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SOME CHARACTERISTICS OF THE HYPERREACTIVITY TO BACTERIAL LIPOPOLYSACCHARIDE INDUCED IN MICE BY *TRYPANOSOMA CRUZI* INFECTION

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Mice infected with T. cruzi, *Y strain, acquire a high level of susceptibility to the effects of bacterial gram-negative LPS. The LD50 of adult female SW mice to LPS from* S. typhosa, *decreases from 450 to 2,5mcg 10-12 days after* T. cruzi *infection. This hyperreactivity to LPS induced by* T. cruzi *presents all the characteristics of that found in infection caused by many other agents. During the acute phase of experimental infection with* T. cruzi *Y strain, mice generally die with a hypovolemic shock very similar to that induced in uninfected animals injected with an adequate dose of bacterial endotoxin. There is evidence for and against the hypothesis that LPS absorbed from the intestinal tract may be involved in the mechanism of death of mice during the acute phase of* T. cruzi *infection.*

Hyperreactivity towards lipopolysaccharide (LPS) from gram-negative bacteria has been described in a variety of conditions in different animal species, including man. Among agents that can potentiate this hyperreactivity, which may decrease the LD50 dose of LPS as much as 100-1000 fold, include infection by virus, bacteria, protozoa and helminths; administration of several chemical agents and various treatments such as irradiation, neonatal thymectomy, adrenalectomy, parabiosis, graft-versus-host reactions and pregnancy (Becker & Rudbach, 1978; Chedid, 1973; Clark, 1978; Cooperstock, Tucker & Baublis, 1975; Ferluga, Doenhoff & Allison, 1979; Howard et al., 1959; Peavy, Baugher & Musher, 1979; Shands, Miller & Martin, 1969; Shands & Senterfitt, 1974; Suter, 1962; Suter & Kirsanow, 1961; Suter, Ullman & Hoffman, 1958). Here we present the results of experiments carried out to study some aspects of this hyperreactivity induced in mice by *T. cruzi* infection, as well as the eventual cooparticipation of this LPS in the mechanism of death of infected animals.

MATERIAL AND METHODS

Mice: swiss albino, random-bred, female mice, weighing 20 ± 2g were used in all experiments. Animals were housed in groups of 10/cage and provided with standard pellet and water "ad libitum".

Lipopolysaccharide: LPS from *Salmonella typhosa* (Difco Chemical Co.) was a Westphal phenol-water preparation. LPS was suspended in sterile nonpyrogenic 0.85% NaCl and kept frozen at -20°C. Its LD50, calculated by the method of Reed & Muenchen (1938), was found to be 450mcg by intraperitoneal (i.p.) injections.

Trypanosoma cruzi: T. cruzi, Y strain trypomastigote, was maintained by serial passage in SW mice.

T. cruzi **infection**: each animal was i.p. injected with 0.2ml of a suspension containing 2 x 10^4 *T. cruzi* blood-form trypomastigotes. The degree of parasitemia was determined 5, 7 and 9 days after infection. The survival time of the animals was registered during 30 days.

Hyperreactivity to LPS induces by *T. cruzi:* mice were i.p. infected with 2 x 10^4 blood-forms of *T. cruzi*, Y strain. After regular intervals thereafter, groups of five mice were challenged i.p. with serial dilutions of LPS. Uninfected mice received similar doses of LPS (Table I). Deaths resulting in 24h from LPS were recorded and the 50% lethal dose (LD50) determined.

Levels of LPS in serum of mice infected with *T. cruzi:* venous blood was aseptically collected from mice 7 and 12 days after *T. cruzi* infection. The serum was transferred to pyrogen-free glass tubes and heated at 100°C/10 min (Levin, Tomasulo & Oser, 1970; Reinhold & Fine, 1971). Several twofold dilutions were prepared. 0.1 ml samples were added to an equal volume of limulus amebocyte lysate (Mallinckrott) and the tubes incubated at 37°C for 60 min. The tubes were read immediately afterwards.

