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Immunization Against Experimental Chagas' Disease by Using Culture Forms of *Trypanosoma cruzi* Killed with a Solution of Sodium Perchlorate

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Protection against infection with virulent blood (trypomastigote) forms of *Trypanosoma cruzi* was accomplished in mice by immunization with culture (mainly epimastigote) forms killed by treatment with sodium perchlorate. Sodium chloride, used instead of sodium perchlorate, with all other conditions kept the same, failed to kill all the organisms, indicating that the effects of the perchlorate anion were not simply ionic or osmotic, suggesting that they might be chaotropic. A single dose of the immunogen, without adjuvants, was sufficient to significantly protect against the infection. Protection was achieved by either intraperitoneal, intramuscular, or subcutaneous immunization, though the first two routes appeared to be more effective. After challenge, parasitemias were negative in 25, 29, and 17% of the animals immunized intraperitoneally, intramuscularly, and subcutaneously, respectively.

Resistance to infection with virulent *Trypanosoma cruzi*, the organism causing Chagas' disease in humans, is known to appear after infection with strains or forms of the parasite of little or no virulence (5). This indicates not only that it is possible to immunize against *T. cruzi* infection but also that different strains or forms have either common or similar antigenic determinants. Based on this concept and considering the possible risks involved in immunization with live flagellates (1), several attempts have been made to prepare an effective immunogen with killed organisms. The use of parasites killed by either chemical (10, 13, 14) or some physical (11) means has usually yielded unsuccessful results, due perhaps to the aggresiveness of the reagents or procedures on the antigens. However, partial protection has been reported achieved with culture forms of *T. cruzi* killed by some physical methods (F. C. Goble, J. L. Boyd, M. G. Wehner, and M. Konrath, J. Parasitol. 50(Suppl.):19, 1964) or disrupted at high pressures and low temperatures (6). Live trypanosomes have also been used after exposure to gamma radiation, with variable success (2, 8).

The parasite's antigens might retain their ability to produce a protective immune response if the flagellates were killed by means of a suitable "mild" treatment. Chaotropic ions (4, 7) might serve this purpose since they are known to allow the recovery of biologically active molecules such as antibodies (3) and enzymes (9) from antigen-antibody complexes and multicomponent enzymes, respectively, without major loss in their activities. Similarly, the parasite might be killed in the presence of these ions and its antigens remain unaltered after removal. We have investigated this possibility and will show here that immunization with culture forms of *T. cruzi* killed with a solution of perchlorate anions results in significant protection against infection with virulent trypomastigote forms.

MATERIALS AND METHODS

Animals. Experiments were performed with 25- to 30-day-old male Rockland mice. Strain passages were carried out in 4-week-old mice of the same strain of both sexes. All animals were obtained from a colony maintained at the Immunochemistry Section of this Institute.

Parasites. Both culture and blood forms of Tulahuén strain *T. cruzi* were utilized in this study.

(i) Culture forms. The parasites used to prepare the immunogen were grown in a biphasic medium containing brain-heart infusion (Difco, Detroit, Mich.) and agar (Difco, Detroit, Mich.) in the solid phase, and brain-heart infusion, glucose, and liver extract in the fluid phase. Passages and detection of parasites were performed in the same medium plus 2.5% defibrinated sheep blood added to the solid phase. Cultures were incubated at 28 C.

(ii) Blood forms. *T. cruzi* trypomastigote forms were obtained from the blood of infected mice. The

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lethal dose fifty for Rockland mice, determined by the method of Reed and Muench (15), was 10^3 organisms. The numbers of parasites per milliliter present in the blood of infected animals were determined by a standardized microscopic procedure described previously (12).

Preparation of the antigen. Parasites were obtained from 5-day-old cultures, separated by centrifugation at 2,700 × *g* for 5 min at 4 C, and washed three times with a sterile phosphate-buffered saline solution, pH 7.0 (PBS), under the same conditions. After the last washing the sediment was weighed out and 3 ml of a 3.33 M solution of sodium perchlorate was added per g of wet parasites. This operation was performed in about 10 s at room temperature while continuously stirring with a Vortex-type mixer. The mixture was immediately transferred to another tube and stirred for another 5 min, after which a volume of cold PBS equivalent to the total volume of the suspension was added. The perchlorate ions were then removed by dialysis against PBS at 4 C. The antigenic material will be referred to as *T. cruzi* killed with sodium perchlorate (TCSP). TCSP was fractionated by centrifugation at 20,000 × *g* for 15 min at 4 C. The pellet was resuspended to the original volume of the suspension with PBS and the supernatant was used without further treatment.

