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SOFT-TISSUE CALCIFICATION INDUCED BY RARE EARTH METALS AND ITS PREVENTION BY SODIUM PYROPHOSPHATE

BY

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The increasing utilization of rare earth metals in several branches of industry has stimulated a greater interest in their pharmacology and toxicology. These have recently been the subject of a detailed review (Haley, 1965). Generally, these elements are considered to possess a low to moderately acute toxicity (Haley, 1965), their most characteristic pharmacological action being on blood coagulation (Aiazzi-Mancini, 1927) and on the liver (Snyder, Cress & Kyker, 1959). Here we would like to report on the production of soft-tissue calcification by these metals. The presence of calcium salts in granulomas induced by the intradermal injection of several lanthanides in the guinea pig has been described (Haley & Upham, 1963) as well as the calcifying activity of cerium and lanthanum chlorides (Selye, 1962 ; Gabbiani & Tuchweber, 1965). These two compounds, together with several others (mostly metals) which are called calcergens, elicit calcification at their site of subcutaneous injection in normal animals (Selye, 1962). Previous experiments have shown that all calcergens, whilst soluble in water, precipitate with phosphate or carbonate when added to Tyrode solution in approximately the same amount which produces calcification in live animals (Gabbiani, 1964). To investigate further the value of this test in predicting the calcifying activity of a substance, we examined the solubility of the chlorides of all rare earth elements (except the radioactive promethium) in Tyrode solution and subsequently studied their local and systemic calcifying activity in the living animal.

Since sodium pyrophosphate inhibits calcification induced by lead acetate, one of the most potent calcergens (Gabbiani, 1966), we also examined the possibility of inhibiting, by means of sodium pyrophosphate, the experimental lesions induced by the chlorides of rare earth metals.

METHODS

The compounds tested in our experiments were the trichlorides of scandium, yttrium, lanthanum, cerium, praseodymium, neodymium, samarium, europium, gadolinium, therbium, dysprosium, holmium, erbium, thulium, ytterbium and lutetium (K & K Laboratories Inc, Plainview, N.Y., U.S.A.). First, we diluted 50 mg of each compound in 20 ml. Tyrode solution composed of NaCl (0.8%), KCl (0.002%), $MgCl_2$ (0.001%), NaH_2PO_4 (0.005%), $NaHCO_3$ (0.1%), $CaCl_2$ (0.02%) and having a *p*H of 7.6 as previously described (Gabbiani, 1964).

For the investigation *in vivo,* 470 female Sprague-Dawley rats of the Holtzman strain with a mean initial body weight of 101 g (range 93–105 g) were divided into 47 equal groups for the performance of five experiments as indicated in Tables 1–5. In the first experiment (Table 1) we tested the subcutaneous calcifying activity of rare earth elements. The animals received, each time in 0.2 ml. distilled water, one subcutaneous injection of 10 μg, 100 μg and 1 mg of the various chlorides (in the interscapular, lower thoracic and sacral regions respectively).

In the second experiment (Table 2) we tested the systemic calcifying action of rare earth metals: the various chlorides were injected intravenously in 1 ml. distilled water at the following doses: scandium and therbium chlorides 8 mg ; yttrium and thulium chlorides 10 mg ; samarium, dyprosium, holmium, erbium, ytterbium and lutetium chlorides 12 mg ; lanthanum, cerium, europium, gadolinium, praseodymium and neodymium chlorides 15 mg. These were the highest tolerated dosages as established by preliminary toxicity tests in our animals.

In the third experiment (Table 3), we did a chronological study of the histology of the lesions. One group served as intact control and the other four groups were injected intravenously with 10 mg holmium chloride and killed 1 hr, 5 hr, 24 hr and 4 days after the injection.

For the fourth experiment (Table 4), the animals first received three subcutaneous injections, each time in 0.2 ml., these were: distilled water in the interscapular region ; sodium orthophosphate monobasic (Na_2HPO_4, Fisher Scientific Company, Fairlawn, N.Y., U.S.A.) 2 mg in the lower thoracic region ; and finally sodium pyrophosphate ($Na_4P_2O_7.10H_2O$, J. T. Baker Chemical Co, Phillipsburg, N.J., U.S.A.) 2 mg in the sacral region. These injections were followed immediately by administration of 200 μg of the chlorides in 0.2 ml. distilled water, by the same route and at the same place, so that we could compare in the same animal the action of the different pretreatments.

