

IMMUNITY IN HEARTWATER: I. A PRELIMINARY NOTE ON THE ROLE OF SERUM ANTIBODIES

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ABSTRACT

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Ammonium sulphate precipitated gamma-globulins obtained from sheep immune to *Cowdria ruminantium* (Cowdry, 1925) failed to protect susceptible sheep against artificial infection by this parasite, irrespective of whether the globulin was given simultaneously with the experimental infection, or 6 days later, or after the commencement of the febrile reaction. This finding was supported by the absence of serum antibodies detectable by indirect immuno-fluorescence.

INTRODUCTION

Studies on the nature of the immunity in heartwater have been hampered by the lack of a suitable antigen due to failure to cultivate the causal agent *Cowdria ruminantium* (Cowdry, 1925) artificially. Existing knowledge of the immunity in this disease is based on observations regarding the resistance or susceptibility of animals that have recovered from heartwater and their reaction to subsequent inoculation of infective blood drawn from artificially infected sheep during the febrile reaction.

As Alexander (1931) pointed out, field observations and experimental immunity tests have shown that an immunity does develop in animals that have recovered from either natural or artificial infection. No immunity has as yet been demonstrated in any animal that has not passed through an attack of the disease. He also stated that a complete immunity, conferring resistance to several strains of *C. ruminantium*, only develops after infection with each strain. However, Neitz (1939) was unable to confirm the existence of immunologically different strains and found that there was cross-protection between ten different strains.

The duration of immunity in heartwater is reported to vary from 6 months (Neitz, 1939) to 18 months (Spreull, 1922). Natural reinfection during the period of immunity or declining immunity is an important factor in determining the duration or persistence of immunity. Although it has been shown that after recovery from artificial infection there is a gradual decrease in the degree of immunity, it was still found to be adequate after 4 years to protect against fatal infection under laboratory conditions (Neitz, Alexander & Adelaar, 1947).

The nature of the immunity in heartwater remains obscure. Since the causal agent, in exceptional cases in sheep, has been found to persist in the peripheral blood for periods of up to 60 days after recovery, Neitz *et al.* (1947) considered the immunity to have two phases: a short variable period of premunition followed by a phase of gradually decreasing sterile immunity. These authors also observed that reinfection during the period of declining sterile immunity resulted in the reappearance of the infective agent in the circulating blood and this initiated a repetition of the cycle of premunition and sterile immunity.

The premunition in animals which have recovered from heartwater differs from that in protozoal diseases in that splenectomy does not result in a relapse of the disease. For this reason as well as the inability to demonstrate consistently the persistence of the causal agent in recovered animals, Parrot (1937) differentiates

heartwater from other rickettsial infections in domestic animals [*Ehrlichia canis* (Donatien & Lestoquard, 1935) in dogs, *Ehrlichia ovina* (Donatien & Lestoquard, 1936) in sheep and *Ehrlichia bovis* (Donatien & Lestoquard, 1936) in cattle] in which the state of premunition conforms to the conditions postulated by this author.

Alexander (1931) concluded that protection against heartwater was probably not dependent upon humoral antibodies as the administration of 25 and 50 ml of hyperimmune serum had no effect upon the course of the disease whether it was injected before, after or simultaneously with infective blood. It must, however, be pointed out that this amount of serum is relatively small if one considers that protective passive immunity in malaria, for example, was afforded to monkeys by serum administered at a level of 25 ml per kg body weight and by immune gamma-globulins at a level of 150 mg per kg (Cohen & McGregor, 1963).

In view of the relatively small amounts of serum previously used and because the absence of serum antibodies in heartwater would be exceptional, it was decided to investigate the possible role of antibodies in the immunity to this disease. This entailed determining the protective value of comparatively large amounts of gamma-globulins and attempting to demonstrate the presence of antibodies against *C. ruminantium* in the serum of recovered animals by means of the indirect fluorescent antibody technique. In the absence of methods of cultivating this organism artificially, it was hoped that squash smears prepared from infected brain material could be used as antigen in the latter technique.

MATERIALS AND METHODS

Preparation of γ -globulins

Six fully susceptible two-tooth Merino sheep were immunized against heartwater by three monthly injections of 10 ml citrated infective blood. The serum from which the γ -globulin was prepared was obtained from four of these animals which were bled 3 weeks after the last inoculation. The other two were used as control immune animals, whose immunity was challenged by the intravenous injection of 5 ml citrated infective blood of known virulence 2 weeks after the last inoculation. Serum to prepare normal γ -globulin was obtained from sheep fully susceptible to heartwater.

