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VITAMIN D OVERLOAD AND EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION: PARASITOLOGICAL AND HISTOPATHOLOGICAL ASPECTS

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Abstract—1. Six groups of 45-day-old, 23.0 ± 1.7 g, female Balb/c mice were inoculated intraperitoneally with 63, 252, 440, 630, 2520 or 6300 I.U. of vitamin D for 6 days. A seventh group was inoculated with saline. Each group consisted of 30 animals.

2. All animals inoculated with the doses of 2520 and 6300 and 70% of mice which received 630 I.U. of vitamin D died 21 days after the first administration of the vitamin. The LD_{50} was 630 I.U.

3. The survivors were divided into two groups inoculated intraperitoneally with 5000 trypomastigotes of either Y or CL strain of *Trypanosoma cruzi*.

4. Based on the survival index on day 73 after infection, Vitamin D gave statistically significant protection ($P < 0.01$) for mice inoculated with doses of 63 or 430 I.U. of Y or CL strains, respectively.

5. On histopathological examination, inflammatory reaction and cellular and tissue parasitism were less intense in animals which received higher doses of vitamin D.

6. It is concluded that an overload of vitamin D had a protective effect against CL and Y strains of *Trypanosoma cruzi* infection in Balb/c mice.

INTRODUCTION

The literature on the nutrition–infection relationship as far as experimental Chagas' disease (American trypanosomiasis) is concerned is very scarce. Yaeger and Miller, in a series of papers reviewed by Scrimshaw *et al.* (1969), reported on the effect of specific deficiencies in rats infected with *Trypanosoma cruzi*. They observed that deficiencies of vitamin A, thiamine, pyridoxine and lysine aggravated the disease whereas deficiencies of riboflavin and calcium pantothenate had no effect. Lalonde and Holbein (1984) reported that murine trypanosomiasis (Brazil strain) was more severe if the animal was submitted to an iron overload. Pedrosa *et al.* (1990) showed that in iron deficiency murine Chagas' disease was more severe, not affected, or less severe for CL, YuYu, or Y strain, respectively. Carlomagno *et al.* (1987) showed that severe energy restriction increases significantly the mortality rate in mice infected with *T. cruzi*. Fat free diet protected conventional and germfree mice infected with *T. cruzi* (Santos *et al.*, 1988). Fulgêncio *et al.* (1991) comparing the overload of n-3 and the deficiency of n-6 fatty acids, showed that in n-6 deficient mice, even though the mortality rate was lower, tissue parasitism and parasitemia were higher and phagocytic capacity and hypersensibility reaction were depressed when compared with n-3 overloaded mice. Protein deficiency or overload had no effect on the evolution of experimental Chagas' disease in both germfree and conventional mice (Cintra *et al.*, 1990).

No report has appeared on the effect of vitamin D on experimental Chagas' disease. Vitamin D is hydroxylated, successively, in liver and kidneys, to 1α,25-dihydroxyvitamin D (1α,25$(OH)_2D_3$, calcitriol). Calcitriol induces differentiation of myeloid leukemic cells (Abe *et al.*, 1981) and of cancerous, hematopoietic, and epidermal cells (Suda *et al.*, 1986). Several investigators have reported abnormalities in the immuno-hematopoietic system in children with rickets, the latter being often associated with an increased frequency of infections and impaired phagocytosis by neutrophils. Administration of vitamin D corrects these abnormalities (Stroder, 1975). Nevertheless, vitamin D deficiency or 1α,25$(OH)_2D_3$ receptor defects do not usually cause clear deficiencies of the immuno-hematopoietic system *in vivo*. This finding suggests that, even though calcitriol is not essential for maintaining the immuno-hematopoietic system, it may work synergistically with other growth factors and systemic hormones in the regulation of hematopoiesis and immune responses (Suda *et al.*, 1990).

This paper deals with the influence of vitamin D overload on the evolution of murine American trypanosomiasis using parasitemia, histopathology and

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mortality as parameters to evaluate the course of the disease.

MATERIALS AND METHODS

Vitamin D

Cholecalcipherol (Vitamina D-500) was kindly supplied by Roche Produtos Químicos e Farmacêuticos, Brazil.

