Trypanosoma rhodesiense: Protection in Mice by Inoculations of Homologous Parasite Products

RALPH E. DUXBURY, ELVIO H. SADUN, MAURICE J. SCHÖNBECHLER AND DAVID A. STROUPE

Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D.C. 20012

(Submitted for publication November 20, 1973)

DUXBURY, RALPH E., SADUN, ELVIO H., SCHÖNBECHLER, MAURICE J., AND STROUPE, DAVID A. 1974. Trypanosoma rhodesiense: Protection in mice by inoculations of homologous parasite products. Experimental Parasitology 36, 70-76. Mice were immunized against Trypanosoma rhodesiense (Wellcome strain) with whole lyophilized trypanosomes, with antigens produced by disrupting lyophilized trypanosomes under pressure, and with excretions and secretions of the living parasites. The survival rate in groups of 40 mice inoculated with disrupted trypanosomes and challenged with the homologous strain was 48% with a soluble fraction, and 70% with a particulate fraction of the parasites. There was 95% survival after challenge in a group immunized with lyophilized trypanosomes; none of the controls survived. Results were essentially the same whether or not an aluminum hydroxide adjuvant was used. In subsequent experiments, complete protection was obtained with either crude excretion-secretion (ES) antigens or the particulate fraction of the ES antigen, while 40% of the mice survived challenge after inoculations of ES supernatant fluid. Mice immunized with crude ES antigen failed to survive challenge with a heterologous strain, although their mean survival time was prolonged several days beyond that of the controls.

INDEX DESCRIPTORS: Trypanosoma rhodesiense; Immunization; Antigens, excretion and secretion; Lyophilization; Pressure cell fractionation; Mice.

INTRODUCTION

Rodents, bovines, and subhuman primates have been immunized against trypanosomiasis by inoculating them with attenuated living trypanosomes exposed to ionizing radiation (Sanders and Wallace 1966; Duxbury and Sadun 1969; Duxbury et al. 1972a, b). Irradiated parasites were more effective as immunizing agents than vaccines prepared from dead parasites or their extracts. Attempts by various investigators to induce resistance to trypanosomiasis by artificial immunization have often given poor and conflicting results, although Thillet and Chandler (1957) successfully immunized rats against Trypanosoma lewisi by injecting them with metabolic products of the parasites. Partial protection against T. brucei in mice was reported by Miller (1965), who used trypanosome homogenates prepared in a Hughes press. Some success has also been attained by disrupting the organisms under controlled pressure and temperature. This method was used by Gonzales Cappa et al. (1968) to protect mice against T. cruzi, and by Corradetti et al. (1971) to immunize rats against T. lewisi.
Goble (1964, 1970) used the French pressure cell, ultrasonication, and shaking with glass beads to produce homogenates for immunizing mice against *T. cruzi*. In another study with *T. cruzi*, freeze-dried culture forms inoculated into mice, conferred resistance to subsequent infection with virulent blood forms (Rego 1956).

Theoretically, immunization with attenuated trypanosomes may approach that produced by living nonattenuated parasites in an infection which has been arrested by chemotherapy. This would indicate that the excretions and secretions of the trypanosomes are the antigens most important in inducing protective immunity. Until recently, a serious handicap in using excretion and secretion antigens or disintegrated trypanosomes for immunization purposes was the difficulty in obtaining large numbers of organisms in suspensions relatively free of host blood components. This difficulty was obviated by the introduction of an efficient method for isolating trypanosomes from infected blood by adsorbing particulate blood components onto DEAE cellulose columns (Lanham 1968).

As part of a continuing effort to develop effective vaccines against African trypanosomiasis, experiments were designed to determine if antigens from pressure-disrupted *T. rhodesiense*, or from excretions and secretions of the living trypanosomes could be used to immunize mice against this parasite.

**Materials and Methods**

*Preparation of Parasite Suspensions*

*Trypanosoma rhodesiense* (Wellcome strain) maintained in albino rats 2 (Walter Reed WI, BR strain) was used as the source of immunizing material. The trypanosomes were collected from the blood of heavily parasitized rats on the third or fourth day of infection. Heparin was used as an anticoagulant. The parasites were separated from the blood in a DEAE cellulose column (Lanham and Godfrey 1970) and washed twice by centrifugation at 600g for 20 min in phosphate–saline–glucose buffered pH 8.0 (PSG).

The procedure used for fractionation and extraction of the parasites was as follows. The trypanosomes were separated from the blood and lyophilized at .005 mm Hg. At a later date, 270 mg of the lyophilized parasites in 100 ml PSG were placed in a Sorvall–Ribi Cell Fractionator (Ivan Sorvall Inc., Norwalk, CT) in which they were ruptured by passage through a needle valve under pressure of 40,000 psi at 5 C. The resulting suspension, centrifuged at 27,000g for 40 min, yielded 80 ml of a soluble fraction (S) for use in immunizing mice. The sediment at the bottom of the tube was mixed with PSG in a tissue grinder to suspend a particulate fraction (P) of the trypanosomes for immunizing purposes. The volume of this fraction was brought up to 15.0 ml in PSG.

