Cell-Mediated Hypersensitivity in Rabbits Infected with *Trypanosoma brucei* and *Trypanosoma rhodesiense*

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Animals infected with strains of *Trypanosoma brucei* and *T. rhodesiense* exhibited cutaneous hypersensitivity to intradermal administration of antigen. This reactivity was of two types, an Arthus-type, antibody-mediated reaction which reached maximum intensity 4 hr after injection and a delayed-type, cell-mediated reaction which reached maximum intensity 24 hr after injection. This delayed-type hypersensitivity appeared in rabbits not earlier than 3 weeks after onset of infection. It did not occur in animals which received dead organisms. There was a cross-reaction in both types of reactivity between antigens prepared from *T. brucei* and *T. rhodesiense*. The delayed-type hypersensitivity was transferred to normal rabbits by intravenous inoculation of washed living cells from the spleen of a rabbit which showed delayed hypersensitivity. Dead cells failed to transfer hypersensitivity. The histological picture of the indurated area was typical of a delayed-type reaction.

Recent studies by Mackaness and Blanden (3) have demonstrated that the capacity of microorganisms to survive and multiply within host macrophages is apparently related to the appearance of delayed-type hypersensitivity towards the corresponding microbial antigens. The occurrence of this type of hypersensitivity is also related to the existence of nonspecific changes in macrophage activities which are manifested in increased resistance to these microorganisms, so-called acquired cellular immunity. Neither delayed-type hypersensitivity nor acquired cellular immunity can be transferred to normal animals by the use of serum; they may, however, be transferred by living small lymphocytes such as are contained in spleen or lymph node cell suspensions.

Delayed-type hypersensitivity has been shown to occur in many microbial, fungal, and protozoal diseases. In protozoal diseases caused by hemoflagellates, delayed-type hypersensitivity has been recently reexamined by Gonzalez-Cappa et al. (2) and Seah (6), who studied *Trypanosoma cruzi* infection, and reviewed by Cahill (1) in leishmaniasis.

Delayed-type hypersensitivity has not yet been reported to occur in African trypanosomiasis. This may be due to the general belief that African trypanosomes have no intracellular stages. Recent work by Soltys and Woo (8), Ormerod and Venkatesan (4), and Wery and Kayembe (9), in which the occurrence of leishmanial stages has been demonstrated, suggested to us that delayed-type hypersensitivity may be present in animals suffering from subacute or chronic infection. The results of experiments designed to demonstrate the existence of cell-mediated skin reactions of the delayed type in rabbits infected with trypanosomes of the *brucei-rhodesiense* group are reported in this paper.

**MATERIALS AND METHODS**

*Trypanosomes.* The trypanosomes used in this study consisted of two substrains of *T. brucei* Shinyanga III. One strain referred to as *T. brucei* "M" was frequently syringe-passaged through mice during a period of more than 15 years. The outer substrain, *T. brucei* "R", was syringe-passed 39 times through rabbits. Other strains examined were *T. brucei* TREU 285 and *T. rhodesiense* TREU 334. (These two strains were obtained from W. H. R. Lumsden.) All of the strains had been preserved at −79 C (6) before these experiments.

**Preparation of antigens.* Organisms were isolated by bleeding mice infected with trypanosomes at the peak of parasitemia and separating the trypanosomes from blood by the method described by Soltys (7). The organisms were then washed and resuspended in Alsever’s solution to 10% (v/v).

From this suspension the following antigen solutions were prepared: (i) a suspension of organisms in Alsever’s solution kept at about 4 C for several days
until dead and then frozen; (ii) a suspension of freshly prepared organisms disrupted by sonic treatment; (iii) a suspension of organisms resuspended to 10% (v/v) in distilled water, with NaCl added after 30 min to restore isotonicity; (iv) a suspension of organisms resuspended in Alsever’s solution containing 0.1% beta propiolactone; (v) a suspension heated to 56 C for 1 hr; and (vi) a suspension of organisms resuspended in Alsever’s solution containing 0.5% phenol.

Animals. Cross-bred white rabbits weighing between 2.5 and 4 kg were used.