Trypanosoma cruzi

Two strains of *T. cruzi* were used: CL (Brener and Chiari, 1963) and Y (Silva and Nussenzveig, 1953). They were maintained through successive transfers in mice.

Mice

Forty-five-day-old, 23.0 ± 1.7 g, female Balb/c mice, fed on commercial ration, were used.

Infection with T. cruzi

Blood was drawn from infected mice. Proper dilution with saline was carried out to obtain 5×10^3 parasites in 0.1 ml. Infection was done intraperitoneally.

Determination of parasitemia

This was determined according to the method used by Brener (1962).

Histopathological evaluation

The organs were fixed in 4% formaldehyde solution for at least 48 hr. Fragments (2–4 mm^3) from liver, spleen, lymphonodes, and cardiac and striated muscles were properly processed for paraffin inclusion and cut with a microtome to a width of 5 μm. The slices were dehydrated, diaphanized and stained with hematoxilin–eosin. The slides were examined by only one pathologist who did not have access to the experimental conditions of each group. The slides were codified. After the report had been written, the material was decodified.

Experimental design

The mice were divided into seven groups, each with 30 animals. Six groups were inoculated intraperitoneally with daily doses of 63, 252, 440, 630, 2520 or 6300 I.U. of vitamin D for 6 days. The seventh group was inoculated with saline solution. The LD_{50} was determined on the 21st day after the first inoculation and calculated as described by Reed and Muench (1938). The survivors were divided into three subgroups: the first and the second subgroups were infected with 5×10^3 trypomastigotes of CL or Y strains, respectively; the third subgroup was maintained as controls. Survival was followed up to 73 days after the infection. The dead mice were opened ventrally and put in 4% formaldehyde solution for histopathological evaluation. After day 73 the survivors were killed under ether anesthesia and treated the same way.

Statistical analysis

Survival time and its corresponding percentage were treated through analysis of variance and Chi square test, respectively. The epstat program (T. L. Gustafson, Round Rock, TX, U.S.A.) was used.

RESULTS

The survival index, 15 days after the first administration of vitamin D, in the groups of mice which received 0, 63, 252 or 440 I.U. of vitamin D was 100%; in the group which received 630 I.U., the survival index was 70%. There were no survivors with the doses of 2520 and 6300 I.U. In the described conditions the LD_{50} for vitamin D was 630 I.U. (Fig. 1). The evolution of parasitemia for CL strain is shown in Fig. 2. The highest level of trypomastigotes in the blood was observed with the dose of 630 I.U. and, in decreasing order, 63, 252, 0 and

