

THE EFFECT OF IRON NUTRITIONAL STATUS ON *TRYPANOSOMA CRUZI* INFECTION IN GERMFREE AND CONVENTIONAL MICE

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Abstract—1. Conventional (CV) and gnotobiotic (GN) female CFW mice were infected with the Y strain of *Trypanosoma cruzi*.

2. After infection, both CV and GN groups received injections of iron-dextran or desferrioxamine. Non-injected mice served as controls.

3. The parasitemia was more intense in iron-dextran-treated mice.

4. The iron levels in serum, liver, and spleen were: (a) not decreased by desferrioxamine and (b) increased by iron-dextran treatments.

5. An increase in leukocyte numbers was observed in all GN and CV groups after infection.

6. There was no difference in total iron binding capacity (TIBC) and iron saturation transferrin (IST) between GN and CV mice before infection.

7. In CV groups, after infection, TIBC was decreased whereas the levels of IST were increased; in GN the opposite occurred.

8. Trypanosome-specific IgG and IgM antibody levels were raised in the GN group but not in the CV group.

INTRODUCTION

The nutritional status of the host may affect its relationship with the parasite (Keusch and Farthing, 1986). Iron, among other specific nutrients, plays an important role in the pathogenesis of many diseases. Some evidence favors the hypothesis that mild iron deficiency would limit the proliferation of the pathogen thus protecting the host against infection. On the other hand, some authors suggest that iron deficiency would predispose the host to infection by interfering with the immune system (Weinberg, 1984, 1990; Kent *et al.*, 1990).

The course of experimental infection with *T. cruzi* is affected by nutritional factors. In a series of papers in the 1960s, Yaeger and Miller studied the effect of deficiencies of thiamine, pantothenate, pyridoxine, vitamin A, protein and zinc on the evolution of American trypanosomiasis in rats. Those papers were reviewed by Scrimshaw *et al.* (1968).

Recently, a relative protection against infection with *T. cruzi* in mice submitted to essential fatty acid deficiency was reported (Santos *et al.*, 1992). Almeida *et al.* (1989) showed that the mortality index was higher in animals either deficient or overloaded with

vitamin E as compared to controls fed on a diet containing normal levels of α -tocopherol.

In relation to iron nutriture, an increase in the cellular iron stocks through administration of iron-dextran led to higher mortality in mice infected with the Brazil strain of *Trypanosoma cruzi*; administration of desferrioxamine with the consequent decrease of iron stores led to a reduction of pathogenicity (Lalonde and Holbein, 1984). Results from this laboratory showed that treatment of mice with iron-dextran and further infection with *T. cruzi* resulted in: (1) higher parasitemia and mortality with the Y strain; (2) more pronounced parasitemia with the CL strain; (3) no effect with the YuYu strain. Treatment with desferrioxamine led to: (1) milder disease with the YuYu strain; (2) no effect with the Y and CL strains (Pedrosa *et al.*, 1990a).

The germfree (GF) animal is a good model for studies of the host–parasite relationship. The lack of any antigenic challenge by living organisms allows a specific response to any invading agent. American trypanosomiasis is much more severe in GF than in conventional (CV) mice and rats (Silva *et al.*, 1987) whereas cutaneous leishmaniasis is much more severe in CV mice (Vieira *et al.*, 1987).

In the present work, the effects of iron deprivation and supplementation in CV and GF mice infected

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with the Y strain of *T. cruzi* were evaluated through the determination of parasitological, biochemical, hematological, immunological and histopathological parameters. A preliminary report of this work has been published (Pedrosa *et al.*, 1990b).

MATERIALS AND METHODS

Mice

CV and GF CFW 60-day-old female mice were housed in flexible plastic isolators (Trexler, 1959), handled according to established procedures (Pleasant, 1974) and fed on a commercial diet (Nuvilab, Nuvital, PR, Brazil). The animals were derived from a breeding nucleus of GF mice kindly supplied by Dr Morris Pollard (Lobund Laboratory, University of Notre Dame, U.S.A.). The CV animals used were derived from the GF colony after many generations in an open animal room.

