SHORT ARTICLES

ANTI-LEISHMANIAL ACTIVITY OF IMMUNE GUINEA-PIG SERUM

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IMMUNE reactions to leishmanial infection have been known to exist for many years, but their biological role is not yet clear. Circulating antibody to leishmania has not been regularly demonstrated in human subjects who have recovered from oriental sore or in guinea-pigs that have recovered from *Leishmania enriettii* infection (Adler, 1965; Bryceson *et al.*, 1970). Our previous findings indicated that rabbits immunised with the promastigote form of *L. tropica* developed an anti-leishmanial growth-inhibitory activity in their sera (Rezai, Sher and Gettner, 1969; Rezai, Behforouz and Gettner, 1970). In this communication a factor in the sera of guinea-pigs that had recovered from *L. enriettii* infection is investigated.

MATERIALS AND METHODS

Immune guinea-pigs. Guinea-pigs weighing 250–300 g from our animal breeding house were infected subcutaneously in the ear with 8×10^5 *L. enriettii* amastigotes in 0.2 ml. After recovery, the animals were reinoculated with the same infective dose of leishmania; failure to develop infection was considered the criterion of immunity.

Source of immune and normal sera. Blood was collected from several normal and immune guinea-pigs by cardiac puncture. Normal and immune bloods were pooled separately and defibrinated by shaking with glass beads; the serum was separated by centrifugation.

Source of inoculum. *L. tropica* was obtained from random-bred mice that had been infected with the Rhombomys strain originating from a *Phlebotomus* host in the Isphahan Public Health Centre. *L. enriettii* was given to us by Dr Zuckerman of Hadassa Medical School, Hebrew University, Israel. Both leishmanial strains were grown in modified NNN medium (Lemma and Schiller, 1964) for 3–4 days and used as inoculum for the inhibition studies. After a few in-vitro passages, the stock cultures were passed through guinea-pigs in the case of *L. enriettii* or through mice in the case of *L. tropica*.

Titration of the growth-inhibitory activity. The growth-inhibitory activity was both shown and titrated in modified NNN medium. For this purpose, 1-ml volumes of diluted (or treated) serum were used as the liquid phase in the medium in screw-capped tubes. Each tube was inoculated with 3×10^6 promastigotes and incubated at 26°C for 4 days. Diluted normal serum and saline alone were used as controls. After 4 days of incubation, the concentration of free-living leishmania in each tube was determined by direct count in a haemocytometer.

RESULTS

Growth-inhibitory activity of immune guinea-pig sera. The effect on the growth of *L. enriettii* and *L. tropica* of various dilutions of pooled immune serum, as well as of pooled normal serum is presented in table I, which is based on the results of three experiments in which pooled sera from animals of each group were used. The table shows that sera obtained from animals immunised against *L. enriettii* had some growth-inhibitory activity against *L. enriettii* demonstrable up to a dilution of 1 in 16. The inhibitory activity on the growth of *L. tropica* was much less pronounced, but a minimal effect occurred in a dilution of 1 in 2.

Normal guinea-pig serum also had some inhibitory activity on the growth of *L. enriettii*. This varied with different samples of guinea-pig serum, but its titre did not exceed 1 in 8.

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TABLE I

*Inhibitory activity of immune and normal serum on the growth of Leishmania enriettii and L. tropica**

Leishmanial species	Source of serum	Number of leptomonads† found per ml in serum dilutions of					
		1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64
<i>L. enriettii</i>	Immune	0	0	1.4×10^5	3.8×10^5	2.2×10^6	1.3×10^7
<i>L. enriettii</i>	Normal	5.2×10^4	5.2×10^4	1.7×10^6	‡	‡	‡
<i>L. tropica</i>	Immune	9.6×10^5	1.3×10^6	‡	‡	‡	‡
<i>L. tropica</i>	Normal	1.9×10^6	5.1×10^6	‡	‡	‡	‡

* Inoculum 3×10^6 organisms.

† Mean values of three experiments.

‡ Good growth, approximately the same number as in serum-free controls. (The growth of promastigotes in serum-free control medium varied from 7×10^6 to 2.2×10^7 with an average of about 1.4×10^7 .)

TABLE II

*Effect of heat and absorption on the inhibitory activity of immune guinea-pig serum on Leishmania enriettii**

Treatment	Number of leptomonads found† per ml in serum diluted			
	1 in 2	1 in 4	1 in 8	1 in 16
Untreated immune serum	0	1.3×10^5	1.3×10^5	6.1×10^6
Immune serum heated to 56°C	5×10^6	8.6×10^6	7.2×10^6	6.8×10^6
Heated immune plus unheated normal serum‡	0	0	6.6×10^4	7.3×10^6
Absorbed immune serum§	NT	3.2×10^6	2.4×10^6	3.8×10^6
Untreated normal serum	1.2×10^5	1.2×10^6	1.3×10^7	1.5×10^7
Normal serum heated to 56°C	1.6×10^6	2.8×10^6	1.3×10^7	1.7×10^7
Heated normal serum with 1 in 10 unheated normal serum added	1.0×10^6	3.4×10^6	1.2×10^7	1.6×10^7
Absorbed normal§ serum	8.0×10^6	1.4×10^7	1.5×10^7	1.4×10^7

* Original inoculum 3×10^6 organisms.

† Mean values of three experiments.

