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# Role of 1,25-dihydroxyvitamin $D_3$ in the generation of the acute-phase response in rats with talc-induced granulomatosis

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*Abstract.* Subcutaneous injection of nonspecific irritants such as magnesium silicate (talc) provokes granulomatous inflammation in the rat. Part of the acute phase response (APR) in these animals is the loss of trabecular bone at sites distant from the site of inflammation. To assess the possible involvement of vitamin D in the bone loss, we studied the development of the acute phase response in vitamin D-deprived rats. The serum APR provoked by subcutaneous inflammation in rachitic rats consisted of hypozincemia, hypercupremia, increased alkaline phosphatase activity and adrenocorticotropic hormone (ACTH) concentration, and was similar to that in control animals except for the absence of hypoferremia. Control rats with talc-induced subcutaneous inflammation also had splenomegaly and decreased total and mononuclear peripheral blood cell counts, while subcutaneous inflammation did not induce spleen changes in rachitic rats. Subcutaneous inflammation induced the loss of trabecular bone and decreased the osteoblastic cell count in tibial metaphyses in control animals. Rachitic rats had abundant osteoid on trabecular surfaces, and the number of osteoblasts and osteoclasts was comparable to that of the controls. Subcutaneous inflammation did not affect any of the bone parameters in rachitic rats.
These results indicate that vitamin D plays an important role in the generation of the acute phase response during inflammation, particularly in the induction of spleen and bone cell changes. The discrepancy of the blood on one hand and bone and spleen indices of the APR on the other, indicate that there may be divergent pathways in the generation of the inflammatory response, some of which may be dependent on vitamin D.
*Key words.* Acute phase response; bone loss; inflammation; rickets; talc; vitamin D.

Vitamin D plays a role in the regulation of calcium metabolism. After modification in liver and kidney, $1,25(OH)_2D_3$, the most potent form of vitamin D, acts primarily on cells in intestine, bone and kidney to increase intestinal calcium absorption and reabsorption in kidney, stimulate bone resorption and inhibit bone formation[1,2]. $1,25(OH)_2D_3$ has also been implicated in the regulation of a variety of cells of the immune system[3], including monocytes[4] and lymphocytes[5]. Moreover, activated monocytes and lymphocytes may produce the enzyme 1-alpha hydroxylase which converts $25(OH)D_3$ into its active form[2,6], particularly in granulomatous disease, including sarcoidosis and tuberculosis[7]. Clinical observations also linked vitamin D with blood cells. Children with vitamin D-deficient rickets have anemia and recurrent infections[8]. Such patients have an impaired ability to react to nonspecific inflammatory stimuli[9] and their peripheral leukocytes have decreased motility and an impaired capacity to phagocytize[10].

Subcutaneous injection of nonspecific irritants, such as magnesium silicate (talc), asbestos and agarose, provokes granulomatous inflammation in the rat[11,12]. Talc has a particular clinical significance since its effects in humans were reported after accidental inhalation, contact with powdered surgical gloves and injection of crushed tablets in heroin addicts (for review see ref. 13). Part of the acute phase response (ARP) in rats with talc-induced inflammation is the loss of trabecular bone in tibial metaphysis distant to the inflammatory site[14]. The cellular basis of the bone loss is a reduction of osteoblast number and activity and a retardation of longitudinal bone growth[15]. In order to assess the role of vitamin $D_3$ in the generation of the bone loss accompanying nonosseous inflammatory processes, we studied the development of the acute phase response and bone changes in vitamin D-deprived rats.