For the fifth experiment (Table 5), sodium pyrophosphate was given intravenously in a dose of 5 mg in 1 ml. distilled water. This was immediately followed by the intravenous injection of the chlorides in a dose of 10 mg in 1 ml. water, except for therbium chloride, which was given in a dose of 8 mg.

During the course of experiments the rats were maintained exclusively on Purina Laboratory Chow (Purina Co. of Canada) and tap water. Unless otherwise stated, the experiments were terminated on the fourth day by killing the animals with chloroform. At autopsy, the diameter of the calcified wheal produced by local treatment was measured in mm and the internal organ lesions were judged by inspection with a binocular loupe in terms of an arbitrary scale in which 0=no lesion, 1=just detectable, 2=moderate and 3=most severe lesion, as previously described (Selye, 1962). The values for calcification with standard deviation given in Tables 1, 2, 4 & 5 are each an aggregate score from 10 rats ; a similar scale is used for von Kóssa positivity (Table 3). Specimens of the injected skin and of the affected organs were fixed in alcohol-formol (4 parts of absolute alcohol and 1 part of 10% neutral formalin) for subsequent embedding in paraffin and staining by the PAS stain for polysaccharides, the von Kóssa technique for phosphate and carbonate, and chloranilic acid for calcium (Carr, Rambo & Feichtmeir, 1961 ; Eisenstein, Werner, Papajiannis, Konetzki & Laing, 1961). For the demonstration of lipids, the material was fixed in neutral formalin and subsequently stained with oil red.

RESULTS

Solubility of chlorides of rare earth metals in Tyrode solution and their subcutaneous calcifying activity in vivo.—Table 1 shows the local calcifying activity of the chlorides of rare earth elements after subcutaneous injection in aqueous solution ; it also shows that precipitation occurs in Tyrode solution. The subcutaneous calcification increases with the dose and has the appearance of a well-delimited whitish plaque with no tendency to invade the dermis (Fig. 3 top). Microscopically, calcification (as judged by the positivity to both von Kóssa and chloranilic acid stains) takes place mostly in contact with collagen fibres, and a moderate degree of fibrous reaction with formation of giant cells, may be noted.

TABLE 1
SOLUBILITY IN TYRODE SOLUTION AND LOCAL CALCIFYING ACTIVITY OF RARE EARTHS

Group	Rare earth	Precipitation in Tyrode	Local calcification (mm)		
			10 μg	100 μg	1 mg
1	$ScCl_3$	+	0	3±1·5	14±1·2
2	YCl_3	+	4±1·3	9±0·7	18±0·7
3	$LaCl_3$	+	0	8±0·4	16±0·5
4	$CeCl_3$	+	0	8±0·7	17±0·5
5	$PrCl_3$	+	0	6±0·8	14±0·4
6	$NdCl_3$	+	0	5±0·9	15±0·6
7	$SmCl_3$	+	0	9±0·3	19±0·6
8	$EuCl_3$	+	0	5±0·2	18±0·6
9	$GdCl_3$	+	0	8±0·9	17±0·7
10	$TbCl_3$	+	0·4±0·2	10±0·5	19±0·5
11	$DyCl_3$	+	0	7±0·5	17±0·7
12	$HoCl_3$	+	0	9±0·9	16±0·9
13	$ErCl_3$	+	0	11±0·3	15±0·8
14	$TmCl_3$	+	0	10±0·5	19±0·5
15	$YbCl_3$	+	0	10±0·4	19±0·6
16	$LuCl_3$	+	0	9±0·3	18±0·4

Systemic calcifying action of rare earth metals.—As seen in Table 2, all the chlorides injected, except scandium and lanthanum chlorides, induced calcification of the spleen in various degrees. Upon macroscopic inspection, the organ showed several whitish spots, and the cut surface revealed white rings around the follicles (Fig. 1). Histologically, the calcified material was almost exclusively deposited in the marginal zone and in the