Parasites

T. cruzi, Y strain, was maintained by successive transfers in CFW mice. The infection was carried out as described previously (Silva *et al.*, 1987).

Parasitemia

Mice were bled daily from the tail, starting at the 5th day after infection, for the evaluation of the number of circulating parasites, according to Brener (1962).

Assays

The number of red blood cells and total leukocytes were evaluated. Serum iron and total iron-binding capacity (TIBC) were determined with a commercial kit (Labtest, Belo Horizonte, MG, Brazil) based on the method of Goodwin *et al.* (1966). Spleen and liver iron levels were determined by the phenanthroline method after incineration according to AOAC (1980). Iron saturation transferrin (IST) was determined from the ratio: serum iron/TBIC.

IgG and IgM determination

Trypanosome-specific antibodies in serum samples were identified by an enzyme-linked immunosorbent assay (ELISA) in which the antigen was 4.0×10^4 clean epimastigotes, kindly provided by Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil. The parasites were fixed to the ELISA plate (Hemobag, Campinas, SP, Brazil) by drying a suspension of cells in carbonate/bicarbonate buffer, pH 9.6, onto the wells. The wells were blocked with bovine serum albumin (Biobrás, Montes Claros, MG, Brazil). Horseradish peroxidase conjugated goat anti-mice immunoglobulins (Sigma, St. Louis, MO, U.S.A.) served as conjugate and the peroxidase substrate was *o*-phenylenediamine. The procedure followed was that described by McLaren *et al.* (1980). Optical

absorbance was read at 492 nm with an ELISA-reader.

Experimental design

Eighty CV and 50 GF female mice were inoculated with 1700 blood forms of *T. cruzi*. The infected animals were divided into three groups. One group remained as controls. The remaining two groups were injected intraperitoneally either with 5 mg of iron-dextran (ferric hydroxide dextran complex, Sigma) or with 10 mg of desferrioxamine mesithylate (Desferal, Ciba, Brazil) on days 5 and 6 after infection. These drugs were previously filter-sterilized and introduced into the isolators in sealed ampoules. On days 6, 10 and 15 after infection, some of the animals from all groups were weighed and killed under ether anesthesia. Blood was collected from the axillary plexus. Liver and spleen were removed and weighed. Fragments of these organs were used for iron assays and the remaining of the viscera and carcass were used for histopathological evaluation.

Histopathological examination

Tissue samples were fixed in 4% formaldehyde and processed for paraffin embedding. The histological sections (3–5 μ m) were stained with hematoxylin-eosin and the slides examined by only one person who did not have access to the codification of the animal, whose identification was done only after each report had already been written.

Statistical analysis

The results were compared through analysis of variance followed by the determination of the minimal significant difference (Snedecor and Cochran, 1980). The calculations were made using the EPIS-TAT computer program (T.L. Gustafson, Round Rock, TX, U.S.A.).

RESULTS

Effect of treatment with iron-dextran or desferrioxamine on parasitemia and histopathology

Parasitemia in CV and in gnotobiotic (GN) mice (i.e. monoassociated with *T. cruzi*) are shown in Fig. 1. Control animals showed detectable parasitemia from day 7 of infection and peak parasitemia occurred on day 9. Parasitemia on both GN and CV mice was intensified when these animals were treated with iron-dextran. These results suggest that the increase in iron availability stimulates the proliferation of *T. cruzi* in GN and CV mice. The parasitemia of both CV or GN mice treated with desferrioxamine did not differ from the non-treated control group.

On histopathological examination, no difference between iron-dextran or desferrioxamine-treated mice and their controls could be detected, either in CV or GN animals. However, the disease was more severe in GN than in CV mice as revealed by a more

