through an antagonism with the physiologically essential divalent metals.

INTRODUCTION

It has been shown previously that some physiologically essential divalent metals can suppress the actions of their carcinogetic counterparts. Interactions of this type have been reviewed comprehensively in a recent article by Nordberg and Andersen (26). The most striking findings reported so far include the antagonistic effects of zinc upon cadmium tumorigenicity (6-12, 23, 26, 28, 29, 41) and manganese inhibition of the development of muscle tumors (33, 34) and of in vitro cell transformation (3) induced by nickel subsulfide. More recently, an antagonism between the divalent physiological metals calcium or magnesium and 2 carcinogenic ones (nickel and lead) has been investigated in our laboratory (30). Both calcium and magnesium completely prevent the sharp increase in lung tumor formation in strain A mice induced by nickel and lead acetates (30). The described antagonism may result from a biochemical competition between the essential and nonessential metal cations for some tissue and cellular ligands (18). Such competition has been especially well studied with regard to the metabolism and toxicity of cadmium (16, 17). Hypercalcemia and deficiency of calcium are often observed in workers occupationally exposed to cadmium (14, 31). A low-calcium diet facilitates the intake and retention of cadmium in rats (21, 37) since both metals compete for intestinal absorption (32, 39). In vitro investigations have disclosed that cadmium can replace calcium and zinc in certain enzymatic and carrier proteins (17); decrease the stability of the DNA molecule, which depends on the Ca²⁺, Mg²⁺, and Na⁺ cations (17); and decrease the fidelity of nucleotide incorporation into RNA (17). The latter infidelity can be overcome by increased concentrations of Mg²⁺, the obligatory ion for proper transcription (17). Similarly, calcium may reverse the inhibition of DNA synthesis caused by cadmium in the liver cell culture (13), and calcium and zinc may protect against overall cadmium cytotoxicity (36). All these findings encouraged us to explore whether calcium and magnesium could also affect the carcinogenicity of cadmium.

MATERIALS AND METHODS

Cadmium(II) chloride 2.5-hydrate (CdCl₂), calcium(II) acetate monohydrate (calcium acetate), and magnesium(II) acetate tetrahydrate (magnesium acetate), Fisher-certified reagents, were purchased from Fisher Scientific Co., Pittsburgh, Pa. Injection solutions of those chemicals were prepared in 0.9% NaCl solution and filtered through 0.22-µm Nalgene sterilization filters (NaIgo Co., Rochester, N. Y.). Purina laboratory chow meal was used throughout the experiment to feed the animals or to prepare the special diet containing supplements of either calcium acetate or magnesium acetate. The diet was obtained by blending the meal with 3% (by weight of anhydrous salt) admixture of calcium acetate or magnesium acetate on a Patterson-Kelley Co. V-Blender for 30 min. All diets were stored at room temperature and supplied to the animals ad libitum. Four hundred fifty male Wistar rats, purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and weighing 120 to 150 g, were held after arrival for 2 weeks prior to initiation of the experiment. They were then divided into 18 groups (25 rats/group), housed in polycarbonate cages on a cornocob bedding (3 rats/cage), and finally treated as described in Table 1. CdCl₂ was applied at the nape of the neck as a 0.005 or 0.01 ml solution in amounts of 4 ml/kg of body weight (0.02 and 0.04 mmol of CdCl₂ per kg). Solutions of 0.04 mmol calcium acetate or 1.0 mm magnesium acetate were injected at the same site on the day prior to, the day of, and the day after the CdCl₂ injection, in amounts of 4 ml/kg (0.16 mmol calcium acetate per kg; 4.0 mmol magnesium acetate per kg). Animals receiving CdCl₂ and one of the acetates, or 0.9% NaCl solution, on the same day were given injections of a common solution containing the defined molarities of both compounds. No precipitation was evident in the resulting mixed solutions. Control groups were given 4-ml 0.9% NaCl solution doses per kg instead of CdCl₂. The doses of calcium and magnesium acetates had been established in separate toxicity studies as the maximum tolerated doses.