TABLE 2
SYSTEMIC CALCIFYING ACTIVITY OF RARE EARTHS

Group	Rare earth	Splenic Calcification (Scale 0–3)	Mortality (%)
1	$ScCl_3$	0	0
2	YCl_3	1·7±0·4	10
3	$LaCl_3$	0	30
4	$CeCl_3$	1·5±0·2	60
5	$PrCl_3$	0·6±0·2	40
6	$NdCl_3$	2·2±0·3	50
7	$SmCl_3$	2·1±0·3	0
8	$EuCl_3$	2·2±0·4	20
9	$GdCl_3$	2·7±0·3	30
10	$TbCl_3$	2·4±0·2	10
11	$DyCl_3$	1·0±0·6	30
12	$HoCl_3$	2·3±0·2	30
13	$ErCl_3$	2·8±0·2	10
14	$TmCl_3$	1·6±0·2	10
15	$YbCl_3$	1·0±0·2	10
16	$LuCl_3$	1·1±0·3	0

red pulp (Fig. 2); it was mainly located intracellularly, but also appeared in the extracellular space. A moderate degree of fibrous reaction was also noticeable in the calcified zones. A few foci of cardiac necrosis were evident in the animals treated with erbium, therbium and thulium. Upon histological examination, the Kupffer cells of the hepatic sinusoids revealed the presence of von Kóssa positive material, though this was not constant. Some rats also showed haemorrhagic spots in the thymus, and all the treated

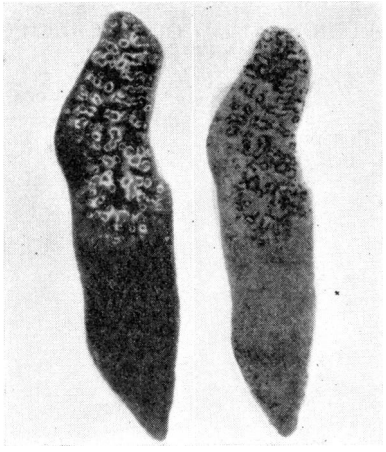

Fig. 1. Splenic calcification induced after i.v. injection of $ErCl_3$. *Left*: At macroscopic examination, the spleen shows white rings of calcified material around the follicles. *Right*: After staining of the spleen "in toto" with the von Kóssa technique, the calcified material appears black.

animals had a strong tendency to bleed ; this observation is in accordance with previous findings. Less commonly, though evident at autopsy, there was discoloration of the liver, typical of fatty degeneration and an increase of lipids after staining with oil red. Few animals showed evidence of hepatic necrosis and calcification.

Chronology of the lesions induced by holmium chloride.—As shown in Table 3, the degree of splenic and hepatic lesions studied by histological means differed at the various time intervals studied. Firstly, we noted the appearance of von Kóssa positive material

TABLE 3
CHRONOLOGY OF THE LESIONS PRODUCED BY $HoCl_2$

Group	Time of Autopsy*	Von Kóssa positivity	
		Spleen	Kupfer cells
1	1 hr	0	0
2	5 hrs	1·0±0·2	0
3	24 hrs	1·9±0·1	3·0
4	4th day	3·0	0·8±0·2

* All animals received at 0 hour $HoCl_3$ 10 mg in 1 ml. water intravenously.

5 hr after the injection of holmium chloride, at the marginal zone between the lymphatic follicles and the red pulp of the spleen. After 24 hr in the same location, the von Kóssa reaction was very pronounced and the chloranilic acid stain demonstrated the presence of calcium salts. At that time the Kupffer cells of the liver appeared to be hypertrophic and filled with von Kóssa positive material (Fig. 2), but only very irregularly stained by chloranilic acd. On the fourth day, while the deposition of calcium salts in the spleen was much increased, the von Kóssa positive material in the Kupffer cells disappeared almost completely.

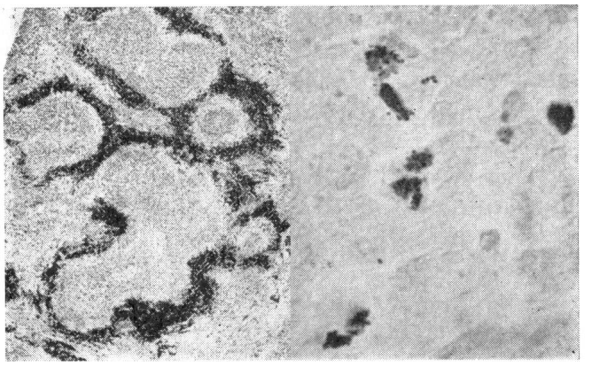

Fig. 2. Spleen and liver lesions induced by rare earths. *Left*: Calcification of the marginal zone and the red pulp of the spleen after i.v. injection of $HoCl_3$ (von Kóssa, ×35). *Right*: Granules of von Kóssa positive material deposited in the Kupffer cells of the liver 24 hr after the injection of $HoCl_3$ (von Kóssa, ×1200).