Injections. All materials were given intraperitoneally unless stated otherwise.

Statistical analysis. The significance of differences between means was established by Student's *t* test. Differences were considered to be significant if $P < 0.05$.

RESULTS

Blood-agar cultures containing up to 150 mg of TCSP showed no signs of growth over an incubation period of 12 weeks, whereas control cultures with single parasites were usually positive after 2 to 4 weeks. Direct microscopic observation of numerous preparations did not reveal the presence of motile organisms. By electron microscopy, treated parasites showed a fairly severe intracellular damage (E. Schuchner and F. Kierszenbaum, unpublished observations). Many had lost their flagella and both the nucleus and the kinetoplast had disappeared as defined structures, but membranes seemed normal.

When the treatment was performed with a solution of sodium chloride instead of sodium perchlorate, with all other conditions kept the same, about 1% of the parasites were still motile after dialysis and cultures of this preparation were positive in 24 to 72 h. These results indicate that the effects of the perchlorate anion on *T. cruzi* are not simply ionic or osmotic.

Although the insoluble and the soluble fractions of TCSP evoked protective immunity against *T. cruzi* infection in mice, the former appeared to be more effective (Fig. 1 and 2).

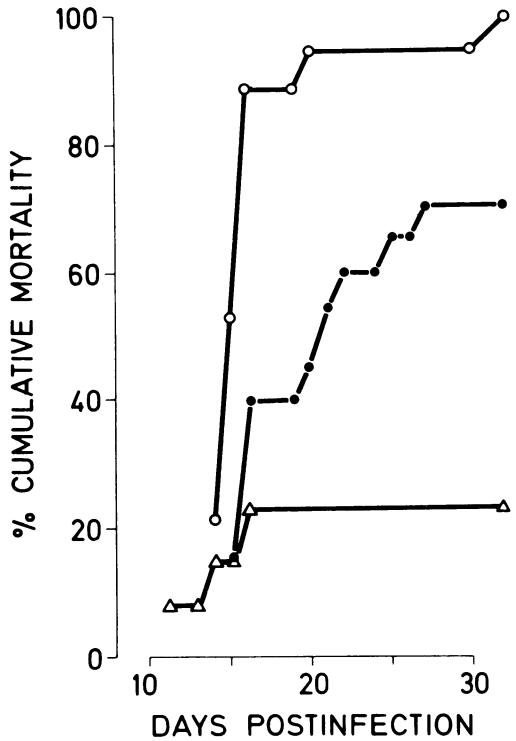


FIG. 1. *Mortality of mice infected with T. cruzi after immunization with soluble or insoluble antigens of TCSP. Symbols:* (○) *Control mice (17) given two doses of 0.4 ml of PBS;* (●) *mice (17) immunized with two doses of 0.4 ml of the soluble fraction;* (△) *mice (13) given two doses of insoluble antigen, each containing 50 mg (wet weight) of killed organisms. The interval between doses was 7 days. Challenge was made with 5 × 10^4 trypomastigotes 20 days after the last dose. In parentheses, the initial numbers of animals.*

Immunized mice surviving after challenge were still alive 15 months later when they were sacrificed for other purposes.

Toxic or deleterious effects were nondemonstrable; thus, immunized mice failed to show either weight loss or mortality over a period of 30 days, after which observation was suspended. Immunization with either fraction caused no exacerbation of the disease (Fig. 1 and 2). Since the supernatant had a slight protective and no exacerbating effect, nonfractionated material was utilized in further experiments.

Significant protection was obtained with either one or two doses of TCSP, each of which contained 50 mg (wet weight) of killed parasites (Fig. 3 and 4). In this experiment none of the mice immunized with two doses of TCSP died after challenge. The animals were sacrificed 15 months after the infection. Four of the 16 mice