Skin testing. Antigens were inoculated intradermally into the shaved skin on the back of infected rabbits in a dose of 0.1 ml. The injection site was observed 2, 4, 6, 8, 24, 48, and 72 hr after injection and examined both for increase in skin thickness as measured by tuberculin calipers and for erythema.

RESULTS

Effect of the method of preparation of trypanosome antigen upon skin reaction to T. brucei. Ten rabbits infected with T. brucei Shinyanga III M and R were skin-tested with antigens prepared by various techniques (see above). The most efficient preparation for the induction of skin reactions was the first antigen solution, prepared by allowing the organism to die at about 4 C. No other method of preparation approached this technique in its efficiency (Fig. 1.) Minor differences were observed in the efficiency of each preparation for the 4-hr reaction relative to the 24-hr reaction.

Period of infection at which skin reactions can be demonstrated. Two groups of normal rabbits, each containing five animals, were infected with the Shinyanga III strain of T. brucei. One group received 2 x 10^8 organisms per animal of the mouse-adapted strain M, and the other group was infected with an identical dose of the rabbit-adapted strain R.

One rabbit in each group was tested each week after infection with antigens of T. brucei M and T. brucei R prepared by the first method (see above). No animal was skin-tested on more than one occasion.

Reactions to injected antigen appeared to be of two types: an initial erythematous reaction which reached maximum intensity 4 to 6 hr after testing, and a delayed reaction which was not necessarily erythematous but which was invariably indurated and reached a maximum intensity 24 to 48 hr after testing (Table 1). These will be referred to as the 4- and 24-hr reactions, respectively.

Skin reactions as a result of acute inflammation occurring 4 hr after testing were considered to be related to the presence of circulating antitrypanosome antibody and consequent local formation at the injection site of antigen-antibody complexes (Arthus reaction.) Reactions of this nature occurred in both M- and R-infected rabbits. They were of maximum severity after 2 weeks of infection, a time known to coincide with peak trypanosome agglutination titers in serum. In contrast, "R" antigens gave relatively poor 4-hr reactions which were of greater severity in M-infected than in R-infected animals.

Skin reactions occurring 24 and 48 hr after infection of antigen were elicited by M antigen in both M- and R-infected animals to a similar extent. These reactions, in contrast to the 4-hr response, were apparently unrelated to serum trypanosome agglutinin levels and were found to be of maximum intensity in animals tested 4 weeks after onset of infection. In contrast to the reaction to M antigen, the 24-hr skin reaction to R antigen was invariably weak, irrespective of the infecting strain of the organism. The results are summarized in Fig. 2.

Passive transfer of 24-hr skin reactions to normal rabbits. To clarify the nature of the immunological process participating in these skin reactions, attempts were made to transfer skin reactivity
from infected to normal rabbits. Rabbits infected with each of the four strains which showed marked 24-hr skin reactions were killed, and their spleens and a cardiac blood sample were removed. Spleen cells were suspended in 2.5 ml of sterile saline; erythrocytes present in this suspension were lysed by exposure to 7.5 ml of cold distilled water for 40 sec before restoring isotonicity with 2.5 ml of 3.5% NaCl solution. The remaining cells were washed exhaustively. Samples of these cells were then killed either by freezing and thawing five times or by heating to 56° C for 30 min. Normal recipient rabbits received intravenously 3 ml of serum, 3 ml of 50% live spleen cells, or 3 ml of 50% dead spleen cells. Twenty four hours later, each animal received homologous antigen intradermally and was examined 4, 24, and 48 hr later. Skin reactivity at 24 hr was transferred only by live spleen cells from rabbits infected with T. rhodesiense, T. brucei Shinyanga III R, and T. brucei TREU 285. Live spleen cells were not able to transfer 24-hr skin reactivity to T. brucei Shinyanga III M. This may have resulted from a marked parasitemia in the donor animal which depressed skin reactions by saturation and blockage of effector cells and also possibly from a blocking effect caused by the presence of aglutinating antibody. This was reflected in a strong 4-hr reaction in animals which received both serum and live cells.

**Histology of 24-hr skin reactions to antigen.** Twenty-four-hour skin reactions in infected rabbits were characterized by extensive induration with little evidence of acute inflammation. Strong reactions presented a slightly raised indurated area with a blanched central area.

