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In vitro Effects of Specific Immune Blood on Certain Blood and Tissue Flagellates.*

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In a study of the *in vitro* effects of specific immune sera upon several species of *Leishmania*, Kligler[1] found that when high concentrations of the serum are added to cultures, the homologous organisms are lysed while the heterologous organisms are not affected. He further noted that the exposure of cultures of organisms to dilute homologous sera caused them to agglutinate but did not inhibit their growth.

Malamos[2] found that the addition of immune serum to cultures of the *Leishmania* tended to retard their growth to some extent, but that immune serum did not have any greater action on homologous organisms than on other species of *Leishmania*.

In the course of our investigations on the antigenic composition of the blood and tissue flagellates it was observed that when young, actively growing cultures of *Trypanosoma cruzi* were added to a culture medium which contained homologous immune blood the organisms did not survive.

To obtain a clearer understanding of this reaction a series of experiments was planned (1) to determine the extent to which this inhibitory or lethal effect of immune blood is consistently associated with the various species of blood and tissue flagellates, (2) the specificity of the reaction (3) and the nature of the factor or factors responsible for this phenomenon.

Materials and Methods. Immunization of Rabbits. Rabbits were immunized with killed cultures and with living cultures of *L. brasiliensis, L. donovani, L. tropica,* and *T. cruzi.* Antigens were prepared according to the method described by Senekjie[3] and were administered by a schedule which has been previously outlined (Senekjie and Lewis[4]). One week after the agglutinin titers had risen sufficiently the animals were bled, employing strict aseptic technics. From 7 to 10 cc of blood were collected into each of 2 tubes, one of which contained 1 cc of sterile 3.5% sodium citrate.

Blood was similarly collected from normal rabbits.

Preparation of Media. 1. *Immune whole blood leishmania agar medium.* To different batches of leishmania agar (Senekjie[3]) at 45°C, sufficient immune citrated blood of rabbits immunized with antigens of *L. brasiliensis, L. donovani, L. tropica,* and *T. cruzi* was added to make 6 to 9% of the respective media. The media were tubed and made into slants.

One set of each medium was preheated in a water bath at 56°C for 30 minutes, another set at 70°C for 30 minutes, and the remaining sets were not heated.

2. *Immune erythrocyte-normal serum leishmania agar medium.* Erythrocytes of the rabbits immunized with antigens of *L. donovani* were washed 4 times in sterile physiological salt solution. The washed cells were then resuspended in an equal volume of normal rabbit serum. Ten cc of this mixture

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1 Kligler, I. J., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1925-26, **19**, 330.

2 Malamos, B., *Arch. f. Schiffs. u. Tropen-Hyg.*, 1937, **41**, 416.

3 Senekjie, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 56.

4 Senekjie, H. A., and Lewis, R. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 17; *Am. J. Trop. Med.*, in press.

were added to 90 cc of leishmania agar at 45°C. The medium was then tubed and slanted. Similarly, medium was prepared with the washed erythrocytes of rabbits immunized with *T. cruzi* antigens.

3. *Immune serum-normal erythrocyte leishmania agar medium.* Media were prepared with the serum of rabbits immunized with *L. donovani* and *T. cruzi* antigens. Five cc of the respective immune sera were mixed with 5 cc of washed erythrocytes of normal rabbits and each mixture was added to 90 cc of leishmania agar at 45°C. The media were put into tubes and slanted.

4. *Non-immune whole blood leishmania agar medium.* To leishmania agar at 45°C sufficient citrated or defibrinated normal rabbit blood was added to make 10% of the medium, which was then dispensed in tubes and made into slants.