‡ Equal amounts of normal and immune serum were mixed and subsequently diluted to give the dilution of immune serum shown.

§ For method of absorption see text.

NT = Not tested.

The role of complement. Immune and normal rabbit sera were diluted 2-fold up to 1 in 16 with saline and incubated for 30 min. at 56°C in a waterbath, in order to inactivate complement. The heated sera were tested for anti-leishmanial activity on *L. enriettii* in complement-free medium. Table II shows that this treatment of immune serum caused loss of the anti-leishmanial activity. Activity was, however, restored following the addition of equal amounts of unheated normal serum in the same dilutions as the inactivated serum. The anti-leishmanial activity of normal serum was partially removed by heating at 56°C, but could not be restored by the addition of equal amounts of a dilution of 1 in 10 of normal guinea-pig serum.

Effect of absorption. To determine whether the anti-leishmanial activity of immune or normal sera could be removed by absorption with the specific organisms, the following experiment was set up. Immune guinea-pig serum, 1 in 4, and normal serum, 1 in 2, were

FIGURE.—Effect of immune and normal sera on the growth of the promastigote form of leishmania. ●—● Leishmania in normal serum; □—□ leishmania in immune serum.

absorbed with a small amount of packed cells of *L. enriettii* (6×10^7 per ml) for 1 hr. The serum was separated by centrifugation and the absorption procedure repeated once more with a fresh suspension of *L. enriettii*. The absorbed serum was tested for anti-leishmanial activity in various dilutions in modified NNN medium. The untreated immune serum had some inhibitory activity up to a dilution of 1 in 8, while after absorption there was almost full growth of the leishmania in a 1 in 4 dilution (table II).

Effect of serum on amastigotes. Amastigote forms of leishmania (4×10^6 per ml) were exposed to dilutions of 1 in 3 of normal and of immune serum and incubated at 26°C. The numbers, as well as any morphological changes in the amastigotes, were determined at various intervals after incubation. The amastigotes exposed to immune serum showed loss of a defined cell outline, partial disintegration, and some decrease in the total numbers compared with the cells in the presence of normal serum.

The time at which the anti-leishmanial effect could be first observed was determined by the following experiment. One ml of a dilution of 1 in 16 of pooled sera from immune guinea-pigs was used as the liquid phase in modified NNN medium. The tubes were inoculated with 5×10^6 promastigotes and incubated at 26°C. At various time intervals after inoculation, 0.05-ml samples of the liquid phase of each tube were removed and the number of leishmania counted. The results of this experiment are shown in the figure. During the first 5 hr there was a reduction in the number of leishmania in both the immune and the normal

serum, but the degree of lytic activity shown by the reduction of the organisms was considerably greater in the immune serum. After 24 hr the number of leishmania in the immune serum was nil whereas in normal serum it had increased to 8×10^6 .

DISCUSSION

Our results are strongly indicative of the existence of an antibody or antibody-like substance in immune guinea-pig sera which specifically inhibits the growth of *L. enriettii*. The inhibitory activity was not found in a dilution exceeding 1 in 32. The substance was not only active against the promastigote form of leishmania, but also showed cytotoxicity toward amastigotes. Normal guinea-pig serum showed some inhibitory activity to the growth of *L. enriettii*, but this activity was markedly less than that of immune sera.

As the inhibitory activity of immune serum disappeared after heating at 56°C and was restored upon addition of normal guinea-pig serum, it would appear that the inhibitory factor requires complement for its action. The actual substance is heat stable at 56°C. Specific absorption was effective in the removal of this activity. The dependence on complement, heat stability and absorbability of this substance are all characteristics suggesting conventional antibody.

The mechanism of immunity to intracellular parasites is not clearly understood. Most investigators believe that cellular factors are responsible for the immunity to these parasites (Bray and Bryceson, 1968; Mackaness, 1969; Patterson and Youmans, 1970). Others have presented evidence indicating that the antibody-macrophage system is responsible for immunity to some intracellular bacteria (Jenkin and Rowley, 1963; Rowley, Auzins and Jenkin, 1968).

Those who hold that only cellular factors are responsible for immunity to leishmania have based their conclusions on indirect evidence, such as the development of delayed skin reactivity, inhibition of macrophage migration by the specific antigen, lymphocyte transformation *in vitro* and the absence of demonstrable antibody in immune guinea-pigs (Bryceson *et al.*, 1970). It is generally agreed that leishmania infections induce a state of delayed hypersensitivity, but one cannot conclude that this is the basis of immunity. Previously, antibody had not been detected in either man or animals after recovery from leishmanial infections. Our present findings suggest strongly that guinea-pigs that recover from leishmaniasis develop antibody. The role of this antibody in the mechanism of immunity to leishmanial infection is at present under investigation. Our observations on the histopathology of the lymph-nodes in guinea-pigs infected with *L. enriettii* also support the finding of an antibody response (Rezai *et al.*, 1972).

SUMMARY

A substance with the characteristics of a specific antibody was found in immune guinea-pig sera. It had inhibitory activity towards the growth of promastigotes and cytotoxicity to the amastigote form of *Leishmania enriettii*. The inhibitory effect was shown by direct counting of viable leptomonads. The cytotoxicity of immune sera on amastigotes was evaluated on the basis of morphological changes as well as of a decrease in the number of the organisms. The substance responsible was found to be heat stable and complement dependent. The activity could be removed by the specific absorption.

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