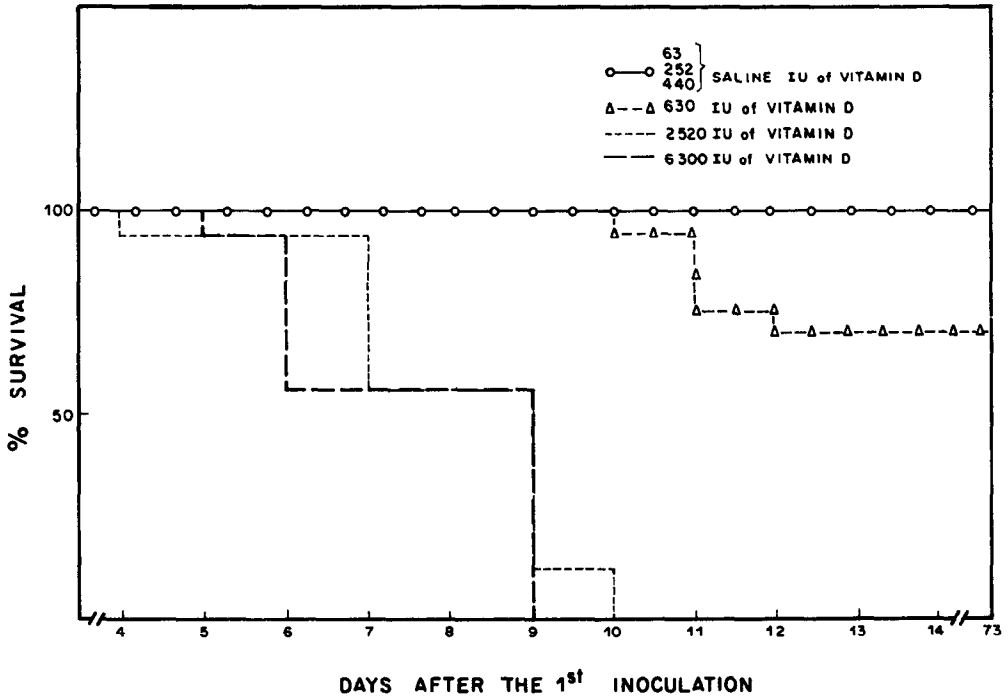

Fig. 1. Survival of groups of Balb/c mice treated with saline, 63, 252, 440, 630, 2520 or 6300 I.U. of vitamin D. Each group contained 30 animals.

440 I.U. of vitamin D. The parasitemia curve seems to show a peak on day 20. This may be explained by the fact that few animals, with high levels of parasites, remained alive. For Y strain, the highest levels of parasitemia were observed for the control group and the group which received 440 I.U. and, in decreasing order, 252, 630 and 63 I.U. of vitamin D (Fig. 3).

As far as the survival index is concerned, there was a statistically significant protection ($P < 0.01$) for mice inoculated with Y strain with a dose of vitamin D of 63 I.U. when compared with the other groups in which there were no survivors after 28 days of infection (Fig. 4). For CL strain, there was a statistically significant protection ($P < 0.01$) with the dose of 440 I.U. of vitamin D when compared with the other groups. After 73 days, the survival indexes were 0, 20 and 8% for mice inoculated with 250, 440 and 630 I.U. of vitamin D (Fig. 5). The significant protection of 440 and 63 I.U. observed, respectively, for CL and Y strains was confirmed by Chi square test on comparison with the control groups. Analysis of variance confirmed that, for both strains, the survival time was statistically smaller for all groups when compared with the groups for which some protection was conferred.

The histopathological evaluation showed signs of infection by *T. cruzi* in cardiac and skeletal muscles, in the spleen mononuclear macrophagic system, and in the lymphonodes of all groups. Inflammatory reaction and cellular and tissue parasitism were less intense in the animals which received higher doses of vitamin D (Fig. 6a,b). No differences between the different strains were detected. No morphological signs of vitamin D intoxication were found. On polarized light microscopy, small but numerous foci of myocardial calcification in the animals which died, but not in the ones which survived, could be observed. The higher the dose of vitamin D, the higher the effect (Fig. 6c).

DISCUSSION

To be sure to elicit hypervitaminosis D in mice, the intraperitoneal route was chosen. Wilgram