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of both salts under the described conditions. The experiment lasted 110 weeks. Over that period of time, the body weights, survival, tumor occurrence, and necrotic observations were recorded. Tissues were preserved in 10% formalin and examined by light microscopy. The incidence of injection site tumors and survival data were analyzed with the computer program developed and described by Thomas et al. (35). The unadjusted analysis of proportions of tumor-bearing rats was done by the $x^2$ test utilizing the Cochran-Armitage test for trend, if appropriate. Mortality-adjusted comparisons of tumor incidences in rats at risk were made both graphically and analytically. Computer-derived Kaplan-Meier survival curves (19, 35) were used to plot the complementary tumor incidence curves for the injection site tumors, since these were fatal lesions. Comparisons among treatments were made by Cox’s test and/or the generalized Kruskal-Wallis test (35). These methods of statistical analysis all gave essentially the same results. Therefore, in most instances, wherever the $p$ value is given, it is based on the $x^2$ test for Cox’s analysis. The tumors of the testes and of the pancreas, observed incidentally, were analyzed statistically by the $x^2$ test. Since the tumor yields from the corresponding groups of rats, receiving 0- (Groups 1 and 10), low- (Groups 4 and 13), and high- (Groups 7 and 16) CdCl$_2$ doses, were essentially identical in both the feeding and injection studies, for statistical purposes, the results from those groups were combined.

**RESULTS**

No acute toxic effects of the CdCl$_2$ injections or of the calcium acetate and magnesium acetate treatments were observed. No rats died over the first 36 experimental weeks, when the first injection site tumor developed. However, the body weight gain data indicated a temporary depressive effect of the high-CdCl$_2$ dose on the growth of rats up to the 12th week after injection (compare curves in Charts 1 and 2, C, with those in Charts 1 and 2, A and B). At the seventh experimental week, for example, the mean body weight of the placebo-injected rats was 375 ± 10 g. The body weight of the low-dose CdCl$_2$ recipients was 350 ± 8 g, and that of the high-dose CdCl$_2$-injected rats equaled 326 ± 5 g, irrespective of the calcium acetate or magnesium acetate treatment. The difference between the body weights of the controls and the high-CdCl$_2$-dose recipients was statistically significant, at $p < 0.01$. None of the treatments investigated exerted consistent significant effects on the survival of the experimental animals (Table 2).

The cumulative incidences of the injection site tumors are shown in Charts 3 and 4; the final tumor incidences are listed in Table 2. As can be seen, CdCl$_2$ at both dosage levels produced a significant tumor response at the injection site. The final yields of the injection site tumors in rats receiving CdCl$_2$ alone were practically the same for both doses: 33% of animals at risk (24% of the initial number of rats) in the low-dose groups and 34% (28%) in the high-dose groups versus 0% in the control 0.9% NaCl solution-injected groups (Charts 3 and 4; Table 2). There were statistically highly significant differences between the control group and either experimental group ($p < 0.0008$). Dietary calcium acetate and magnesium acetate exerted no statistically significant effect on those values despite some dissimilarities among the curves in Chart 3 ($p > 0.7$ for the differences among curves in Chart 3A; $p > 0.2$ for Chart 3B; $p = 1$ for Chart 3C).

Injections s.c. of calcium acetate in rats which received either dose of CdCl$_2$ also had no statistically significant effect on the incidence of injection site tumors ($p > 0.4$; Chart 4, B and C). Two injection site tumors which developed in the control rats at the site of calcium acetate injection (Chart 4A) appeared statistically nonsignificant compared to the other rats which were not given CdCl$_2$ ($p > 0.2$). Administration s.c. of magnesium acetate, however, completely prevented the development of tumors at the injection site, with $p < 0.02$ for the low-CdCl$_2$ dose and $p < 0.01$ for the high-CdCl$_2$ dose (Chart 4, B and C).