## *Materials and methods*

*Animals.* Female Fisher rats, 8–10 weeks old, weighing $200 \pm 10$ g, were used in all experiments. Rachitic rats were produced by keeping pregnant mothers in conditions without ultraviolet (UV) light, and feeding them with a vitamin D-depleted diet (Altromin C1017, Lage, Germany; mineral content: Ca, 9.5 mg/kg, P, 7.5 mg/kg)[16]. Tap water was given ad lib. Pups were weaned at 22 days of age onto their mothers' diet and housed in the conditions without UV-light. For the experiment, the animals were caged individually, were fed the vitamin D-depleted diet and drank tap water ad lib. Age-matched controls were kept under the same

experimental conditions, except for a 12-hour light and dark cycle and a standard diet (Altromin C1000, Lage; mineral content: Ca, 9.5 mg/kg, P, 7.5 mg/kg). Subcutaneous inflammation was provoked by injections of 800 mg sterile talc (magnesium silicate, $Mg_3H_2(SiO_3)_4$; Merck, Darmstadt, Germany) suspended in 1 ml isotonic saline solution at four different locations on the back of the animal; inflammation sites were not located near the skeleton. Control animals received saline solution only. Weight gain and food intake were recorded daily. Since talc-injected rats consume less food than the controls[15], animals without subcutaneous inflammation were pair-fed with talc-injected ones to exclude possible effects of decreased food intake on bone growth. Rats were sacrificed seven days after talc injection. Blood was drawn from the abdominal aorta, and serum separated by centrifugation and stored at −20°C until analyzed. The spleen was dissected out and weighed.

*Histological analysis.* Tibial proximal ends were fixed in 70% ethanol, dehydrated in increasing ethanol concentrations, embedded undecalcified in methylmetacrylate, and cut lengthwise into two equal halves. Sections measuring 3 µm were cut from the resulting plane with a microtome (Reichert 1516, Heidelberg, Germany) and stained with modified Goldner's stain. Bone morphometric parameters were measured at a magnification of 300× using the Zeiss II ocular grid (Zeiss, Jena, Germany) and semiautomatic image analyzer (Morphomat 10, Opton, Heidelberg, Germany). Trabecular bone and osteoid volume were measured as the percentage of total osseous space occupied by cancellous bone or osteoid, respectively, in the area 2 mm distal to the growth cartilage-metaphyseal junction (GCMJ), as described elsewhere[11–15]. The numbers of osteoblasts and osteoclasts were counted within a 1 mm-wide trabecular area, with cortical bone forming lateral boundaries and the upper boundary 1.25 mm distal to the GCMJ. Results were expressed as number of cells per $mm^2$ [11–15].

*Biochemical analysis of the serum.* Serum calcium, magnesium, iron, copper and zinc concentrations were determined by atomic absorption spectrometry (AAS, Pye Unicam, Cambridge, MA). Inorganic phosphate was measured using a Technicon Autoanalyzer, and alkaline phosphatase activity was measured using p-nitrophenol as a substrate, both as described elsewhere[14].

Adrenocorticotropic hormone (ACTH) and osteocalcin concentrations were determined by radioimmunoassay (RIA) using commercial RIA kits, ACTH-PR and OSTK-PR, respectively (CIS, Gif-sur-Ivette, France). Concentration of corticosterone was also measured by a radioimmunoassay (RSL Inc., Carson, USA). Electrophoresis of serum proteins was performed on cellulose acetate gel membranes, and their relative amounts were determined by densitometry (14).

*Differential Blood Cell Count.* Polymorphonuclear and mononuclear (lymphocytic and monocytic) fractions of the white blood cell compartments were determined by counting at least 100 cells per rat, after peripheral blood had been smeared on a glass slide, air-dried, and stained with May-Grünwald-Giemsa stain.

*Results*

The concentration of circulating $1,25(OH)_2D_3$ in rachitic rats was below the detection limit of the assay (data not shown), indicating a fully developed rickets. Rachitic animals weighed less than intact controls, but the difference was not significant ($168.0 \pm 9.7$ g in rachitic vs. $178.0 \pm 16.0$ g in control rats). As shown in earlier studies[12–15], subcutaneous inflammation induced anorexia and weight loss in both rachitic and control rats (data not shown).

Control rats with subcutaneous inflammation induced by talc injection presented a typical serum acute phase response[14]: decreased zinc and iron concentrations, increased copper and ACTH concentrations (table 1), increased $alpha_1$-, $alpha_2$- and beta-globulin and decreased albumin and gama-globulin serum protein electrophoretic fractions (data not shown), and increased alkaline phosphatase activity (table 1). Vitamin D deprivation induced a decrease in serum copper concentration (table 1). The serum APR provoked by subcutaneous inflammation in rachitic rats was similar to that in control animals, except for the absence of hypoferremia (table 1).