Anti-Lipid A serum: free lipid A was obtained by hydrolysis of *S. thyphosa* LPS in 1% acetic acid at 100°C for 2h (Mattsby-Baltzer & Kaijser, 1979). Lipid A was solubilized by treatment with 0.25M NaOH at 56°C for 60 min. Mouse erythrocytes were coated with this lipid and repeatedly injected i.p. in mice to obtain specific anti-lipid A antibodies. The collected antibodies gave a final hemagglutinating titer of 1:640.

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Hemoconcentration during *T. cruzi* infection: indirect evaluation of hematocrit variation of mice infected with *T. cruzi* was made by measuring hemoconcentration of 10 µl blood samples collected serially from the retro-orbital vessels during infection. The values of hemoconcentration was expressed as percent of variation of hemoglobin concentration after infection (Harris & Fulton, 1958).

Reversion by hydrocortisone of hemoconcentration induced by *T. cruzi* infection: similar blood samples for evaluation of hematocrit variation were collected from a group of infected mice daily injected s.c. with hydrocortisone acetate (6mg/animal).

Protective effect of different substances on *T. cruzi* infection: groups of mice i.p. infected with 2×10^4 blood forms of *T. cruzi*, Y strain, at different periods of time received separately one of the following agents (see Table III): Hydrocortisone acetate, Promethazin hydrochloride, Heparin, Polymyxin B sulphate, Indomethacin and ZnC12. All substances obtained from Sigma Chemical Co.

Destruction of intestinal flora: groups of mice received a standard sterilized pellet diet and water containing Neomycin sulphate (50mg/ml), Sulfadiziazine (150mg/ml) and Phthalysulfathiazole (100mg/ml). The drug mixture was administered until the number of bacterial intestinal colonies was reduced to 2-3 per plaque, when the animals were i.p. challenged with 2×10^4 *T. cruzi*, Y strain. The drug was maintained during the first 40 days of *T. cruzi* infection. The degree of parasitemia and the animal survival were registered.

Survival of mice passively immunized with antibodies to Lipid A: mice antiserum containing hemagglutinating antibodies to lipid A (titer 1:640) was injected in mice infected with 2×10^4 *T. cruzi* parasites. Each animal received i.v. 0.3ml of antiserum on days 3, 5, 7 after infection.

Survival of mice actively immunized with LPS: the influence of active immunization to LPS in the survival of mice with *T. cruzi* infection was studied in animals that received 6 weekly i.p. injections of 5mcg of heated (100°C/30 min) LPS, *(S. typhosa)* adsorbed on mouse red blood cells. The titer of hemagglutinating anti-LPS antibody, at the moment of challenge with *T. cruzi* trypomastigote, was found to be 1:320.

Liver histology: liver slides were obtained soon after death and fixed in 10% neutral formalin. Sections were obtained by hematoxylin-eosin and the periodic acid Schiff (PAS) reaction for glycogen.

RESULTS

Hyperreactivity to LPS induced by *T. cruzi*: our studies showed the infection with *T. cruzi* increases the reactivity of SW mice to the lethal effect of LPS. As can be seen from Table I, the LD50 or LPS i.p. administered was reduced from 450 to 2.5mcg 10 to 12 days after infection. This hyperreactivity to LPS that can already be shown 4 days after *T. cruzi* infection, increases gradually, reaching its maximum around 10-12 days and persisting until the moment of animal's death.

TABLE I

Heperreactivity to LPS of mice intraperitoneally infected with 2×10^4 blood form *T. cruzi* (Y strain). Mortality determined within 24 hours after intraperitoneal injection of LPS (*S. typhosa*). As can be seen the LD 50 to LPS gradually decreased from 450 to 2.5 mcg during the course of infection

Mice *	Days after infection with *T. cruzi*	LD 50 of LPS (*S. typhosa*) (mcg, i.p.)
Normal	0	450
Infected	4	80
	6	20
	8	7
	10	4
	12	2.5

* Mean of five animals.