For collection of excretion and secretion (ES) antigens, washed trypanosomes from the column were suspended in Medium 199 (Microbiological Associates Inc., Bethesda, MD), to which 1.5 g glucose/100 ml of the medium had been added, and kept for 20 hr in a refrigerator at 5 C. All the parasites were intact, and most of them remained motile at the end of this time. The concentration of parasites was approximately 6 x 10^7/ml. On the following day, the suspension was processed in a refrigerated centrifuge at 1000g for 30 min; the supernatant fluid was removed, and centrifuged again at 17,000g for 20 min. This supernatant solution (200 ml) was subjected to pressure dialysis in 1/4 in. cellulose tubing at 19 psi at 5 C, and reduced to 11.0 ml. Three types of immunizing inoculations were prepared.
from this material: 6.0 ml were used as crude excretion-secretion antigens (ESC), and 5.0 ml was centrifuged at 200,000g for 3 hr to produce a supernatant fluid (ESS) fraction and a particulate (ESP) fraction. The ESP fraction was resuspended in 12.0 ml of Medium 199, placed in an ultrasonicator (Raytheon, well type), and given 2 exposures of 14 min each at 10 kHz.

Animal Inoculations

Four groups of mice (Walter Reed ICR, BR strain, 20–25 g) were inoculated intraperitoneally with 0.1 ml of the S fraction, the P fraction, the ESC fraction, or crude lyophilized *T. rhodesiense* (10 mg/ml) in saline. Some of the mice in each group were inoculated subcutaneously with 0.4 ml aluminum hydroxide as an adjuvant along with the immunizations. A second immunizing inoculation was given 3 weeks after the first. These mice and a group of healthy control animals were challenged 2 weeks after the second immunization with 1000 *T. rhodesiense* of the same strain used for immunization. The same schedule of immunizing and challenging inoculations was followed in mouse protection tests with the ESS and ESP fractions. The effectiveness of the immunizations was determined by examining blood from the tail veins of all the mice 5 days per week, for 30 days. The surviving animals were kept for several months for rechallenge and other experimental use.

RESULTS

In 2 experiments which included 170 mice, the animals were immunized with the S and P fractions obtained in the Sorvall-Ribi Cell Fractionator, with lyophilized *T. rhodesiense*, and with crude excretion-secretion products (ESC) of the trypanosomes. The results of the 2 experiments, combined and summarized in Table I, show that a high degree of acquired resistance was produced in the mice by all 4 types of immunizing inoculations. In contrast to the controls, all of which died as a result of the infection after an average of 4.8 days, a majority of the treated mice survived the challenging infections and remained parasite-free throughout the experiment. Among the mice inoculated with the 2 fractions obtained in the Sorvall–Ribi Cell Fractionator, 70% of those that received the P fraction were protected, as compared to 48% of the group immunized with the S fraction. There was 95% survival among the mice inoculated with lyophilized *T. rhodesiense*, and 100% survival in the group that received inoculations of the

### TABLE I

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. mice</th>
<th>Immunization</th>
<th>% survival</th>
<th>Mean survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>30</td>
<td>Buffered saline</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>II</td>
<td>40</td>
<td>Disrupted trypanosomes (S fraction)</td>
<td>48</td>
<td>6.1</td>
</tr>
<tr>
<td>III</td>
<td>40</td>
<td>Disrupted trypanosomes (P fraction)</td>
<td>70</td>
<td>9.3</td>
</tr>
<tr>
<td>IV</td>
<td>40</td>
<td>Lyophilized trypanosomes</td>
<td>95</td>
<td>7.4</td>
</tr>
<tr>
<td>V</td>
<td>20</td>
<td>Excretion–secretion (ESC) antigens</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>
ESC antigens. Partial protection, indicated by prolonged survival time, was obtained in the mice that failed to survive challenge. The mice of Groups II, III, and IV that died after challenge, had lived an average of 6.1, 9.3, and 7.4 days, respectively, as compared to 4.8 days for the controls in Group I. The mice in the first experiment had been separated into 2 groups, 1 which received inoculations with aluminium hydroxide adjuvant, and 1 which did not. Results with the 2 groups were essentially the same, and therefore the use of the adjuvant was discontinued. The results were pooled in Table I.

Because of the promising results obtained with the ESC antigens in the first 2 experiments, a third experiment was set up to include supernatant fluid (ESS) and particulate antigens (ESP) separated from the ESC by ultracentrifugation. Forty mice were separated into 4 equal groups to test the immunizing effects of these fractions. Groups II, III, and IV were given two 0.1 ml intraperitoneal inoculations of ESC, ESS, and ESP, respectively, at 1 week intervals. Group I, the controls, was inoculated according to the same schedule with 0.1 ml Medium 199. All the mice were challenged with 1000 T. rhodesiense of the homologous strain 2 weeks after the second immunization. The results (Table II) revealed that all the untreated control animals died from the infection after an average of 4.5 days. Complete protection from the challenging inoculation was induced in the mice that had been immunized with the ESC (Group II) and with the ESP (Group IV). A lesser survival rate (40%) was obtained in the mice of Group III that received immunizations of ESS. However, even in the mice of this group that died, the mean survival time was increased almost 2 days over that of the controls (Group I).