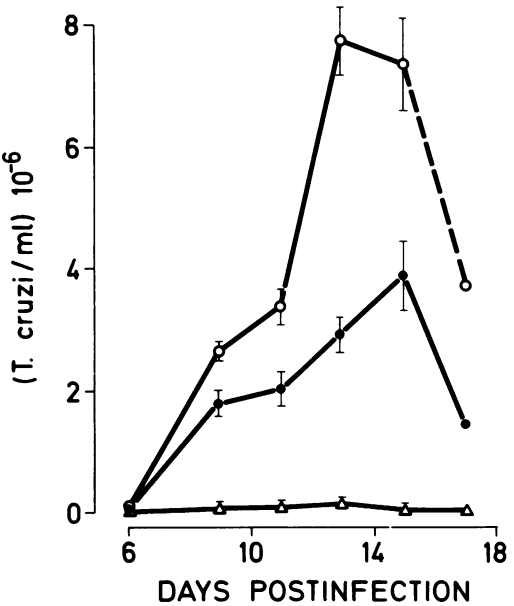
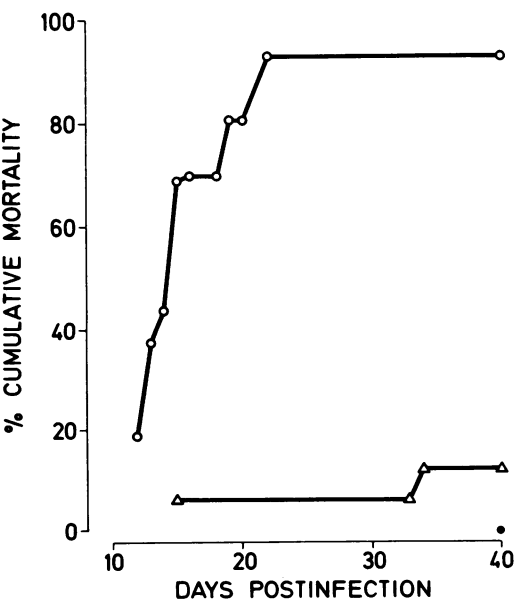


FIG. 2. *Parasitemia of mice infected with T. cruzi after immunization with soluble or insoluble antigens of TCSP. Symbols are as described for Fig. 1 and the data correspond to the same animals whose mortality is shown in that figure. Points and vertical bars represent mean parasitemia and standard error, respectively, of mice surviving at the indicated time. On day 17 postinfection only two control mice were alive. From day 9 on, differences between mean values obtained from mice immunized with the insoluble fraction and those of the control group are statistically significant ($P < 0.001$). Animals given the soluble portion showed significant differences on days 11 ($P < 0.05$) and 13 ($P < 0.001$).*



receiving a single dose of TCSP never showed parasites in their blood. In contrast, all of the mice of the control group showed high parasitemias. No individual follow-up was performed with the animals that received two doses of TCSP.

To establish whether protection with TCSP can be produced by other routes than the intraperitoneal, immunization was carried out either intramuscularly or subcutaneously. Here, the dose of TCSP was lowered to 25 mg (wet weight) in view of successful exploratory trials. Significant protection was achieved by either route of immunization, though it was somehow better when TCSP was given intramuscularly (Fig. 5 and 6). The numbers of animals with systematically negative parasitemias repre-

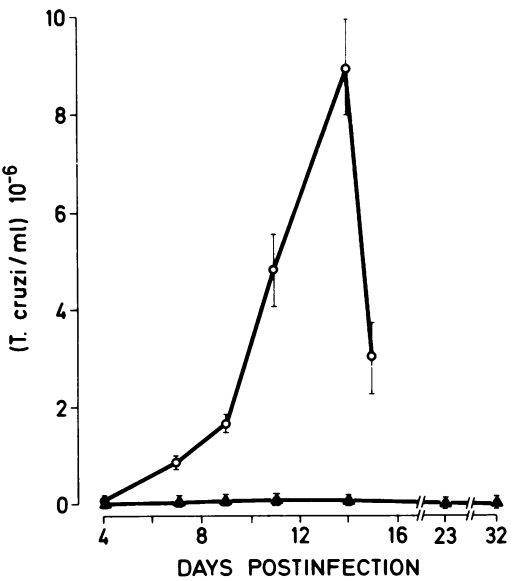


FIG. 4. *Parsitemia of mice infected with T. cruzi after immunization with one or two doses of TCSP. Symbols are as described for Fig. 3 and the data were obtained from the same animals whose mortality is shown in that figure. From day 7 on, all differences between experimental and control values are statistically significant ($P < 0.02$). Note that closed circles and open triangles overlap.*

FIG. 3. *Mortality of mice infected with T. cruzi after immunization with one or two doses of TCSP. Symbols: (Δ) Mice (16) immunized with a single dose; (●) mice (5) given two doses; (○) control group (16) injected with PBS. The interval between doses was 7 days and each contained 50 mg (wet weight) of killed parasites. All animals were infected with 5.2. × 10^4 trypomastigotes 20 days after the last dose. In parentheses, the initial numbers of animals. The single closed circle over day 40 represents 0% mortality of mice given two doses of TCSP.*