Control rats with subcutaneous inflammation had normal serum calcium and increased serum phosphate concentrations (table 1, ref. 11–15). Rachitic rats had hypophosphatemia and normal calcium concentration, while the concentration of magnesium was decreased (table 1.). Subcutaneous inflammation abolished the effects of rickets on mineral concentrations, reverting hypophosphatemia and hypomagnesemia of rachitic animals into hyperphosphatemia and hypermagnesemia (table 1; $p < 0.001$ and $p < 0.01$ vs. control, respectively).

A significant decrease in white blood cell (WBC) count was observed in control rats with subcutaneous inflammation. Mononuclear cell number was decreased, while the number of polymorphonuclears remained unchanged (table 1). Vitamin D deprivation did not have any effect on the peripheral WBC count, and subcutaneous inflammation in rachitic rats induced a decrease in total and mononuclear WBC similar to that in control animals (table 1). Spleen weight increased significantly as a consequence of subcutaneous inflammation in control, but not in rachitic rats (table 1).

The serum APR of control rats with subcutaneous inflammation was parallelled by the loss of trabecular bone in tibial metaphyses (table 2, ref. 14). The number of osteoblasts was decreased, while that of osteoclasts did not change (table 2, fig. B). Rachitic rats had abundant osteoid on trabecular surfaces, but the

Table 1. Serum mineral and trace element concentrations, alkaline phosphatase activity, ACTH concentration, number of white blood cells (WBC), and spleen weight of control and rachitic rats with subcutaneous granulomatosis 7 days after talc injection

| | Control | Talc sc. | p vs. control | Rickets | p vs. control | Rickets + Talc sc. | p vs. rickets |
|---|---|---|---|---|---|---|---|
| Ca (mM) | 2.4 ± 0.2 | 2.3 ± 0.1 | | 2.6 ± 0.4 | | 2.1 ± 0.1 | <.05 |
| P (mM) | 1.6 ± 0.1 | 2.4 ± 0.2 | <.01 | 0.6 ± 0.1 | <.01 | 3.7 ± 1.0 | <.001 |
| Mg (mM) | 0.9 ± 0.1 | 1.0 ± 0.1 | | 0.6 ± 0.1 | <.05 | 1.5 ± 0.2 | <.01 |
| Zn (μM) | 20.4 ± 2.1 | 8.1 ± 2.1 | <.001 | 21.3 ± 2.1 | | 1.5 ± 0.2 | <.001 |
| Cu (μM) | 26.2 ± 1.2 | 44.5 ± 12.6 | <.05 | 16.3 ± 0.9 | <.01 | 53.4 ± 4.4 | <.001 |
| Fe (μM) | 46.4 ± 12.6 | 5.4 ± 2.4 | <.001 | 41.4 ± 7.4 | | 41.5 ± 14.5 | |
| Alkaline phosphatase (U/L) | 69.8 ± 10.5 | 126.4 ± 40.8 | <.05 | 211.8 ± 29.6 | <.01 | 173.8 ± 28.5 | <.05 |
| ACTH (pg/ml) | 384 ± 35 | 625 ± 260 | <.01 | 361 ± 96 | | 729 ± 286 | <.01 |
| WBC ($10^9$/L): mononuclears | 11.3 ± 0.8 | 4.9 ± 0.7 | | 10.8 ± 0.9 | | 7.2 ± 0.5 | <.005 |
| Polymorphonuclears | 2.8 ± 0.8 | 2.4 ± 0.6 | | 2.1 ± 0.8 | | 2.0 ± 0.6 | |
| Spleen weight (g/kg b.w.) | 2.1 ± 0.2 | 4.3 ± 0.4 | <.001 | 2.1 ± 0.1 | | 2.4 ± 0.3 | |

Values are means ±S.D.; 5 rats per group. Experiment was repeated twice with similar results. Statistical difference between experimental groups was calculated using Mann-Whitney U-test.