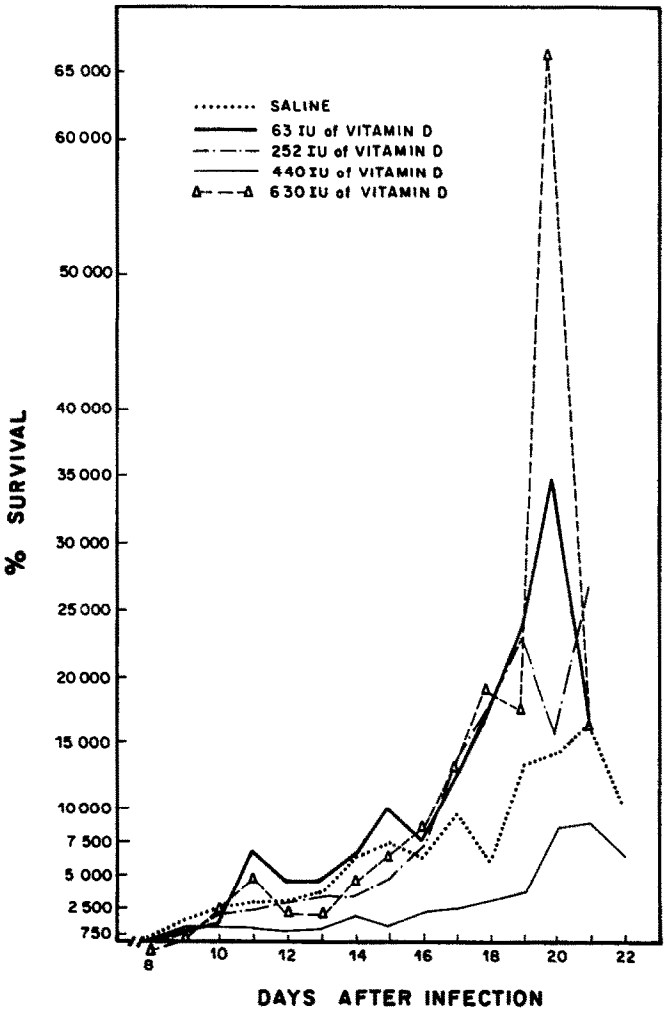

Fig. 2. Parasitemia of groups of Balb/c mice treated for 6 days with daily doses of saline, 63, 252, 440 or 630 I.U. of vitamin D and further inoculated with 5×10^3 trypomastigotes of CL strain of *Trypanosoma cruzi*. Each group contained 8–10 animals.

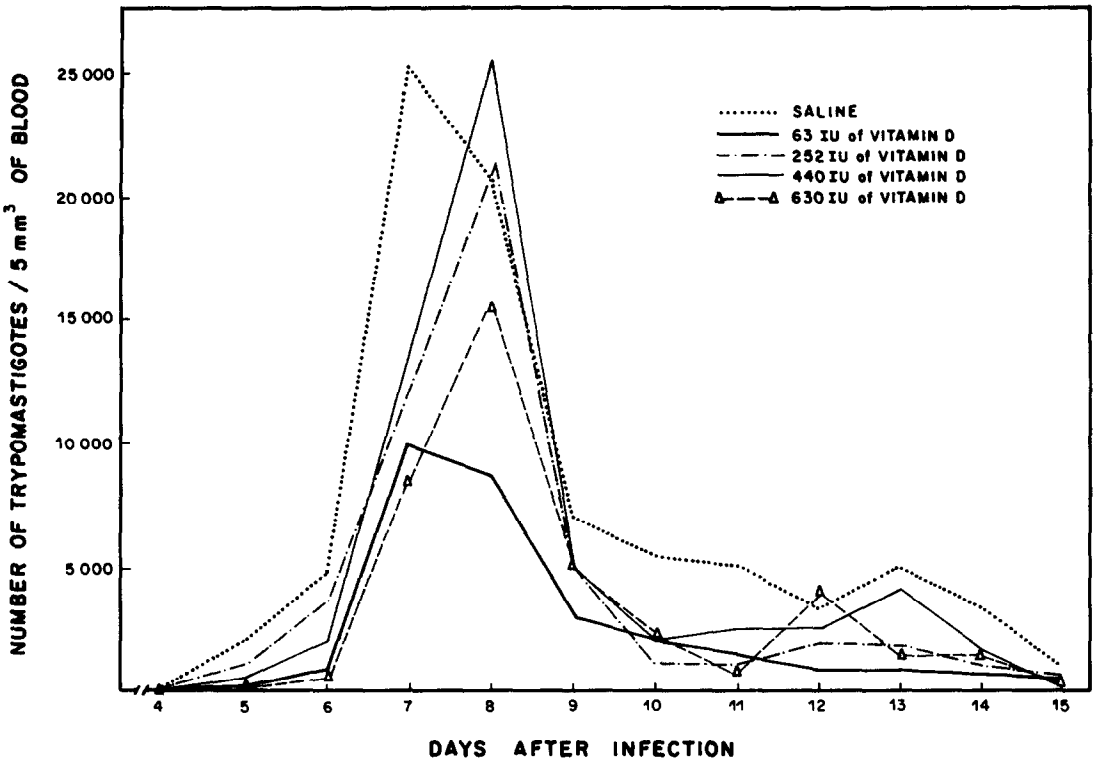

Fig. 3. Parasitemia of groups of Balb/c mice treated during 6 days with saline, 63, 252, 440 or 630 I.U. of vitamin D and further inoculated with 5×10^3 trypomastigotes of Y strain of *Trypanosoma cruzi*. Each group contained 8–10 animals.