CdCl$_2$ treatment at both dose levels produced testicular tumors in rats (Table 2). The final yields of testicular tumors were much higher than those of the injection site tumors. The incidences were practically equal for both doses of CdCl$_2$ investigated (Table 2): 86% in the rats receiving the low-CdCl$_2$ dose and surviving more than 52 weeks; and 85% in the corresponding high-dose rats. It is noteworthy that the incidence of testicular tumors in the control rats was unexpectedly high: 30% in rats receiving no carcinogen and surviving more than 52 weeks. The incidences of testicular tumors in animals sacrificed at the termination of the study were 44, 100, and 79% in the control (Groups 1 and 10 combined), the low-dose (Groups 4 and 13 combined), and high-dose CdCl$_2$ (Groups 7 and 16 combined) groups, respectively. The corresponding tumor incidences in rats dying between 52 and 109 experimental weeks were 6, 75, and 94% of the control, low-dose, and high-dose groups, respectively. Thus, both doses of CdCl$_2$ significantly decreased the latent period and increased the final yield of testicular tumors in rats. Administration of calcium acetate or magnesium acetate along with CdCl$_2$, by both dietary or s.c. routes, had little effect on the occurrence of testicular tumors (Table 2). However, according to the Cochran-Armitage test, which considers the final incidence of tumors in the original number of animals, the
Inhibition of Cadmium Carcinogenesis

Chart 1. Body weight gain curves of rats given injections s.c. of a single dose of CdCl₂ or 0.9% NaCl solution and fed a 3% calcium acetate-containing diet, a 3% magnesium acetate-containing diet, or an ordinary diet for 2 weeks before and 2 weeks after the injection. A, control rats given injections of 0.9% NaCl solution; B, rats given injections of 0.02 mmol of CdCl₂ per kg; C, rats given injections of 0.04 mmol of CdCl₂ per kg. Curves 1, rats fed an ordinary diet; Curves 2, rats fed calcium acetate; and Curves 3, rats fed magnesium acetate. A slight, but statistically significant depression of the body growth was noticed only for rats given injections of the high-CdCl₂ dose up to the 12th week of the experiment (C, p < 0.02). Neither calcium acetate nor magnesium acetate had any significant effect on the growth rate.

Chart 2. Body weight gain curves of rats given injections s.c. of a single dose of CdCl₂ or 0.9% NaCl solution and 3 doses of 0.16 mmol of calcium acetate per kg, 4.0 mmol of magnesium acetate per kg, or 0.9% NaCl solution on the day before, the day of, and the day after the CdCl₂ injection. A, control rats given injections of 3 doses of 0.9% NaCl solution in place of the other chemicals; B, rats given injections of 0.02 mmol of CdCl₂ per kg; C, rats given injections of 0.04 mmol of CdCl₂ per kg. Curves 1, rats given injections of 0.9% NaCl solution; Curves 2, rats treated with calcium acetate; Curves 3, rats treated with magnesium acetate. Statistically significant differences were found only between body weight gain in the control rats and rats given 0.04 mmol of CdCl₂ per kg (up to the 12th week; p < 0.02). Calcium acetate and magnesium acetate had no significant influence on the effects of CdCl₂ or on the growth of control animals.

Tumor incidence in rats given injections of the high-CdCl₂ dose and fed calcium acetate (Table 2) was significantly lower than in the high-CdCl₂ dose-treated rats fed ordinary diet (p < 0.0003) or the magnesium acetate diet (p < 0.02). No other protective effects against testicular tumorigenesis by CdCl₂ could be observed in rats treated with either calcium acetate or magnesium acetate (Table 2).

All tumors observed in these studies were classified histologically (Table 2). Such examination revealed that the tumors at the injection site in the 300 rats treated with CdCl₂ included: 35
Twenty-five animals per group were given injections of CdCl₂ or 0.9% NaCl solution. They were simultaneously treated with dietary or injected calcium acetate or magnesium acetate as described in Table 1. All surviving animals were sacrificed 110 weeks later.

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The tumors observed were predominantly fibrosarcomas, undifferentiated sarcomas, and histiocytic sarcomas. The effect of time on tumor development is shown in Charts 3 and 4.

Except for 3 mesotheliomas, all testicular tumors were interstitial cell tumors.

All tumors were adenomas except for single adenocarcinomas in Groups 2, 6, 16, and 18, and for 3 adenocarcinomas observed in Group 12.