Table 2. Trabecular bone and osteoid volume, number of osteoblasts and osteoclasts in tibial metaphysis, and osteocalcin serum concentration in control and rachitic rats with subcutaneous inflammation 7 days after talc injection.

| | Control | Talc sc. | Rickets | Rickets +Talc sc. |
|---|---|---|---|---|
| Trabecular bone (%) | 20.9 ± 3.9 | 11.2 ± 4.2* | 24.4 ± 7.7 | 21.5 ± 3.0 |
| Osteoid (%) | 0 | 0 | 7.1 ± 3.7 | 10.1 ± 5.0 |
| Osteoblasts (No/mm$^2$) | 151.4 ± 50.8 | 50.1 ± 19.3* | 148.9 ± 62.7 | 100.7 ± 26.2 |
| Osteoclasts (No/mm$^2$) | 17.4 ± 3.3 | 16.9 ± 2.7 | 14.9 ± 5.7 | 13.0 ± 4.1 |
| Osteocalcin (ng/ml) | 3.4 ± 0.2 | 2.0 ± 0.2** | 3.2 ± 0.2 | 4.1 ± 1.4 |

Values are means ±S.D.; 5 rats per group. The experiment was repeated twice with similar results.
*$p < 0.01$ and **$p < 0.05$, significant effect of talc injection, Mann-Whitney U-test.

numbers of osteoblasts and osteoclasts were not significantly altered compared to those of control rats (table 2, fig. C). Subcutaneous inflammation did not affect any of the bone parameters in rachitic rats (table 2, fig. D). The concentration of osteocalcin, a specific osteoblastic marker, was decreased in talc-injected control rats and unchanged in rachitic and talc-injected rachitic rats (table 2).

*Discussion*

Acute phase response (APR) includes a broad spectrum of immunological, endocrine, hematological, neurological and metabolic responses manifested in nearly every organ and tissue[17]. Our previous work on subcutaneous talc-induced non-osseous inflammation in rats indicated that bone loss is an important part of the APR in these animals[14,15]. In the present study, we show that vitamin $D_3$ plays a crucial role in the generation of bone loss during APR.

$1,25(OH)_2D_3$ exerts pleiotropic effects on bone cells in vivo and in vitro (reviewed recently in ref. 18). Injected in vivo, $1,25(OH)_2D_3$ increases osteoclast numbers in rodents. On the other hand, mature osteoclasts do not have receptors for $1,25(OH)_2D_3$[19,20] and do not respond to it in vitro[21]. $1,25(OH)_2D_3$ also appears to modulate activities of the immune cells, including stimulation of their maturation and release of osteoclast-activating cytokines[22–24], which may be responsible for the involvement of bone in inflammation. Lack of the effect of vitamin $D_3$ deficiency on the number of osteoclasts in our study indicates a complex pathophysiological mechanism in rickets. We have shown previously that the number of osteoclasts did not change during the course of inflammation in talc-induced granulomatosis, whereas their function, assessed as the resorption of calcified cartilage in tibial metaphysis, was transiently decreased[15]. Functional characteristics of osteoclasts from rachitic animals were not elucidated in this study, so that conclusions about their possible functional impairment cannot be drawn. One possible explanation for the normal number of osteoclasts in rachitic rats may be local factors produced by activated osteoblasts,