(1958) have reported that rats survived for up to 8 months on a balanced diet containing 140,000 I.U. of vitamin D/100 g of diet. Zane (1976) found that intragastric administration of 50,000 I.U. of vitamin D for 7 days caused no noticeable damage to mice. The effect of genetic factors on the susceptibility to hypervitaminosis D was discussed. A dose of 1800 I.U. of vitamin D for long periods of time may be toxic for susceptible strains. On the other hand, in cases of

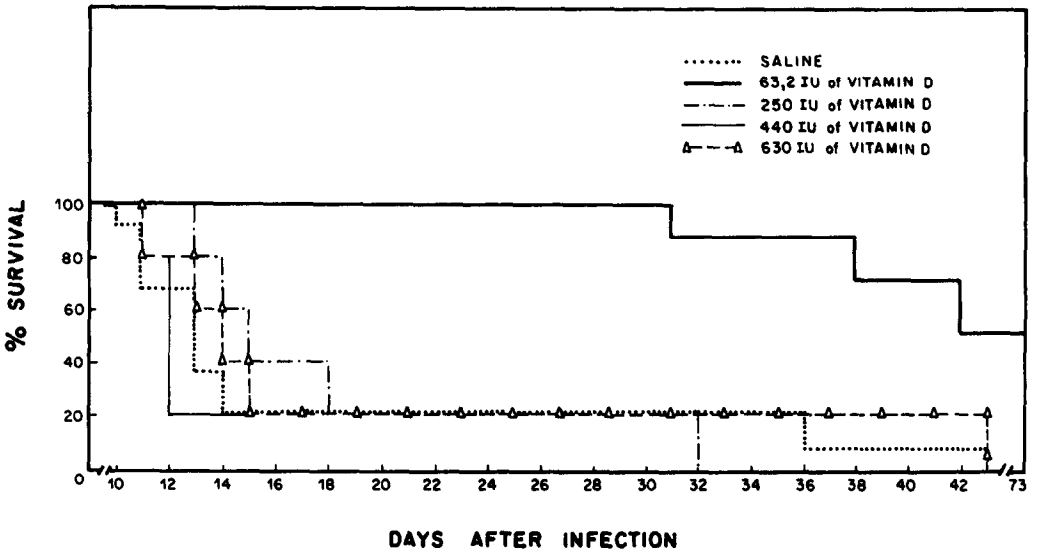

Fig. 4. Survival of groups of Balb/c mice treated for 6 days with saline, 63, 252, 440 or 630 I.U. of vitamin D and further inoculated with 5×10^3 trypomastigotes of Y strain of *Trypanosoma cruzi*. Each group contained 8–10 animals. Statistically significant differences were found only between the group which received 63 I.U. of vitamin D and all the groups through analysis of variance for survival time and Chi square test for percentage of survivors.

refractory rickets, doses as high as 100,000 may be tolerated.

Preliminary experiments indicated that most animals submitted to a vitamin D overload died before the day 15 after the last dose. In the present work, the overload of vitamin D was elicited by injection of cholecalciferol in addition to the dietary vitamin D. The levels of parasitemia were comparable to the ones reported by Pedrosa *et al.* (1990) for mice. The peaks for CL and Y strain were 15,750 and 25,000 trypomastigotes/5 ml of blood, respectively, whereas Pedrosa *et al.* (1990) reported values of 5000 and 6000, respectively, with an inoculum five times smaller. Analysis of parasitemia and mortality indicates that doses of 440 and 63 I.U. have a protective effect against CL and Y strains of *T. cruzi*, respectively. The differences obtained between these strains may be explained by their distinct characteristics (Tafuri, 1987).

The results reported herein are probably due to the known relationship between vitamin D and the immune system, as follows: (1) the description of a receptor for vitamin D in monocytes and in activated T and B lymphocytes (Provvedini *et al.*, 1983; Bhalla *et al.*, 1983); (2) the finding that calcitriol would limit the activation of T cells and the cytotoxic activity of macrophages in the inflammation sites (Rigby, 1988).

The histopathological data indicate milder inflammation and lower tissue parasitism in mice which received high doses of vitamin D, independently of the strain of *T. cruzi*. Apparently, the overload of the vitamin favored the host which overcame the acute phase and reached the chronic phase of the disease. In other words, the control mice died in the acute phase whereas the ones which received vitamin D died or were killed in the chronic phase. This was true for both Y and CL strains and may explain the histopathological differences found.