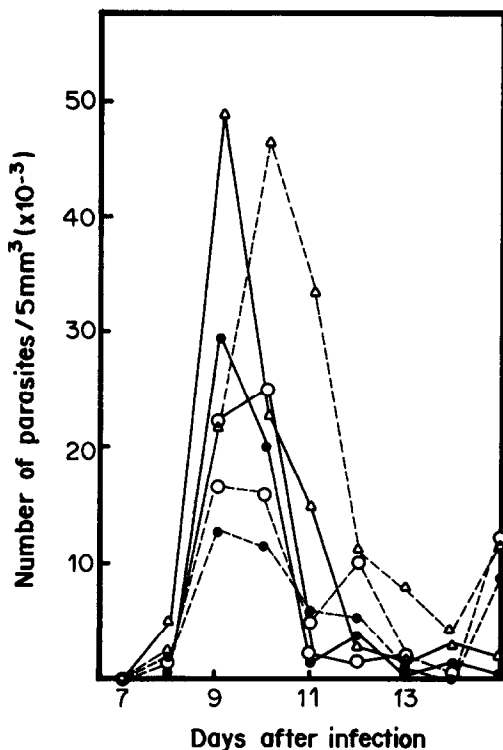


Fig. 1. Parasitemia in control (○), desferrioxamine-treated (◐), and iron-dextran-treated (Δ) conventional (—) and gnotobiotic (---) mice inoculated with 1700 *Trypanosoma cruzi* (blood forms). No. of animals: from 7 to 10 day, 10-14 and 9-10; from day 11 to 15, 4-8 and 4-6 for conventional and gnotobiotic mice, respectively.

intense cell and tissue parasitism, confirming previous results (Silva *et al.*, 1987).

Effect of desferrioxamine or iron-dextran on the iron status of the host after infection with T. cruzi

The effect of the treatments with desferrioxamine and iron-dextran on serum levels of iron of infected animals were evaluated (Fig. 2A). CV and GN animals treated with desferrioxamine did not show significant differences on serum iron when compared with untreated controls at the same day after infection. Treatment of CV animals with iron-dextran slightly elevated the levels of serum iron; this increase was only detected on day 15 post-infection. In GN animals the levels of iron were higher in dextran-treated mice than in controls reaching a maximum on day 6 post-infection.

Before infection and on day 6 after inoculation with *T. cruzi*, the iron content in liver of GF controls was higher than of their CV counterparts. Desferrioxamine-treated GN mice showed lower levels of iron in liver than GN controls 10 days after infection but not on day 15 when there was an increase in the iron levels. No such alterations were observed in the CV group. Treatment with iron-dextran enhanced the levels of iron in livers of GN and CV infected

mice (Fig. 2B) on days 15 and 10 after infection, respectively.

The iron contents in spleen are shown in Fig. 2C. Before infection these contents were the same in CV and GF animals. After infection, these levels were significantly raised in CV but not in GN mice. Treatment with desferrioxamine did not produce any alteration in iron levels when compared to infected controls, both for CV and GN mice. On treatment with iron-dextran, there was an elevation of iron levels in spleens on day 15 after infection in CV mice and on days 10 and 15 in GN mice.

Effect of the infection with T. cruzi on serum iron, total iron binding capacity (TIBC), and iron saturation transferrin (IST)

Table 1 shows that the levels of serum iron, TIBC and IST were the same in GF and CV controls before infection. In the course of the disease, serum iron levels were not altered in the CV group; however, in GN mice a rise in this parameter was observed. In the CV group, TIBC levels were decreased 15 days after infection whereas IST levels increased after day 10 of infection. In GN animals, TIBC increased sharply after 10 day of infection and the IST decreased at day 15.

Effect of the infection with T. cruzi on the levels of IgG and IgM

The evolution of antibody levels in each group is shown in Fig. 3. The basal levels of antibodies were the same for both GN and CV groups. After infection, the levels of these immunoglobulins were significantly raised in the GN group, this did not occur in the CV group. When compared with their CV counterparts, the GN mice had higher titers of IgG and IgM, 15 days after infection.

Effect of desferrioxamine, iron-dextran and the infection with Trypanosoma cruzi on the number of red blood cells and leukocytes

Infection by *T. cruzi* caused a reduction in numbers of red blood cells in CV control mice, 15 days after infection (Table 2). This difference was not observed in animals treated with desferrioxamine or iron-dextran. There was an earlier and more intense reduction of the red blood cell number in control GN mice. Desferrioxamine- or iron-dextran-treated GN animals also showed a marked reduction in red blood cell number in the course of infection.