Characteristics of acute *T. cruzi* infection: the levels of blood LPS of animals during acute *T. cruzi* infection, as detected by the lymulus amebocyte lysate oscilate between 0.18 to 1.8mcg/ml around the 7th day and reaching 9mcg/ml around the 12th day (Table II). The development of hemoconcentration during acute *T. cruzi* infection, as revealed by hemoglobin concentration in serial samples of blood was already observed 6 days after infection, thereafter increasing gradually until the 12-14 days. As can be seen (Fig. 1), hydrocortisone almost completely reverted the hemodynamic characteristics of shock due to the hemoconcentration. The hepatic lesion found in mice dying of *T. cruzi* infection is represented by hepatocyte necrosis, depletion of cellular glycogen and degenerative reactions.

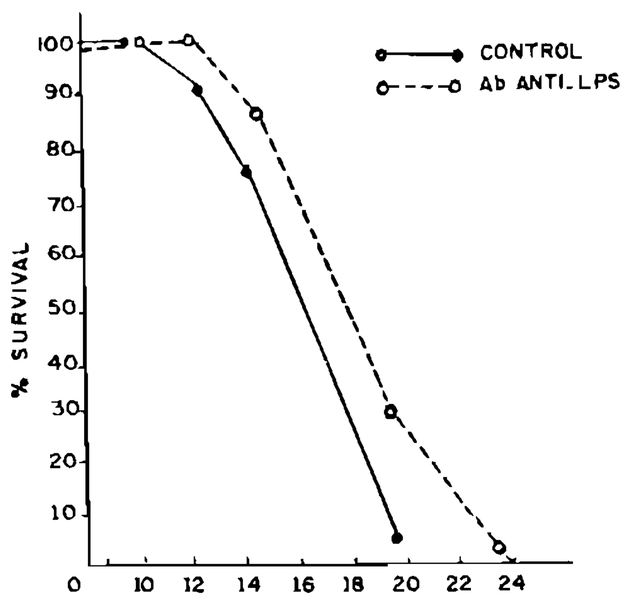
TABLE II

Levels of serum LPS of mice infected with *T. cruzi*. Titration by limulus amebocyte lysate on days 0, 7, 12 after infection

Mice	Days after *T. cruzi* infection	Serum LPS * (mcg/ml)
Normal	0	<0.18
Infected	7º	0.18 – 1,80
	12º	>9.0

* Mean of five animals.

Cooparticipation of LPS in the mechanism of death of animals with acute *T. cruzi* infection: mice with reduced levels of bacterial intestinal flora, after adequate chemotherapy and then infected with *T. cruzi*, Y strain, showed prolonged survival period and a reduced percentage of cumulative death when compared to untreated mice (Fig. 2). The presence of serum antibodies induced against LPS gave no protection against death caused by *T. cruzi* infection (Fig. 3). Also the passive administration of lipid A antiserum 3, 5, 7 days after infection offered no protection (Fig. 4). It was not possible to prolong the survival time of mice, during acute phase *T. cruzi* infection, with drugs that block biologic effects of LPS (Heparin, Endomethacin, Polymyxin B, Promethazine or zinc chloride) (Table III).