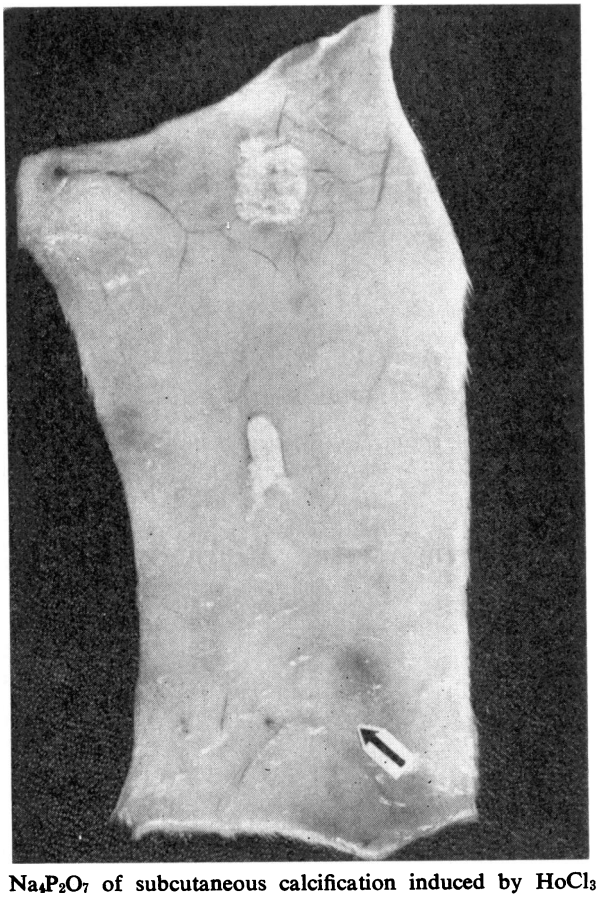

Fig. 3. Inhibition by $Na_4P_2O_7$ of subcutaneous calcification induced by $HoCl_3$ s.c. Reversed skin showing. *Top*: Site treated with $HoCl_3$ and distilled water. *Middle*: $HoCl_3$ and Na_2HPO_4. *Bottom (arrow)*: $HoCl_3$ and $Na_4P_2O_7$.

Prevention of the local calcifying action of rare earth metals by sodium pyrophosphate.—As shown in Table 4, the calcifying activity of rare earth metals was not changed by the addition of distilled water or sodium orthophosphate. However, the addition of pyrophosphate completely inhibited the calcification induced by the various chlorides (Fig. 3).

TABLE 4

PREVENTION BY $Na_4P_2O_7$ OF THE SUBCUTANEOUS CALCIFICATION INDUCED BY RARE EARTHS

Group	Rare earth	Cutaneous calcification (mm) at site of H_2O	Na_2HPO_4	$Na_4P_2O_7$
1	$HoCl_3$	10±0·9	6±0·5	0
2	$GdCl_3$	7±1	6±0·8	0
3	$DyCl_3$	10±0·5	7±0·5	0
4	YCl_3	10±0·5	6±0·6	0

Prevention of the systemic calcifying action of rare earth elements by sodium pyrophosphate.—As can be seen in Table 5, splenic calcification induced by the chlorides of rare earth elements was prevented by sodium pyrophosphate (Fig. 4). On histological examination, the spleen and liver appeared normal. The mortality rate induced by rare earth metals was not influenced by pyrophosphate administration.

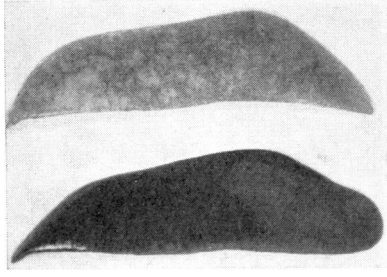

Fig. 4. Inhibition by $Na_4P_2O_7$ of splenic calcification induced by $HoCl_3$ i.v. *Top*: Spleen of an animal injected with $HoCl_3$ i.v. *Bottom*: Inhibition of splenic calcification in an animal injected with $HoCl_3$ and $Na_4P_2O_7$ i.v.

TABLE 5

PREVENTION BY $Na_4P_2O_7$ OF THE SYSTEMIC CALCIFICATION INDUCED BY RARE EARTHS

Group	Rare earth	Splenic calcification (Scale 0–3) None	Na-Pyro	Mortality % None	Na-Pyro
1	YCl_3	2·8±0·2	0·6±0·3	40	30
2	$GdCl_3$	2·2±0·3	0	0	10
3	$TbCl_3$	2·2±0·2	0·1±0·1	10	30
4	$DyCl_3$	2·8±0·2	0·3±0·2	10	30
5	$HoCl_3$	2·9±0·1	0·1±0·1	0	20

DISCUSSION

Among the pharmacological effects of rare earth elements, the anticoagulant action, the induction of fatty liver and the irritating effect on the skin and ocular tissues are well known (Haley, 1965). However, little has been reported on their calcifying activity.