Table 3 shows the evolution of blood leukocytes in the different groups of animals. After infection, a rise in leukocyte number could be observed in all GN and CV mice. This rise in leukocyte number was less intense in GN mice when compared with their CV counterparts, with the exception of GF controls at day 15 after infection.

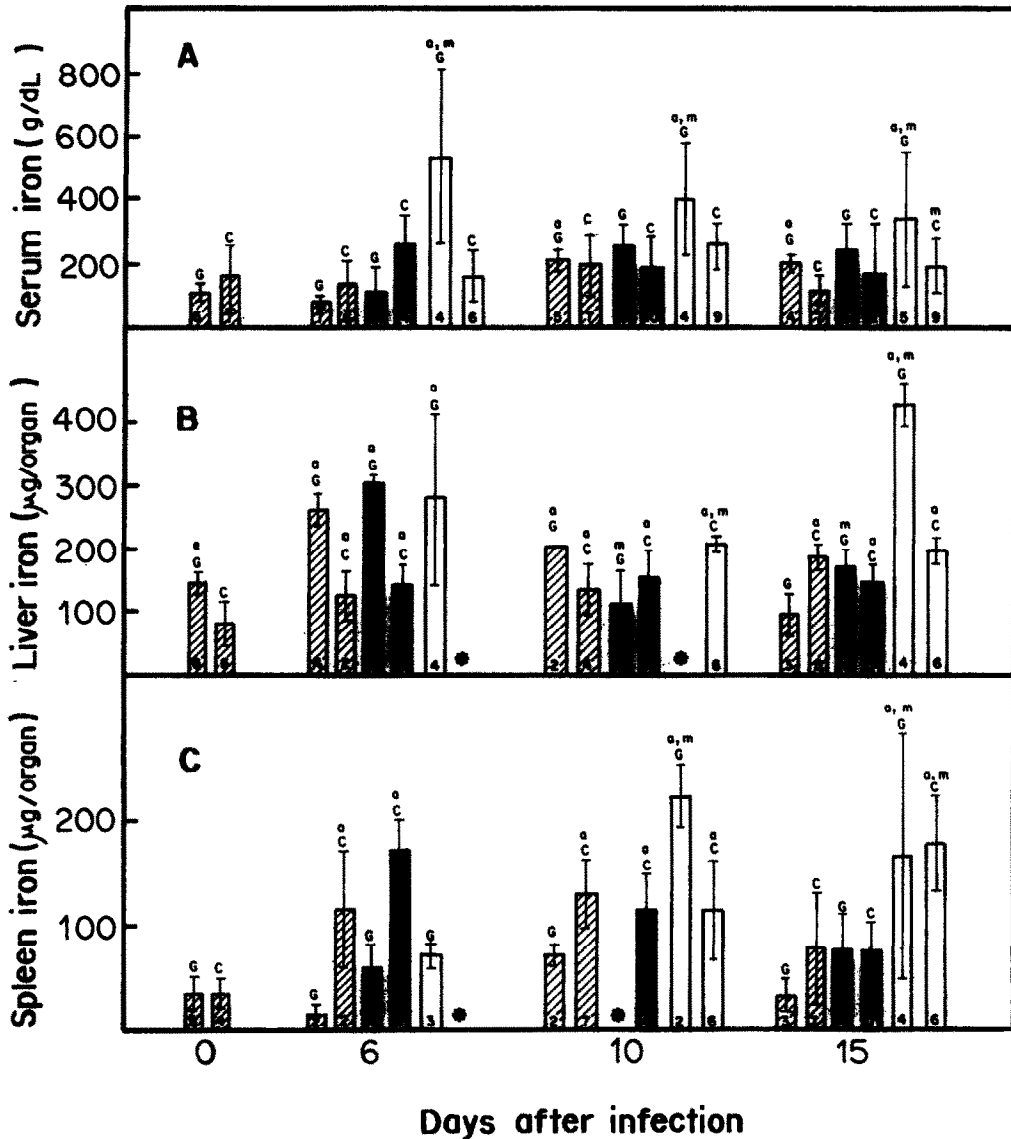


Fig. 2. Mean levels of iron in serum ($\mu\text{g}/\text{dl}$) (A), liver ($\mu\text{g}/\text{organ}$) (B), and spleen ($\mu\text{g}/\text{organ}$) (C) in gnotobiotic (G) and conventional (C) control (▨), desferrioxamine-treated (■) or iron-dextran-treated mice (□). Assays were performed before infection or on days 6, 10, and 15 after infection. Statistical differences ($P < 0.05$) are indicated as follows: a, in relation to day 0 within the same group; e, in relation to their conventional counterpart at day 0; m, in relation to their control counterpart at the same day of infection. The number of animals in each group are inside the vertical bar. Vertical lines represent the standard deviations of the means. *Not done.