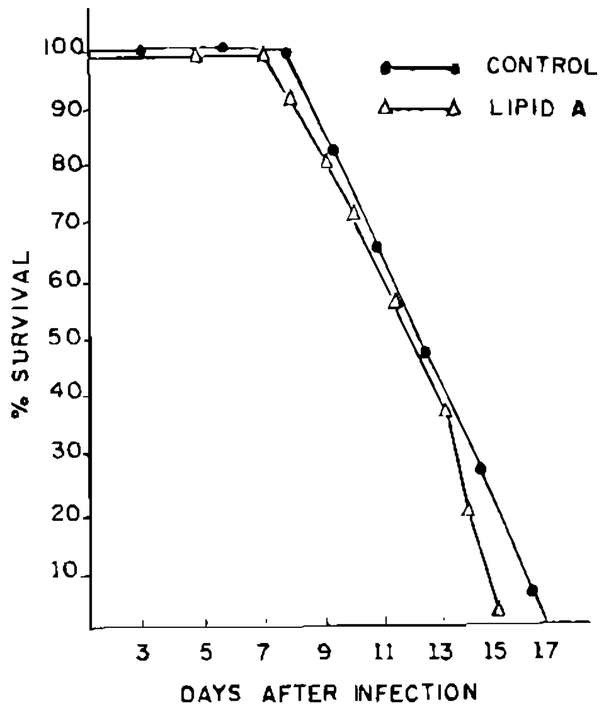


Fig. 1: mean percentage variation of hemoglobin concentration in different days after challenge with 2×10^4 blood *T. cruzi*, (Y strain) as compared with samples collected in a group of infected mice daily injected s.c. with hydrocortisone acetate (6mg/animal). As can be seen the corticoid completely blocked the development of hemodynamic aspects of shock during *T. cruzi* infection. Fig. 2: survival of mice previously treated with oral antibiotics and with their intestinal gram-negative flora greatly reduced. Animals challenged with 2×10^4 blood *T. cruzi* (Y strain). Fig. 3: survival of mice actively immunized with LPS *(S. typhosa)* and challenged with 2×10^4 blood *T. cruzi* (Y strain). The mean titer of serum hemaglutination antibody to LPS at the moment of challenge was found to be 1:320. Fig. 4: survival of mice passively immunized i.v. with 0,3ml of lipid a antiserum on days: 3, 5 and 7 after infection with 2×10^4 *T. cruzi*.

DISCUSSION

T. cruzi infection with Y strain increases greatly the susceptibility of adult SW mice to the letha effect of LPS from gram-negative bacteria, thus corroborating the results obtained by Kierszenbaum using Tululahuén strain (Kierszenbaum & Budzko, 1973; Kierszenbaum & Saavedra, 1972). In a representative experiment (Table I) with *S. typhosa* LPS administered i.p., the LD50 was found to decrease from 450 to 2.5mcg, 10-12 days after infection. This hyperreactivity could even be seen 4 days after infection, when 50% of the animals can be killed with 80mcg of LPS. This suscetibility gradually increases to reach its maximum 12 days after infection and persists until the moment of death. The hyperreactivity to LPS

TABLE III

Schedule for the different substances used in the protection against death during *T. cruzi* infection

Substance	Route	Days in which the substance was injected after *T. cruzi* infection					Total amount injected
		1	2	3	4	5	
Hydrocortisona acetate	S.C.	+	–	–	–	+	6 mg
Prometazine hydrochloride	S.C.	+	+	+	+	+	250 mcg
Heparin	I.P.	+	+	+	+	+	3.000 mcg
Polymyxin B sulphate	I.P.	+	+	+	+	+	300 mcg
Indometacin	S.C.	+	+	+	+	+	500 mcg
ZnC12	S.C.	+	+	+	+	–	1.000 mcg
Saline	S.C.	–	–	–	–	–	–

induced by *T. cruzi* presents all the characteristics of that found in infections caused by other agents (virus, bacteria, protozoal, helminths etc). During the acute phase of experimental infection with *T. cruzi* Y strain, mice generally die with a hypovolemic shock very similar to that induced in uninfected animals injected with an adequate dose of bacterial endotoxin. In both situations the animals present cold extremities, erected furs, tachycardia, tachypneia, pronounced adynamia and reduced peripheral blood volume. The development of hemoconcentration, as revealed by the increase of hemoglobin concentration in serial samples of blood, can be seen six days after *T. cruzi* infection, thereafter increasing gradually to reach a plateau 12-14 days later. Hydrocortisone, a substance that normally antagonizes the hemoconcentration of the infected animals, increases significantly the leves of blood and tissue parasites. The hepatic lesions found in mice during *T. cruzi* infection, were similar to those described in animals inoculated with an adequate dose of LPS. Lesions much more severe (hepatocyte necrosis, depletion of cellular glycogen, extensive degenerative phenomena) were found in animals infected with *T. cruzi* and subsequently injected with LPS. Acute *T. cruzi* infection alone can also induce both a moderate elevation of blood transaminase and a pronounced hypoglycemia. Subsequent LPS injections greatly enhance these changes (data no shown).