All tumors were adenomas except for single adenocarcinomas in Groups 4, 5, 6, and 18, and for 2 adenocarcinomas found in Groups 9, 11, and 15.

fibrosarcomas; 10 undifferentiated sarcomas; 6 histiocytic sarcomas; 4 osteosarcomas; one basal cell carcinoma; one sebaceous cell carcinoma; 3 histiocytes; 3 fibromas; and one schwannoma. The testes of the same rats contained 206 interstitial cell tumors and one mesothelioma. Tumors in other organs included: 70 pancreatic tumors (48 acinar plus 22 islet cell tumors); 77 adrenal tumors (51 medullary pheochromocytomas plus 26 cortical tumors); 63 pituitary gland tumors (all chromophobe adenomas); and single liver, thyroid, and mammary gland tumors. Among the 150 control rats which were not given CdCl₂, the following types of tumors were found (Table 2): at the injection sites of 0.9% NaCl solution, calcium acetate, or magnesium acetate, one fibrosarcoma and 2 schwannomas; in the testes, 31 interstitial cell tumors and 2 mesotheliomas; in the pancreas, 41 tumors (38 acinar plus 3 islet cell tumors); in the adrenals, 35 tumors (25 pheochromocytomas plus 10 cortical tumors); and in the pituitary gland, 37 chromophobe adenomas. Single hepatic, thyroid, and mammary gland tumors were found among the control animals. Except for the testis and the injection site, none of the organs examined developed a tumor incidence significantly different from that of control animals. Similarly, except in the pancreas, none of the treatments altered the relative
than in rats not receiving CdCl2 (3 of 137 or 2.2%) and surviving tumors appeared in the CdCl2-injected rats (22 of 259 or 8.5%).

A significantly higher incidence of pancreatic islet cell tumors appeared in the CdCl2-injected rats than in rats not receiving CdCl2 (3 of 137 or 2.2%) and surviving tumors appeared in the CdCl2-injected rats (22 of 259 or 8.5%).

Proportions of the histological types of tumors within any single organ. A significantly higher incidence of pancreatic islet cell tumors appeared in the CdCl2-injected rats (22 of 259 or 8.5%) than in rats not receiving CdCl2 (3 of 137 or 2.2%) and surviving more than 1 year, the time of appearance of the first such tumor (p < 0.02). Also, of the 86 acinar cell tumors observed in the present study, 13 (0.52 tumors/rat) appeared in a single group that received 3 successive injections of calcium acetate (Table 2, Group 11). The remaining CdCl2-untreated animals developed an average of 0.22 ± 0.06 (S.E.) acinar tumors/rat, while the CdCl2-treated rats developed 0.18 ± 0.03 tumors/animal. The significance of the elevation in acinar cell tumor formation in the calcium acetate-treated rats remains obscure. CdCl2 treatment did not affect the ratio of malignant to benign tumors seen in the pancreas (Table 2). The great majority of both acinar cell and islet cell tumors were typical well-differentiated adenomas.

**DISCUSSION**

The results of the present investigation demonstrate clearly that the development of tumors at the site of a s.c. CdCl2 injection can be prevented by simultaneous injections of magnesium acetate at the same place. Injections of calcium acetate did not produce such protective effects. On the contrary, the relatively high local toxicity of calcium acetate may have assisted cadmium carcinogenesis at the injection site, although the effects appeared statistically insignificant (Table 1; Chart 4). Dietary administration of either calcium acetate or magnesium acetate was completely ineffective in preventing CdCl2-induced sarcomas. Similarly, both physiologically essential metals appeared to be generally ineffective in preventing CdCl2-induced testicular tumors. The final yields of testicular tumors in the CdCl2-treated groups were not significantly different except among rats fed calcium acetate. The results of the dietary calcium acetate treatment were equivocal. Calcium acetate exerted some protective effects on the testes of rats given injections of the high-CdCl2 dose; those effects of dietary calcium acetate were not noticed at the low-CdCl2 dose. Magnesium acetate, either fed or injected, furnished no protection against testicular tumors induced by CdCl2.

Neither calcium acetate nor magnesium acetate had any noticeable influence on the growth of animals or on the mild inhibition of growth caused by the administration of the high-CdCl2 dose. Nor did they exert any significant effect upon tumor development at sites other than the injection site and the pancreas in either the CdCl2-treated or -untreated rats.

This experiment furnishes the first evidence that CdCl2 gives rise to the development of the pancreatic islet cell tumors. This finding, however, requires further experimental verification.