DISCUSSION

Our results demonstrate that an increase in available iron stimulates the parasitemia in CV and GN mice (Fig. 1), confirming previous results (Pedrosa *et al.*, 1990b). However, the higher rate of proliferation of the parasite with the increase in iron levels was not followed by a more intense tissue parasitism. The short period of experimentation could explain this apparent paradox.

Non-infected GF and CV mice presented the same values for serum iron and TIBC confirming the results

obtained by Donati *et al.* (1969). After infection with *T. cruzi*, the levels of serum iron were not altered and increased in CV and GN animals, respectively (Table 1). However, TIBC levels were reduced and elevated in CV and GN mice, respectively, in the course of infection. Therefore, there is a difference in iron metabolism between GN and CV animals. It is known that *T. cruzi* is capable of suppressing the host immune system during the acute phase of the infection (Kierzenbaum and Sztajn, 1990) what would lead to a reduction in transferrin synthesis by lymphocytes. This immunosuppression might not have occurred in

Table 1. Levels of serum iron, total iron binding capacity (TIBC) and transferrin saturation index (TSI) in conventional (CV) and gnotobiotic (GN) mice infected with 1700 blood forms of *Trypanosoma cruzi*, Y strain

Group	Days after infection	Serum iron ($\mu\text{g/dl}$)	TIBC ($\mu\text{g/dl}$)	TSI (%)
CV	0	155.7 \pm 93.9 (7)	378.3 \pm 84.9 (6)	33.0 \pm 16.2 (6)
CV	6	132.3 \pm 60.1 (6)	309.3 \pm 122.7 (3)	43.3 \pm 23.8 (3)
CV	10	183.9 \pm 89.9 (7)	294.3 \pm 152.7 (4)	72.8 \pm 14.4 ^a (4)
CV	15	102.6 \pm 45.8 (7)	199.1 \pm 71.3 ^a (7)	52.7 \pm 14.6 ^a (7)
GN	0	104.8 \pm 22.2 (6)	318.3 \pm 97.6 (6)	35.8 \pm 14.0 (6)
GN	6	73.8 \pm 10.9 (7)	272.0 \pm 35.8 (4)	28.5 \pm 4.5 (4)
GN	10	205.1 \pm 34.5 ^a (5)	545.3 \pm 55.9 ^{a,e} (4)	36.0 \pm 4.7 ^e (4)
GN	15	202.9 \pm 39.9 ^{a,e} (4)	1129.0 \pm 218.0 ^{a,e} (4)	18.5 \pm 4.0 ^{a,e} (4)

Statistical differences ($P < 0.05$) are indicated as superscripts: a, in relation to day 0 within the same group; e, in relation to the CV counterpart on the same day. The number of mice is indicated in parentheses.

GN mice. Therefore, these lymphocytes became more reactive and synthesized more transferrin.

Differences in the immune response between GN and CV animals were earlier reported by Rogers and Balish (1978): when CV and GF rats were infected with *Candida albicans* and later challenged with phytohemagglutinin and concanavalin A, they observed: (1) suppression of splenocyte response in CV rat; (2) a five-fold increase in blastogenesis in GN rat. These results suggest that disseminated candidiasis is capable of suppressing blastogenesis in CV rat. The absence of antigenic stimuli derived from intestinal microbial flora could affect the immune response to infection with *T. cruzi*. Such differences may be evidenced by observing the levels of *T. cruzi*-antigen bound IgG and IgM in CV and GN mice. The levels of immunoglobulins in the latter were higher than in the former at day 15 after infection (Fig. 3). The GN group, therefore, produced more IgG and IgM than their CV counterparts. Higher titers of immunoglobulins were observed in GN rats infected with *T. lewisi* (Giannini, 1987) and in GN mice infected with *T. cruzi* (Furarah *et al.*, 1991) than in their CV counterparts. This confirms the hypothesis that the difference in levels of TIBC between CV and GN mice may be due to differences in immunological responses.