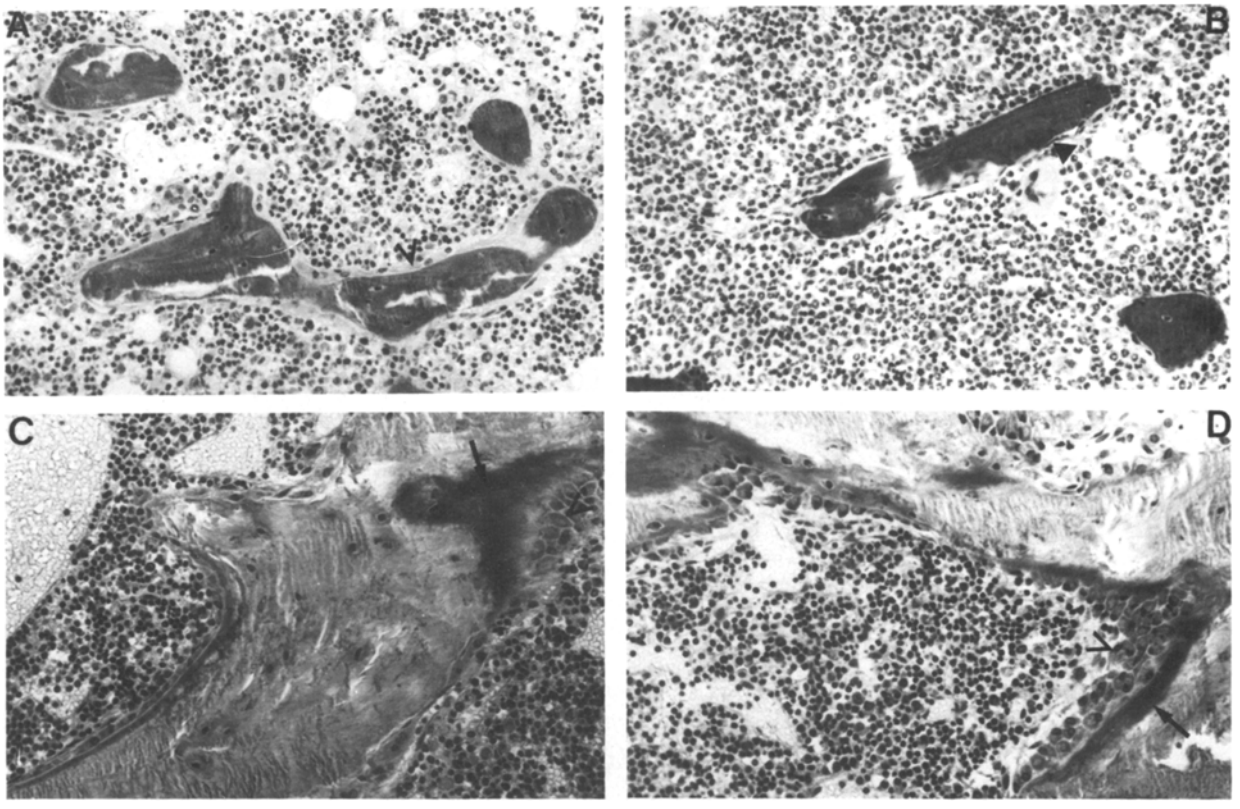


Figure. Histological sections of metaphyseal bone from control and rachitic rats with or without talc-induced inflammation; Goldner's trichrome staining, magnification 100× (A and B) and 150× (C and D). Control rats (A) had metaphyseal trabeculae covered with active osteoblasts (open arrowhead), while trabeculae from the tibial metaphysis of rats with talc-induced subcutaneous inflammation (B) were covered with scarce, flat lining cells (closed arrowhead). Trabecular bone in control rachitic rats (C) and rachitic rats with talc-induced subcutaneous inflammation (D) was covered with abundant osteoid (arrow) and active osteoblasts (open arrowhead). Multilayered osteoblasts in C and D (open arrowheads) are artifacts of a tangential cut along the surface of bone trabecula covered with active osteoblasts.

since it has been shown that they are required for osteoclast formation and activation[23,25]. In fact, the osteoblast may be the most important target of $1,25(OH)_2D_3$ in bone. It is difficult to discern the precise physiological role of vitamin $D_3$ in bone formation, because differences in the responsiveness between species and between culture systems generate contradictory results. The biphasic effects of vitamin $D_3$ on osteoblastic proliferation[26], contradictory results regarding its role in the local hormonal networks, and production of osteoblast-regulatory immune cell cytokines[27] make explanations for the observed effects of inflammation on bone formation in rachitic animals very difficult. Among many possible explanations we favor those implicating the role of vitamin $D_3$ in local immunomodulatory networks. Our current investigation suggests that tumor-necrosis factor (TNFalpha) produced systemically and locally in the bone marrow is responsible for the bone loss in non-osseous inflammation (A. Marušić and S. Vukičević, unpublished data). Recent evidence that $1,25(OH)_2D_3$ potentiates the expression of TNFalpha in lipopolysaccharide-stimulated macrophages[28], supports the role of vitamin D hormone in cytokine expression in the inflammatory microenvironment.