On the basis of previous theoretical considerations and experimental observations, the results of the present investigations are rather unexpected. Since the ionic radius of Cd2+ (0.97 Å) is much closer to the radius of Ca2+ (0.99 Å) than to Mg2+ (0.66 Å) (40), a much stronger biochemical interaction may be anticipated between cadmium and calcium than between cadmium and magnesium. A similar prediction might be made based upon the order of affinity for ligands: zinc > cadmium > calcium > magnesium > potassium (27). Moreover, it is calcium, not magnesium, that has been reported frequently to antagonize cadmium toxicity (13, 15, 20, 27, 36-39). Under the conditions of this experiment, however, calcium failed to protect the tissues against cadmium carcinogenesis. This fact may be attributed to the relatively low dose of calcium which could be safely administered to rats by the s.c. route. That dose, 0.16 mmol calcium acetate per kg for 3 days, was only 12 or 24 times the CdCl2 dose. The corresponding dose of magnesium acetate applied, 4.0 mmol/kg for 3 days, was 300 to 600 times greater than that of CdCl2. A large excess of the competing metal appears to be critical to protect against cadmium toxicity (27). Even such a well-defined cadmium antagonist as zinc can exert its antitumorogenic activity only if administered in high doses, e.g., 100 times those of cadmium (6, 26, 28, 29). Another important factor which might affect the results of this experiment is the very long biological half-life of cadmium, which exceeds 200 days (24, 25). This means that cadmium can persist in animal tissues much longer than its metallic antagonists and resume its toxic action after the latter are excreted. Zinc is again a good example in illustrating such transitory protection. In the experiments of Gunn et al. (6, 8), a single s.c. dose of 0.03 mmol cadmium per kg was injected to rats alone or with 3 daily doses of a total of 3 mmol CdCl2.
zinc per kg. In rats given cadmium alone, typical degenerative injury of the testis developed in 2 days, while in the zinc-treated animals, the onset of the injurious cadmium effects was delayed by 3 to 8 weeks, when the protective level of zinc in the body was lowered by excretion (6–10). One may thus postulate that the inhibition of cadmium carcinogenesis requires sustained, elevated levels of the antagonistic metal within the target tissue. These requirements were met to some extent in our experiment only by magnesium acetate and only at the site of injection. The concentration of magnesium at the injection site, following its administration, may have been high enough to hinder the retention of cadmium in the local tissues. This might be done by either competing for the molecular binding sites or reducing the biochemical reactivity of cadmium in the injection solution. Competition between cadmium and magnesium for the binding sites in skin, s.c. connective tissue, and muscle around the site of injection may also be expected, since magnesium is an integral component of those tissues (1, 4), and cadmium is known to be retained there (23). Thus, if at least a part of cadmium is bound by displacing magnesium, elevated concentrations of the latter should prevent the displacement. Magnesium may also compete with cadmium for any other divalent metal binding sites. On the other hand, it seems possible that concentrated magnesium acetate alters the biological availability of cadmium from the common CdCl₂ solution by forcing hydrolysis of the latter and by suppressing dissociation of the resulting basic cadmium salts in the injection solution (17), which eventually would result in a significant decrease of the cadmium tissue binding. It can be anticipated that the excess amounts of cadmium and magnesium which have not been immediately bound at the site of injection are then quickly transported away and undergo separate redistribution within the body. The nonphysiological excess of magnesium is diluted by body fluids and excreted in the urine in a few hr (1), thus becoming no longer able to prevent cadmium retention and carcinogenicity in any other organ or tissue. To confirm or reject such possibilities, further experiments with radioactive ¹ⁱ¹Cd are currently in progress in our laboratory. The lack of response of cadmium-induced testicular tumor formation to magnesium and calcium treatment can be attributed to possible pituitary involvement in testicular carcinogenesis. A single s.c. injection of cadmium has been shown to suppress androgen levels in testes and serum (22) and to stimulate a persistent hyperplasia of the gonadotropic cells of the anterior pituitary in hamsters (2, 5). A similar response in rats would provide a prolonged proliferative stimulus to the interstitial cells surviving cadmium cytoxicity. If such stimulation by the pituitary gland is involved in cadmium-induced testicular carcinogenesis, it does not appear to be significantly altered by the doses of calcium and magnesium used in these studies.

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