Desferrioxamine, an iron chelator, has been widely used to evaluate the relationship between infection and iron nutriture (Duhr *et al.*, 1989). Two mechanisms are proposed for its action: (1) induction of iron deficiency by limiting its availability within the cell, (2) direct interference with the acquisition

of iron by the parasite (Hershko and Peto, 1988). *In vitro*, this chelating agent is able to remove iron from ferritin, hemosiderin and, to some extent, from transferrin (Keberle, 1964). Desferrioxamine did not affect the levels of liver, spleen and serum iron in either CV or GN mice after infection with *T. cruzi* (Fig. 2), except for GN mice at day 10 after infection. Desferrioxamine did not affect the parasitemia (Fig. 1). These results confirm the ones previously obtained with the Y strain in CV mice even though this strain was more sensitive to iron deficiency (Pedrosa *et al.*, 1990a). Pedrosa *et al.* (1990a) also reported that 40 days after infection, there was a decrease in the levels of iron in spleen and liver of desferrioxamine-treated mice. It is possible that the removal of iron by the chelating agent may be occurring after the period of high proliferation of the parasite which explains the results presented here.

In iron-dextran treated CV mice, there was a slight increase in the levels of serum iron, only detectable on day 15 after the infection. In GN mice, higher levels could be observed after day 6 of infection and remained high up to day 15. Holbein (1980) investigated the destination of iron in mice after intraperitoneal injection of iron-dextran. Two hours later, the levels of serum iron reached its maximum. Histochemical examination of liver and spleen revealed that iron was deposited in cells of the reticulo-endothelial system (Holbein, 1980). It may be concluded that the rise in serum iron in these animals is due to the drug. Our results show that dextran treatment promoted a rise in serum iron in GN mice