In an effort to find out whether or not cross-protection could be induced by this method, 10 mice were immunized with ESC and challenged as in the previous experiment except that the challenging inoculations were made with 1000 T. rhodesiense of a different strain (EATRO 1886). All the mice died as a result of this heterologous challenge (Table III). Again, the mean survival time of the immunized mice (20.6 days) was greater than that of the controls (15.3 days).

**DISCUSSION**

Equivocal results were obtained heretofore, in attempts to induce effective protection against African trypanosomes by vaccination with dead parasites. Present experimental results show that it is indeed possible to prepare parasite products which

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. mice</th>
<th>Immunization</th>
<th>% survival</th>
<th>Mean survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>Medium 199</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>ES (crude)</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>ES (supernatant fluid)</td>
<td>40</td>
<td>6.3</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>ES (particulate)</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>
TABLE III

Development of Immunity against Trypanosoma rhodesiense (Wellcome) in Mice Inoculated Twice with Excretion–Secretion (ES) Antigens, and Challenged 2 weeks after Immunization with a Different Strain of Trypanosoma rhodesiense (BATRO 1886)

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. mice</th>
<th>Immunization</th>
<th>% survival</th>
<th>Mean survival time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>Medium 199</td>
<td>0</td>
<td>15.3</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>ES (crude)</td>
<td>0</td>
<td>20.6</td>
</tr>
</tbody>
</table>

possess an effective immunizing capacity when inoculated into mice. Since previous investigators working with *T. cruzi* (Goble *et al.* 1964; Seneca *et al.* 1966; Gonzalez Cappa *et al.* 1968) and with *T. lewisi* (Thillet and Chandler 1957; Yasuda and Dusanic 1969; Corradetti *et al.* 1971) showed antigenic differences among somatic antigens and excretory–secretory products, the relative effectiveness of these antigens was tested. Results of our experiments indicate that a considerable protection can be induced by inoculation with lyophilized trypanosomes, and with organisms which have been disrupted under pressure.

The best protection was induced with excretion–secretion products. The term excretion and secretion (ES) antigens was suggested for the antigens produced by incubating parasites in various media or physiological salt solution (Campbell 1955). Presumably, as long as the parasites are alive, they are producing excretions and secretions which will be suspended or dissolved in the fluid in which the parasites reside. Thillet and Chandler (1957) demonstrated that metabolic products of blood stream forms of *T. lewisi* inoculated into rats, produced a protective immunity to a subsequent challenge inoculation. Their results were confirmed by Yasuda and Dusanic (1969). Although ES antigens have not been used widely in immunization against protozoan infections, their role in producing acquired immunity against helminth infection has been demonstrated frequently (Campbell 1955; Chute 1956; Thomson 1956, 1963; Chipman 1957; Soulsby 1957; Sadun and Lin 1959; Levine and Kagan 1960; Ewert and Olson 1961). When these products were subjected to high speed centrifugation, it was found that the sedimented fraction was primarily responsible for inducing this protection. It was postulated (Duxbury and Sadun 1969) that since the dose of irradiation needed to suppress normal division and infectivity is only a small fraction of that required to kill the trypanosomes, irradiation may enable one to take advantage of the special immunological properties of the living parasites. The results of the present investigations, indicating that maximal protection is induced by the use of excretory and secretory antigens, extend and support this hypothesis. In general, our experimental results show that by fulfilling several requirements, it is possible to prepare a metabolic antigen with effective immunizing capacity against the homologous strain. It is unlikely that antigenic variation occurred in these experiments, since the infections were passaged in the rats twice weekly. The antigen protected mice against fatal infections with a virulent homologous strain of *T. rhodesiense*, did not require the addition of adjuvants, and had no obvious undesirable effects on the host. Although with some antigens the protection against the
homologous strain was absolute (100% of the inoculated mice survived), there was only a mild protection as evidenced by a prolonged survival time when a heterologous strain was used for the challenge. No protein determinations or other chemical analyses were made of the various antigen preparations. This will be the subject of a subsequent study.

ACKNOWLEDGMENTS

We thank SP6 Dennis D. Broud, Department of Bacterial Diseases, and Herald R. Langbehn, Department of Medical Zoology, Walter Reed Army Institute of Research, for their assistance in antigen preparation. This paper is contribution No. 2 from the International Laboratory for Research on Animal Diseases, Nairobi, Kenya and No. VII in the series “Experimental Infections with African Trypanosomes.”

REFERENCES


MILLER, J. K. 1965. Variation of the soluble antigens of Trypanosoma brucei. Immunology 9, 521-528.