The eventual coparticipation of LPS in the mechanism of death during acute *T. cruzi* infection was studied in different experiments. It was shown that the levels of blood LPS reaches 9mcg/ml 10-12 days after infection, a period in which the LD50 to LPS became reduced to 2.5mcg/ml. One of the conclusions to be reached is that 10 days after infection the blood levels of LPS may be sufficiently high to kill the animals.

The LPS found in the blood of infected mice is probably derived from the intestinal tract. Indeed, mice with reduced level of bacterial intestinal flora, after adequate chemotherapy and subsequently infected with *T. cruzi*, showed a prolonged survival period and a reduced percentage of cumulative death when compared to untreated mice (Fig. 2). It should be stressed that there are not yet sufficient data about the biochemicals and biologicals properties of the lipid fraction of the LPS extracted from *T. cruzi* by Kette-ridge (1978) and Goldberg et al. (1983). Thus, we are not in a condition to appreciate the eventual co-participation of this LPS in the phenomena herein described.

Polymyxin B, a cyclic polypeptide antibiotic, which binds tightly to lipid A of endotoxins and is capable of abrogating many of their biologic activities (From, Fong & Good, 1979), shows no effect on death rate of mice with acute *T. cruzi* infection. The question of whether endotoxins participate in the mechanism of death during acute *T. cruzi* infection could not be solved by the experiments carried out in this paper. There is no doubt that during the acute phase of this infection the LD50 of *S. typhosa* can be reduced to low levels (2.5 mcg/ml). This amount is far below that found in animal blood 10 to 12 days after infection (9mcg/ml). There is, however, several arguments for and against this assumption: 1) the LPS blood levels of the infected mice are sufficiently high to kill them because of the increased susceptibility to LPS induced by *T. cruzi;* 2) mice with acute *T. cruzi* infection generally die with hypovolemic shock caused by the endotoxemia; 3) it is possible to prolong the life of animals acutely infected with *T. cruzi* by reducing the levels of bacterial intestinal flora with adequate chemotherapy; 4) the hepatocyte lesions found in mice with *T. cruzi* infection or injected with LPS are indeed very similar; 5) hydrocortisone almost completely revests the hemodynamic characteristics of shock due to the hemoconcentration induced by *T. cruzi* infection or LPS.

The following arguments can be formulated against the above hypothesis: 1) the antibodies against LPS or lipid A give no protection against death induced by *T. cruzi* infection; 2) polymyxin B, which is capable of binding tightly to lipid A, thus blocking many of the biologic activities of LPS, gives no protection against lethal hypovolemic shock that occurs during acute *T. cruzi* infection; 3) several substances that block the biologic effects of LPS (Heparin, Indomethacin, Promethacin, zinc chloride) do not prolong the survival of mice with *T. cruzi* infection.

RESUMO

Camundongos infectados com a cepa Y do *T. cruzi* adquirem elevado grau de reatividade ao LPS de *S. typhosa.* A LD50 de camundongos SW, adultos, fêmeas, com peso em torno de 20g, decresce de 450 para 2,5mcg ao fim de 10-12 dias de infecção. Esta hiperatividade ao LPS induzida pelo *T. cruzi* apresenta todas as características daquela encontrada nas infecções causadas por muitos outros agentes. Durante a fase aguda da infecção experimental com *T. cruzi,* cepa Y, os camundongos geralmente morrem com choque hipovolêmico muito semelhante ao ocasionado por dose adequada de endotoxina bacteriana. Há evidências a favor e contra a hipótese de que os LPS liberados do trato intestinal estejam envolvidos no mecanismo de morte do camundongo durante a fase aguda da infecção pela cepa Y do *T. cruzi.*

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