The coexistence of normal serum levels of osteocalcin, specific osteoblast protein, abundant osteoid and unchanged numbers of osteoblasts in the histological picture is not uncommon in rickets, both in animals and in humans[1,20–31]. The increase in serum osteocalcin concentration reflects bone formation at a given time and correlates more closely with skeletal healing in rachitic bone disease[29–31]. The activity of serum alkaline phosphatase, a nonspecific indicator of osteoblastic activity[1], was increased in all groups except in control controls. In talc-injected animals this increase was a part of the acute phase response, as the predominant form of alkaline phosphatase in these animals is a liver isoenzyme[8], whereas in rachitic rats the increase in activity could also be explained by the activation of protein synthesis in the liver[1].

The relationship between the talc-induced inflammation, spleen changes and vitamin $D_3$ remains obscure. Spleen has been shown to be the crucial point in the events leading to immunosuppression observed in rats

with talc-induced inflammation[13]. As immunocompetent cells mature in the spleen during inflammation[13,32], the absence of vitamin $D_3$ in rachitic animals may have impaired this physiological cellular response in the spleen and caused subsequent splenomegaly.

The experimental model of inflammatory reaction in rachitic animals disclosed several other interesting pathophysiological mechanisms. The importance of vitamin D in the differentiation of monocytic/macrophage cell lineage of the bone marrow and in the secretion of cytokines is well documented[3-5,8-10,33-35]. In our study, vitamin $D_3$ deprivation did not change the number of mono- and polymorphonuclear white blood cells, nor did it affect the characteristic inflammatory WBC response. This is not surprising in view of the findings that vitamin $D_3$ primarily affects functional maturation of monocyte/macrophage cell lineages, both *in vivo*[8-10] and *in vitro*[33-35]. It is common to find normal numbers but abnormal function of white blood cells in rachitic patients[8-10]. Functional characteristics of the white blood cells were not investigated in our model, but the less developed granulomatous envelope around talc injection sites (data not shown, ref. 15) may indicate functional impairment of WBC. The effects of inflammation on the mineral balance in rachitic rats (reversal of hypophosphatemia and hypermagnesemia of rickets into hyperphosphatemia and hypermagnesemia) are obscure at the moment and probably reflect complex pathophysiological interactions between the two disease states.

Hypophosphatemia and normocalcemia of rachitic rats was probably due to the secondary hyperparathyroidism, while hypophosphatemia and secondary hyperparathyroidism may both have contributed to the development of hypomagnesemia[1]. Mineral and trace element disbalance in vitamin D deficiency may be influenced by alteration of metallothionein metabolism. Metallothioneins have been implicated in the protection of the host against exogenous stimuli and $1,25(OH)_2D_3$ has been shown to stimulate the expression of the metallothionein gene[36].

In conclusion, the results presented in this study indicate that vitamin D plays an important role in the acute phase response to inflammation in a normal organism. Spleen and bone are primary targets of its action, while most of the serum indices of the inflammatory acute phase response and the number of peripheral white blood cells are independent of it. This indicates that there may be divergent pathways in the generation of the inflammatory response. One pathway, including the activation of protein synthesis and redistribution of trace elements, may not be dependent on vitamin D, whereas the other, including bone and spleen responses, may be dependent on the vitamin. The molecular mechanisms of vitamin D action on bone and other systems involved in inflammatory response are unclear. Potential mediators may be heat shock proteins, which have been shown to protect cells from elevated temperatures and other environmental stresses[37]; vitamin D plays a specific role in modulating HS response in monocytes and related cells[38].

*Abbreviations used in the text:* ACTH – adrenocorticotropic hormone; APR – acute phase response; WBC – white blood cells.

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