Areas showing skin reactions after 24 hr were removed and examined histologically. The reactions were characterized by a diffuse infiltration of the injection site with small mononuclear cells. Towards the center of each lesion, there was a collection of large mononuclear cells which were probably macrophages. Polymorphonuclear neutrophils were present in small numbers in the lesions examined, but histological evidence of acute inflammation, edema, and vascular dilatation was absent. Since this neutrophil accumulation was greatest in reactions which had been positive 4 hr postinfection, it is possible that it represented the remains of an Arthus reaction. Pyroninophilic cells were never observed.

**Skin reactivity in rabbits immunized with dead trypanosomes.** Trypanosomes suspended in Alsever’s solution (10%, v/v) were left at 4°C until dead. A 2-ml amount was injected subcutaneously into normal rabbits twice weekly for 4 weeks. At the end of this time, animals were skin-tested in the usual way. Reactions, when present, reached maximum severity 4 hr after testing, and all had declined to insignificance by 24 hr.

**Specificity of the skin reaction.** Rabbits were infected with $2 \times 10^3$ cells of T. rhodesiense TREU 334, T. brucei Shinyanga III R, or T. brucei TREU 285. Six weeks after the onset of infection, each animal was tested with antigens prepared from T. brucei M, T. brucei TREU 285, and T. rhodesiense TREU 334 (prepared by the first method). The animals were examined 4, 24, and 48 hr after injection of antigen. Rabbits

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**Table 2. Cross-reactivity of 24-hr skin reaction to trypanosoma strains in infected rabbits**

<table>
<thead>
<tr>
<th>Test antigen</th>
<th>T. rhodesiense</th>
<th>T. brucei &quot;M&quot;</th>
<th>T. brucei TREU 285</th>
<th>Non-infected rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. rhodesiense</td>
<td>++++</td>
<td>+++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>T. brucei &quot;M&quot;</td>
<td>++</td>
<td>+++++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>T. brucei TREU 285</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Symbols: ++++, >200% increase in skin thickness; ++, 100 to 200% increase; +, 50 to 100% increase.
infected with *T. rhodesiense* reacted to *T. brucei* Shinyanga III M, to *T. rhodesiense*, and to *T. brucei* TREU 285 (Table 2). Similar results were observed in rabbits infected with *T. brucei* Shinyanga III M and *T. brucei* TREU 285.

These preliminary results indicated that the 24-hr skin reaction did not possess a high degree of species specificity.

**DISCUSSION**

In the infected animals which received intradermal antigen, skin reactions present 24 hr later possessed the essential criteria of delayed-type hypersensitivity. Thus, they were transferable to normal animals only by living cells and not by serum; they were characterized histologically by mononuclear cell infiltration, and they reached maximum intensity 24 hr after testing. In addition, these reactions could only be elicited in animals infected with living organisms. Since viable intracellular forms of these organisms are known to occur, it is possible that a state of acquired cellular immunity co-exists with 24-hr skin reactivity.

Cutaneous hypersensitivity tests, although relatively simple to perform, have rarely been employed in the diagnosis of hemoflagellate infection. This has been mainly due to a lack of understanding of the life cycle of many of these organisms and to the complexity of antigenic variation which many exhibit. However, the Leishmanin intradermal test has been extensively employed in studying the incidence and distribution of kala-azar. This test is a cutaneous hypersensitivity reaction, possibly cell-mediated, which is examined 48 hr after intradermal inoculation of antigen. The antigen used is a phenol-killed suspension of cultured leptomonads with a standard dilution of $6 \times 10^8$ to $10 \times 10^8$ parasites per ml (1).

In the investigations reported here, it was noted that a phenol-killed antigen was less efficient in inducing skin reactivity than a preparation of "cold" killed organisms in Alsever's solution. This latter antigen was probably much richer in metabolic products (exoantigens) and free cell sap.

The delayed skin reactions reported here did not exhibit marked species specificity in common with many other immunological tests for trypansomiasis. Nevertheless, the simplicity of the procedure may lend itself to practical use in the diagnosis of trypanosome infection. Although our work inevitably is preliminary, it may assist in defining an area in which further investigation would be profitable.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**