It is known that these metals are deposited in bone (Jowsey, Rowland & Marshall, 1958) and costochondral junctions (Asling, Hamilton, Axelrod-Heller & Louie, 1952), but the possibility that they could participate actively in the process of calcification has not been mentioned. Previous work has shown that two rare earth elements, cerium and lanthanum, induce connective-tissue calcification at the site of their subcutaneous administration (Selye, 1962). For this reason, the two metals were classified among the so-called calcergens or direct calcifiers (Selye, 1962). The experimental lesion induced by these substances has been shown, by means of both chemical and X-ray diffraction techniques, to consist of calcific deposits which are mainly in the form of hydroxyapatite crystals (Selye, Gabbiani & Tuchweber, 1964 ; Jean & Desaulniers, 1965 ; Moss & Urist, 1964).

The present investigation demonstrates that all the rare earth elements induce connective-tissue calcification at the site of subcutaneous injection. Their insolubility in Tyrode solution increases the value of this test in predicting the possible calcifying activity of a metallic compound. While subcutaneous calcification is produced by metals of different groups (Selye, 1962), the induction of splenic calcification after intravenous administration appears to be typical of the lanthanides. The nature of calcified deposits is demonstrated by a positive von Kóssa reaction and chloranilic acid stain. The von Kóssa reaction has long been considered specific for calcified deposits but demonstrates only the anions (e.g., phosphate and carbonate) which are precipitated with calcium (Pearse, 1960). Chloranilic acid stain is, on the other hand, considered specific for the calcium ion (Carr, Rambo & Feichtmeir, 1961 ; Eisenstein, Werner, Papajiannis, Konetzki & Laing, 1961).

Calcification starts in the macrophages of the marginal zone and invades the red pulp, forming a ring around the follicles. On the fourth day, the calcified material is found both intracellularly and extracellularly. Intracellular calcification is rarely observed either under pathological or experimental conditions (Selye, 1962 ; Gabbiani & Tuchweber, 1963). The similarity between the localization of calcium salts and that of acid phosphatase in the normal spleen (Gomori, 1941) is striking. On this basis, one could speculate upon the participation of this enzyme in the pathological process. It is known from the literature that intravenously injected rare earth metals are localized in the spleen and reticulo-endothelial system in great amounts (Aiazzi-Mancini, 1927 ; Laszlo, Ekstein, Lewin & Stern, 1952 ; Durbin, Williams, Gee, Newman & Hamilton, 1956 ; Haley, 1965). It is difficult, however, to understand why calcification is produced only in the spleen.

Based only on the von Kóssa reaction, without a positive chloranilic acid stain, it seems unlikely that the material deposited in the Kupffer cells of the liver represents a calcium salt. It is possible that a complex formed by the injected metal with an anion (such as phosphate, carbonate or others) is responsible for the positive von Kóssa reaction.

Finally, it seems noteworthy that the calcifying activity of rare earth elements is inhibited by administration of sodium pyrophosphate. However, this ion does not change the mortality rate induced by the rare earth metals. Pyrophosphate possesses *in vitro* an antagonistic effect on the growth of apatite crystals (Fleisch & Neuman, 1961), while *in vivo* it inhibits the soft-tissue calcification induced by lead acetate (Gabbiani, 1966). It is conceivable that pyrophosphate exerts its influence by strongly complexing the rare earth elements.

It is difficult to explain at the present time the mechanism by which rare earth metals induce calcification but, in any event, we may conclude that they possess both a local and a systemic calcifying activity, and that the lesions thus produced can be prevented by the administration of sodium pyrophosphate.

SUMMARY

1. Previous experiments have shown that compounds capable of inducing calcification, when placed in contact with tissues of normal animals, are insoluble in Tyrode solution. This test was performed to examine the possible calcifying activity of the chlorides of rare earth elements and was positive for all of them.

2. Furthermore, in living animals, rare earth metals induced an accumulation of calcium salts at the site of their subcutaneous administration and, except for scandium and lanthanum, produced splenic calcification upon intravenous injection.

3. All these calcifying lesions were prevented by administration of sodium pyrophosphate.

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