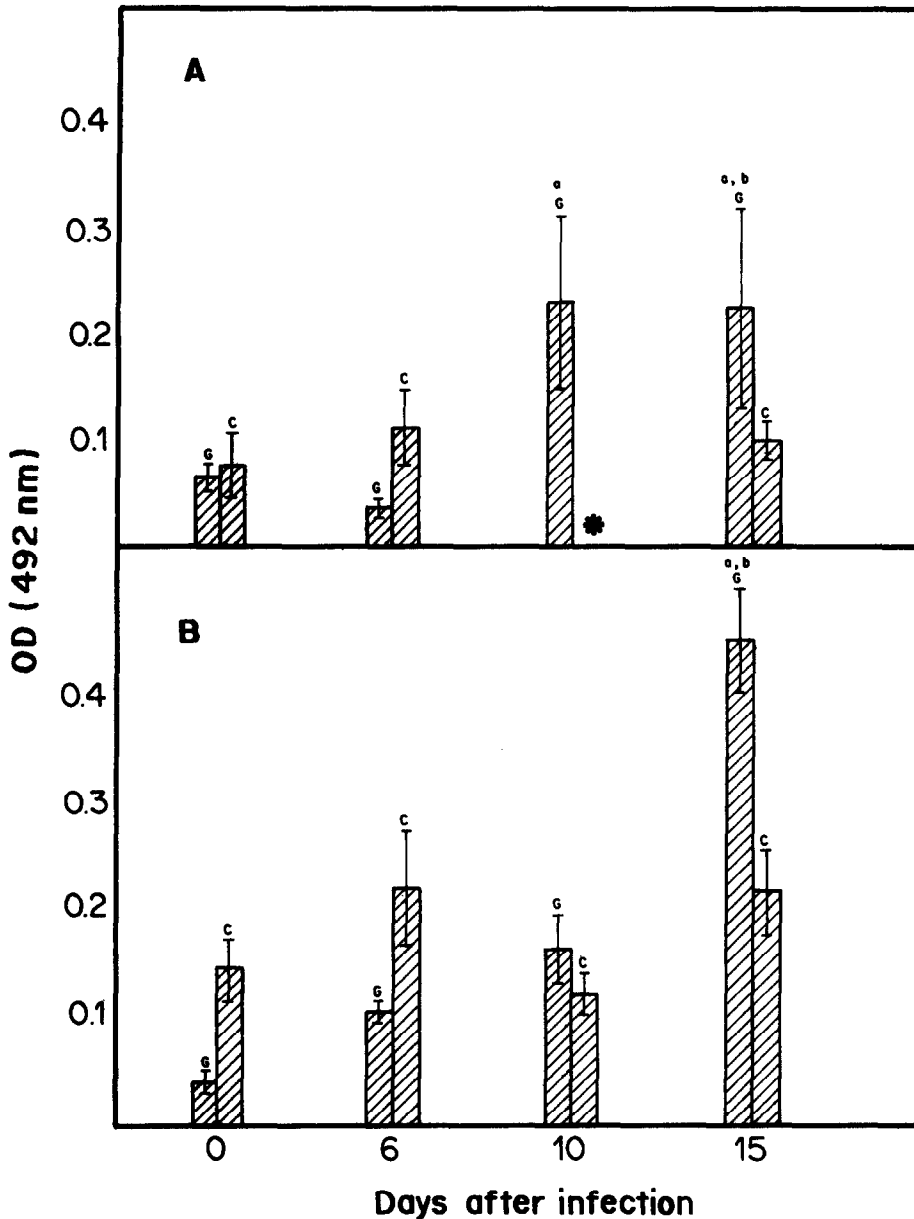


Fig. 3. Mean levels of IgM (A) and IgG (B) determined by ELISA in control gnotobiotic (G) and conventional (C) mice. Assays were performed before infection and on days 6, 10 and 15 after infection. Each bar represents an average of three to six samples. Statistical differences ($P < 0.05$) are indicated as follows: a, in relation to day 0 within the same group; b, in relation to the conventional counterpart at the same day of infection. Vertical lines represent the standard error of the means. *Not done.

(Table 1). It seems, therefore, that the uptake of iron from iron-dextran is more efficient in GN than in CV animals after intraperitoneal injection. On the other hand, the removal of serum iron by the reticulo-endothelial system seems to be less efficient in the former group of animals. This corroborates the data of Donati *et al.* (1969) who observed that the clearance of radio-iron from plasma was markedly slower in the GF mouse, suggestive of a direct or indirect effect of the flora on the reticulo-endothelial system which could explain the difference in serum iron levels

between GN and CV mice. Treatment with iron-dextran promoted an elevation of the levels of spleen iron in CV and GN mice, 15 and 10 days after the infection, respectively (Fig. 2C). An increase in liver iron content in both groups was also observed (Fig. 2B). The increase in iron content in these organs positively affected the proliferation of the parasites (Fig. 1).

No significant difference was found between CV and GN animals as far as blood red cell count is concerned. After infection, there was a reduction in erythrocyte

Table 2. Number of red blood cells/mm³ in conventional (CV) and gnotobiotic (GN) mice treated with desferrioxamine (D), iron dextran (F) or control groups (C)

Group	Days after infection			
	0	6	10	15
CV-C	1590 ± 147 (5)	1183 ± 956 (5)	1280 ± 540 (5)	1090 ± 448 ^a (3)
CV-D		1605 ± 174 (4)	1429 ± 231 (5)	1147 ± 120 (4)
CV-F		1137 ± 461 (5)	1562 ± 319 (4)	1365 ± 171 (4)
GN-C	1772 ± 225 (5)	949 ± 350 ^{a,e} (7)	684 ± 133 ^{a,e} (5)	619 ± 268 ^{a,e} (4)
GN-D		1040 ± 91 ^{a,e} (5)	613 ± 184 ^{a,e} (6)	456 ± 268 ^{a,e} (5)
GN-F		1521 ± 556 (5)	949 ± 316 ^{a,e} (4)	521 ± 159 ^{a,e} (4)

Assays were performed before infection with 1700 blood forms of *Trypanosoma cruzi*, Y strain, and on days 6, 10 and 15 after infection.