The histopathological evaluation under polarized light was used to detect any difference among the animals which died and the ones which survived in the groups overloaded with vitamin D. Small and frequent calcification foci in the myocardium of animals which died from infection were observed. This may be explained by the known action of calcitriol in the metabolism of calcium. Specific calcitriol-binding proteins have been described in the myocardium (Thomasset *et al.*, 1982) and in cardiac myoblast cells (Simpson *et al.*, 1985). Geertinger and Sorensen (1970) had demonstrated that high doses of vitamin D produced calcinotic lesions in myocardium and coronary and kidney arteries and in other sites. Tshibangu *et al.* (1975) showed that doses of 900,000 I.U./kg/day were lethal to rats due, probably, to the above-mentioned lesions besides neoplasic processes. These results suggest an inverse correlation between myocardial calcium deposition and resistance to *T. cruzi* of both strains.

The results reported herein corroborate previous works describing the beneficial effects of vitamin D overdoses in patients with tuberculosis (Davies *et al.*, 1987; Cadranel *et al.*, 1988). In this case, a more intense synthesis of calcitriol is proposed and a corresponding liberation of γ-interferon in the site of infection would activate macrophages and stimulate natural killer cells leading to destruction of *Mycobacterium tuberculosis*. It is possible that a similar mechanism might be occurring in the infection by *T. cruzi* in mice submitted to a vitamin D overload. The results reported here suggest an effect of vitamin D in the course of murine infection with *T. cruzi*.

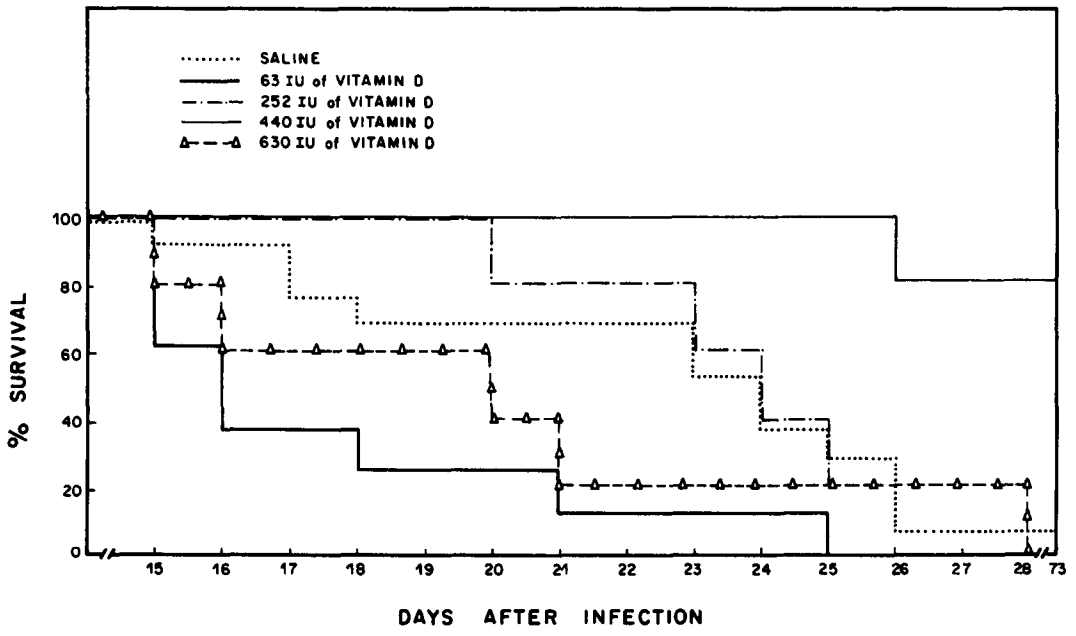

Fig. 5. Survival of groups of Balb/c mice treated for 6 days with saline 63, 252, 440 or 630 I.U. of vitamin D and further inoculated with 5×10^3 trypomastigotes of CL strain of *Trypanosoma cruzi*. Each group contained 8–10 animals. Statistically significant differences were found only between the group which received 440 I.U. of vitamin D and all the groups through analysis of variance for survival time and Chi square test for percentage of survivors.