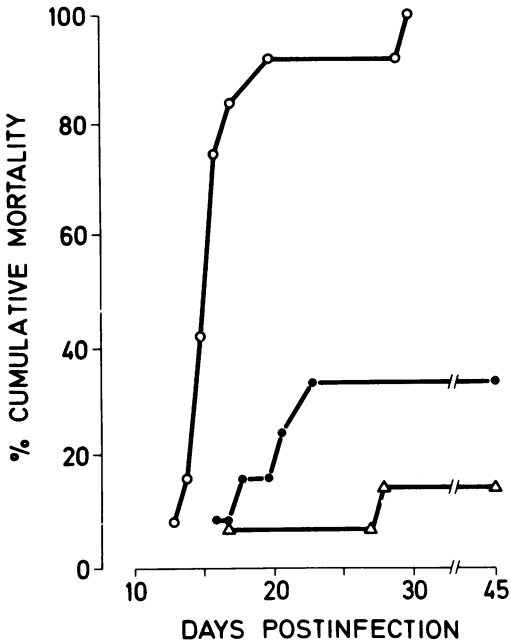


FIG. 5. *Mortality of mice infected with T. cruzi after immunization with TCSP by different routes. Symbols:* (O) *Control group (12) given PBS both intramuscularly and subcutaneously;* (Δ) *mice (14) immunized intramuscularly;* (●) *mice (12) immunized subcutaneously. The immunizing dose contained 25 mg (wet weight) of killed organisms. In parentheses, the initial numbers of mice. The infective dose, 5.1 × 10⁴* trypomastigotes, was given 19 days after immunization.

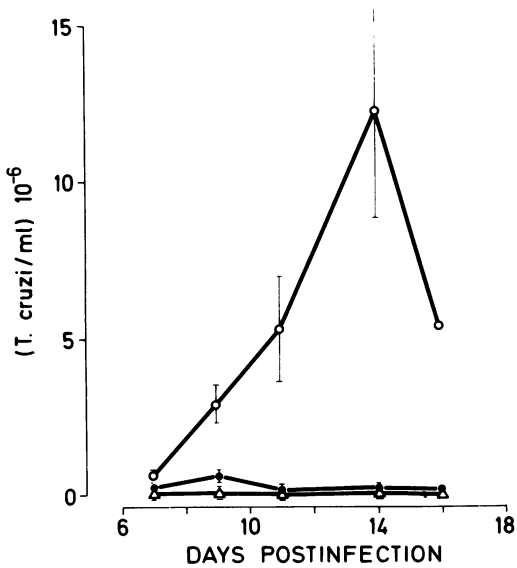


FIG. 6. *Parasitemia of mice infected with T. cruzi after immunization with TCSP by different routes. Symbols are as described for Fig. 5 and the data were obtained from the same animals whose mortality is shown in that figure. All differences between experimental and control values are statistically significant ($P < 0.01$).*

sented 29 and 17% of the groups given TCSP intramuscularly and subcutaneously, respectively. Parasitemias were detectable and reached high levels in all of the control mice. Survivors were still alive 5 months later when they were sacrificed.

DISCUSSION

The results show that significant protection against *T. cruzi* infection can be accomplished in mice by immunization with culture forms of the parasite killed with a solution of sodium perchlorate. The antigenic material comprised killed organisms whose cellular structure was severely damaged. However, in contrast with other chemical means utilized in the past, always unsuccessfully, perchlorate ions seem to exert a "mild" effect on the parasite's antigens. The perchlorate anion is second in the scale of activity of chaotropic ions, which are known to cause reversible molecular alterations (3, 7, 9) probably by disruption of hydrophobic bonding and water structure (4). The fact that chloride ions, which are nonchaotropic, were incapable to kill 100% of the flagellates under the same conditions indicates that the effects of the perchlorate may not be simply ionic or osmotic and suggests that they might be chaotropic. It is conceivable that antigenic determinants of *T. cruzi* are reversibly altered in the presence of perchlorate ions. Alternatively, these determinants may undergo no change during the exposure. In either case they remain immunogenic.

TCSP appeared to be very efficacious in producing protective immunity against *T. cruzi* infection. This was inferred from both the high degree of protection obtained with a single dose without using adjuvants and the percentages of immunized animals with negative parasitemia after challenge. Moreover, the average peak parasitemias of protected and nonimmunized mice differed in some cases by several hundredfold.

Immunization of mice with *Trypanosoma brucei* (an extracellular trypanosome) killed by sodium perchlorate was also found to protect against infection with the same organism (F. Kierszenbaum and P. H. Lambert, unpublished data). Similarly, the use of either perchlorate or other chaotropic ions may prove useful in preparing antigenic materials from other organisms.

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