General Methods. All media were incubated at 37°C for a period of 24 hours to determine bacterial sterility. Prior to inoculation each tube was overlaid with 1 cc of sterile physiological salt solution. The inoculum in each case was 0.25 cc of a rich suspension of the flagellates from cultures 3 to 5 days old. The effects of various constituents of immune blood were studied in a series of experiments by inoculating a single species of flagellate into several tubes of culture media, of which one tube contained the homologous immune material, and each of the other tubes contained a particular type of heterologous immune substance. For example, *L. brasiliensis* was inoculated into a medium containing homologous immune material and into other media containing anti-donovani, anti-tropica, and anti-cruzi immune material respectively, and a similar procedure was employed for *L. donovani, L. tropica* and *T. cruzi.* The experiments were run in duplicate using blood from 2 rabbits similarly immunized, and each experiment was repeated 3 times. Microscopic examinations of the unstained cultures were made at intervals of 1 to 2 days. Morphological changes and the amount of growth were noted. When viable organisms could not be demonstrated by microscopic examination, subcultures were made on the regular leishmania blood agar medium as an additional test of viability.

The control culture medium in these experiments consisted of non-immune whole rabbit blood in leishmania agar, with an overlay of physiological saline.

Results. Effect of immune whole blood in leishmania agar medium. In these experiments the different tubes of media contained a particular kind of immune whole blood. Following the inoculation of these media with each species of organism the cultures were examined over a period of 3 weeks at intervals of 1, 2, 2, 2, 3, 7, and 4 days. The results observed in the duplicate experiments were practically identical in each instance.

The effects of unheated immune whole blood were studied first. These experiments revealed that when the organisms were grown in the homologous immune blood media they showed varying degrees of inhibition depending upon the species, while those grown on heterologous immune blood media manifested no inhibition. The propagation of *L. donovani* was almost completely inhibited from the first day while *T. cruzi* showed a complete inhibition after 24 hours. The organisms first lost their motility, then became agglutinated, swelled to 2 to 5 times their normal size, rounded up, and finally developed complete hyalinization of the internal structure, so that the nucleus, parabasal body, axoneme, undulating membrane or flagellum could not be seen. In the case of *L. donovani* this phenomenon was very marked but less so than in the case of *T. cruzi.* When such forms were placed on the regular leishmania blood agar medium, no propagation was obtained, indicating that the organisms had been killed. *L. brasiliensis* and *L. tropica* on the homologous immune blood media showed a moderate inhibition over a period of 2 weeks. This reaction was most noticeable in the first 2 to 4 days. Such organisms had a tendency to agglutinate and were not actively motile, but remained viable as long as organisms grown on the heterologous immune blood media.

Following the demonstration of the inhibitory or lethal effect of unheated homologous immune blood on certain species of organisms, a series of experiments was carried out which was designed to elucidate the factors respon-

sible for this action. These included an evaluation of the role of complement, studies on the thermostability of the inhibitory factors, and the determination of whether the factors are contained in the erythrocytes or the serum of immunized animals.

In the experiments to determine whether complement is necessary for the inhibitory or lethal effect of immune blood, the media containing immune whole blood were pre-heated at 56°C for 30 minutes. The results observed in these experiments were comparable in every respect to those encountered when unheated immune blood was used. Thus, complement was found to be unnecessary in the production of the inhibitory or lethal effect.

The preceding experiments demonstrated that the inhibitory or lethal factors are not destroyed by pre-heating at 56°C for 30 minutes. The thermostability of these factors was also determined by pre-heating the homologous immune whole blood medium at 70°C for 30 minutes. When this was done *L. donovani* survived for 2 weeks although the growth was poor, while *T. cruzi* remained viable for only 4-7 days. However, *L. brasiliensis* and *L. tropica* grew more abundantly than on the same medium, unheated or pre-heated at 56°C, but definitely less than in the control medium containing normal blood. These results indicate that the inhibitory or lethal factors are partially stable at 70°C. Unfortunately, the thermostability could not be tested at higher temperatures because of the danger of inactivating the growth-promoting factors. These factors are present in the serum and are known to be inactivated at 100°C. (Senekjie and Lewis[4]).

Effects of immune serum-normal erythrocytes and immune erythrocytes-normal serum in leishmania agar medium. In these experiments the distribution of the inhibitory or lethal factors in the blood was studied. This was done by inoculating *L. donovani* and *T. cruzi* into media containing homologous immune serum and normal erythrocytes, and into medium containing homologous immune erythrocytes and normal serum. The growth of *L. donovani* and *T. cruzi* was completely inhibited when the medium contained the homologous immune serum and normal erythrocytes. However, growth was normal and abundant when they were inoculated on a medium containing immune erythrocytes and normal serum.