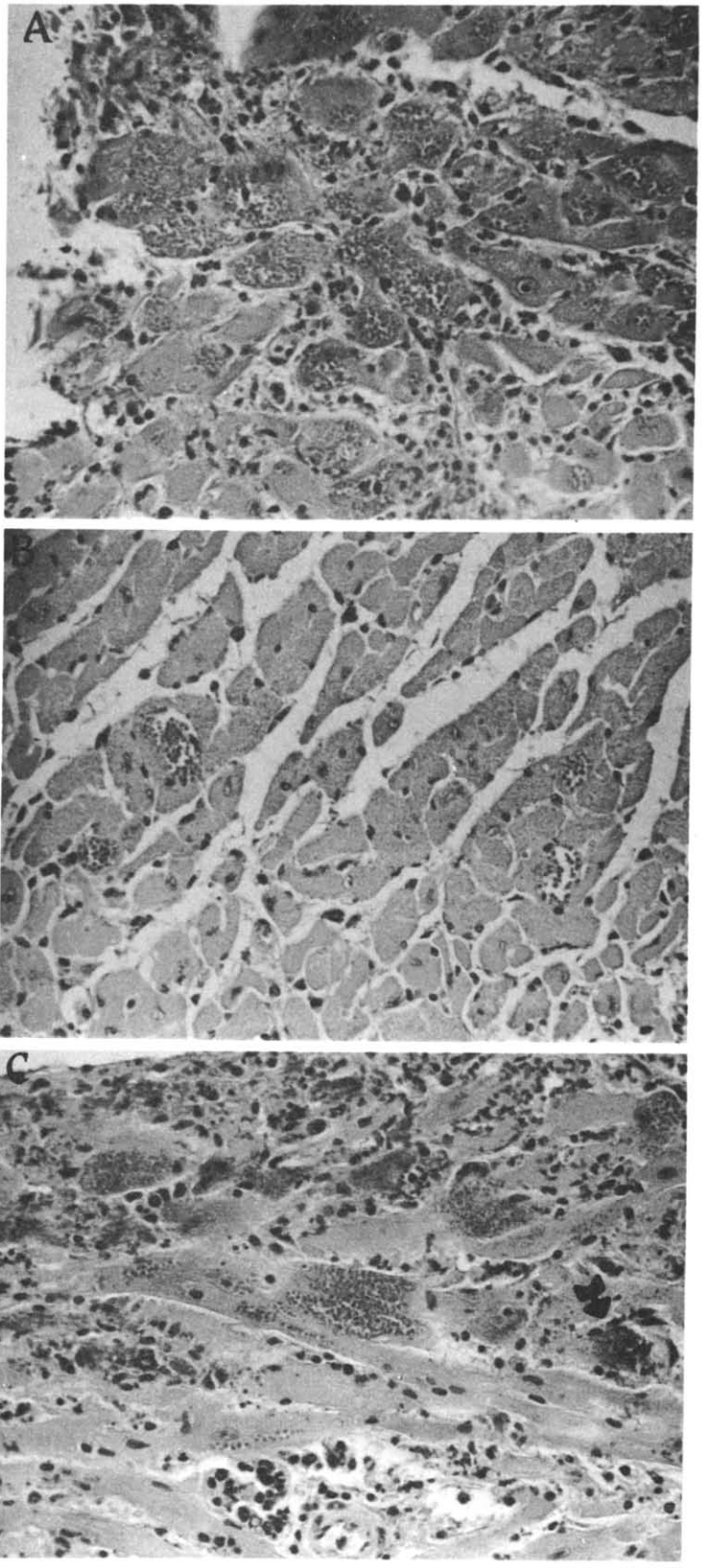

Fig. 6. Microphotographs of myocardium slices from *Trypanosoma cruzi* (CL strain) infected mice inoculated with different doses of vitamin D. (A: 5000 I.U., B: 50,000 I.U.) C: foci of calcification (arrow) from an animal which died 15 days after the sixth daily inoculation with 70,000 I.U. of vitamin D.

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