Statistical differences ($P < 0.05$) are indicated as superscripts; a, in relation to day 0 within the same group; e, in relation to the CV counterpart on the same day. The number of mice is indicated in parentheses.

numbers in both groups (Table 2). Transitory hematological alterations have been described in various infections with trypanosomes which have been attributed to different factors: hemodilution, bone marrow injury, destruction of red blood cells and alteration of their turnover (Cardoso and Brener, 1980). In iron dextran-treated GN mice, a reduction in the number of red blood cells was observed after day 10 of infection. In control and desferrioxamine-treated GN mice the red blood cell numbers were altered after day 6 of infection. A marked anemia in the GN group could be explained by the findings of Hashimoto and

Hashimoto (1968) who observed a smaller hematopoietic activity in GN than in the CV rat. Furthermore, the low IST in GN mice may have contributed to the decrease of the red blood cell count in these animals as compared with their CV counterparts.

An elevation of the number of leukocytes was observed in all CV and GN groups as infection progressed (Table 3). These results contradict the ones obtained by Cardoso and Brener (1980) who reported leukopenia in CV albino mice infected with 10⁴ trypomastigotes. This discrepancy may be explained by the different inoculum size or by the difference

Table 3. Number of leukocytes/mm³ × 10⁻³ of blood in conventional (CV) and gnotobiotic (GN) control mice (C) or in mice treated with desferrioxamine (D) or iron dextran (F)

Group	Days after infection			
	0	6	10	12
CV-C	8.26 ± 2.12 (5)	22.6 ± 0.8 ^a (5)	37.1 ± 15.7 ^a (5)	35.4 ± 4.1 ^a (4)
CV-D		35.2 ± 14.5 ^a (5)	34.5 ± 13.0 ^a (5)	56.4 ± 9.5 ^a (3)
CF-F		25.3 ± 17.9 ^a (5)	51.9 ± 22.4 ^{a,m} (5)	54.8 ± 4.5 ^{a,m} (4)
GN-C	4.6 ± 1.7 ^e (1)	6.3 ± 2.4 ^e (7)	12.2 ± 3.8 ^{a,e} (5)	33.5 ± 8.3 ^a (4)
GN-D		16.2 ± 4.9 ^a (5)	10.8 ± 3.4 ^a (5)	13.3 ± 3.5 ^{a,m} (5)
GN-F		6.4 ± 1.5 (5)	17.3 ± 7.0 ^a (4)	20.0 ± 6.6 ^{a,m} (5)

Assays were performed before infection with 1700 blood forms of *Trypanosoma cruzi*, Y strain, or on days 6, 10 and 15 after infection.

Statistical differences ($P < 0.05$) are indicated as superscripts; a, in relation to day 0 within the same group; e, non-infected GN in relation to the CV counterpart on the same day; m, in relation to the control counterpart on the same day. The number of mice is indicated in parentheses.

in mice strains. In GN control, desferrioxamine- and iron-dextran-treated mice, the rise in leukocyte number was less pronounced than in their CV counterparts, except for the GN control group on day 15 after infection. Anosa and Kaneko (1983) reported leukocytosis with lymphocytosis, monocytosis, eosinopenia and basophilia in *T. brucei*-infected mice. One may suppose that in GN mice, the leukocyte proliferation occurs at a slower rate. On day 15 after infection the levels of leukocytes in GN mice reached the ones of CV animals but treatment with desferrioxamine or iron-dextran hindered this recovery. In CV mice there was a rise in leukocyte numbers from 3- to 7-fold in all groups.

We have shown that GN mice differ from their CV counterparts in: (1) their response to infection by *T. cruzi*, (2) iron metabolism in different conditions of iron nutriture and (3) red blood cell and leukocyte numbers. These results demonstrate that, under the conditions described, desferrioxamine was not capable of producing alterations in the iron status of both GN and CV mice, and therefore, did not interfere with the uptake of iron by *T. cruzi*. Iron-dextran, on the other hand, did elevate the levels of iron in the host and, therefore, its availability, what facilitated the proliferation of the parasite.

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