Discussion. When various blood and tissue flagellates are grown in a medium containing homologous immune whole blood or serum, a distinct inhibitory or lethal action occurs in cultures of *T. cruzi* and *L. donovani*, and a less marked action occurs in those of *L. brasiliensis* and *L. tropica*.

The factors responsible for this inhibitory or lethal effect are not clearly understood. The present study indicates that they are (1) specific in their action, (2) present in immune serum but absent in immune erythrocytes, (3) absent in non-immune blood, (4) able to act in the absence of complement, and (5) partially inactivated by being heated at 70°C for 30 minutes. The most likely hypothesis is that the effect is due to such humoral factors as antibodies.

If the effect is due to antibodies, these observations have much fundamental significance, since they demonstrate a type of lytic antigen-antibody reaction that does not depend on the presence of complement. Complement is essential in bactericidal and hemolytic reactions. Although complement-fixing antibodies are produced in various protozoan infections (see Culbertson[5] for a review), the present study indicates that complement is not essential for the action *in vitro* of these inhibitory or lethal factors. Moreover, this effect is apparently not due to ablastin, since this antibody does not exert its effect *in vitro* (Taliaferro[6,7]).

Summary and Conclusions. The blood of rabbits immunized with various blood and tissue flagellates has been found to exert an inhibitory or lethal action on cultures of the homologous organisms. This effect was very marked on cultures of *Trypanosoma cruzi* and *Leishmania donovani*, but was slight on those of *L. brasiliensis* and *L. tropica*. These factors are associated with the blood serum,

[5] Culbertson, J. T., *Immunity Against Animal Parasites*, Columbia University Press, New York, 1941.

[6] Taliaferro, W. H., *J. Exp. Med.*, 1924, **39**, 171.

[7] Taliaferro, W. H., *Am. J. Hyg.*, 1932, **16**, 32.

are thermostable at 56°C, but are partially destroyed at 70°C.

The inhibitory or lethal action is observed *in vitro* in a medium prepared with the immune whole blood or immune serum on which the homologous organisms are grown. The reaction takes place in 3 stages, viz.,

(1) The organisms lose their motility and become agglutinated.

(2) They round up and become markedly swollen.

(3) Finally, the internal structure undergoes hyalinization which ends in the death of the protozoon.

Complement is not necessary for this reaction. This inhibitory or lethal factor appears to differ from ablastin.

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Penicillin Inhibitor from Staphylococci Which Have Developed Resistance to Penicillin in the Human Body.*†

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In previous reports[1,2] from this laboratory it was pointed out that strains of coagulase-positive staphylococci from human patients varied in their *in vitro* sensitivity to penicillin. None of these naturally resistant strains had been previously exposed to penicillin. Three strains of staphylococcus were made highly resistant to penicillin by exposing the organisms to increasing concentrations of penicillin. At that time, it appeared that acquired *in vitro* resistance to penicillin, like acquired sulfonamide resistance, was probably a permanent characteristic of these strains of staphylococcus. These resistant strains were subcultured on veal infusion agar slants every 3 to 4 weeks, and it was surprising to observe that within a year, the property of penicillin insensitivity had been completely lost. This was in contrast to 2 strains which had become resistant in patients as a result of penicillin therapy and still retained their resistance to penicillin after having been handled and subcultured in the same manner. The observations prompted further investigations to determine if staphylococci made resistant to penicillin by exposure *in vitro* differed from strains which had become resistant in the human body following the administration of penicillin.

Previous investigators[3] had shown that an enzyme-like substance called penicillinase, and extracted from *E. coli,* inhibited the action of penicillin. However, such an inhibitor could not be obtained from at least 6 strains of staphylococcus which were sensitive to penicillin, nor from 2 strains of staphylococcus which had been made resistant to penicillin *in vitro*.[4,5,6] Kirby,[7] employing a meth-

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3 Abraham, E. P., and Chain, E., *Nature,* 1940, **146**, 837.

4 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet,* 1941, **2**, 177.

5 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 132.

6 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 135.

7 Kirby, W. M. M., *Science,* 1944, **99**, 452.