The serum was precipitated by adding two volumes of neutral saturated ammonium sulphate solution to one volume each of serum and physiological saline. After centrifugation at 1000 rpm for 15 min the precipitate was dissolved in one volume of distilled water to which one volume of ammonium sulphate was added. After

centrifugation and one further precipitation, the final precipitate was dissolved in distilled water, the volume adjusted to half that of the original volume of serum and the protein content determined by spectrophotometry.

Two-tooth heartwater susceptible Merino sheep were artificially infected with 5 ml citrated infective blood. They were also injected intravenously with varying amounts of normal or immune γ -globulin, administered either simultaneously with the infective blood, or 6 days after infection, or during the temperature reaction. In two of the four sheep that were injected simultaneously with γ -globulin and blood the two substances were administered separately, whereas the other two animals received γ -globulin plus blood that had been mixed and allowed to stand for 2 hours at room temperature before inoculation (Table 1). The morning rectal temperatures of the animals were recorded daily and those that did not develop clinical disease and survived were challenged by an intravenous injection of 5 ml of citrated infective blood 4 to 6 weeks later.

To control the effect of storage at room temperature on infective blood, two separate quantities of 5 ml of infective blood in citrate were allowed to stand at room temperature for 2 hours, before being injected into two susceptible sheep.

Indirect immuno-fluorescence

C. ruminantium organisms in squash smears of brain hippocampus made on glass slides were used as antigen. The brain of a sheep suffering from naturally acquired heartwater was used for this purpose after first ascertaining by means of conventional Giemsa-stained squash preparations that many endothelial cells of the capillaries were heavily parasitized by *C. ruminantium*. Sera from 7 sheep immunized experimentally against heartwater as well as sera from 5 sheep and 8 cattle originating from heartwater enzootic areas were used as test sera. Undiluted sera and serial two-fold dilutions of these sera were allowed to react for 30 min on the hippocampal smears fixed in acetone. After two washings in buffered physiological saline, the preparations were exposed for 30 min to fluorescein conjugated rabbit anti-ovine or anti-bovine gamma-globulins (Pasteur Institute, Paris). Following two further washings, the slides were mounted in buffered glycerine and examined under a Zeiss binocular microscope equipped with a 200 watt Wild mercury vapour burner. A BG 12 exciter filter and a darkfield condenser were used. After completion of the fluorescent microscopic reading the coverslips were removed and the slides stained with Giemsa in order to verify the presence of rickettsial colonies in the particular squash smear used.

TABLE 1 Effects of γ -globulin upon the course of infection by *C. ruminantium*

Sheep No.	Type of γ -globulin	mg protein/kg body weight	Interval after infective blood	Result	Challenge
S1	Immune	144	Simultaneously	Died typical heartwater. Confirmed by positive brain-smear	—
S2	Normal	215	Simultaneously	Died typical heartwater. Confirmed by positive brain-smear	—
S3	Immune	72	6 days	Died typical heartwater. Confirmed by positive brain-smear	—
S4	Normal	120	6 days	Died typical heartwater. Confirmed by positive brain-smear	—
S5	Immune	72	Mixed with infective blood before injection	No reaction	Died typical heartwater. Confirmed by positive brain-smear
S6	Normal	120	Mixed with infective blood before injection	No reaction	Died typical heartwater. Confirmed by positive brain-smear
S7	Immune	20*	1st and 3rd day temp. reaction	Died typical heartwater. Confirmed by positive brain-smear	—
S8	Immune	10*	1st and 3rd day temp. reaction	Died typical heartwater. Confirmed by positive brain-smear	—
S9	Immune	20*	2nd and 5th day temp. reaction	Died typical heartwater. Confirmed by positive brain-smear	—
S10	Immune	10*	2nd and 5th day temp. reaction	Died typical heartwater. Confirmed by positive brain-smear	—
S11	Immune	25	4th day temp. reaction	Died typical heartwater. Confirmed by positive brain-smear	—

*On each of days mentioned

RESULTS

Gamma-globulins

The results summarized in Table 1 show that, with the exception of two cases, both immune and normal γ -globulin failed to affect the development of heartwater subsequent to infection by virulent blood. In two cases in which the γ -globulin had been allowed to react with the infective blood before it was injected, both immune and normal γ -globulin prevented the development of clinical heartwater.

The two control immune sheep challenged by 5 ml citrated infective blood were fully resistant to infection. The two other control sheep injected with infective blood stored at room temperature, both developed and died from heartwater as shown by positive brain smears.

Indirect immuno-fluorescence

Twelve ovine and eight bovine immune sera applied to colonies of *C. ruminantium* in brain squash smears failed to produce specific positive immuno-fluorescence. Rickettsial colonies could be identified in the preparations where both immune and normal sera had been used, but they exhibited only feeble fluorescence of equal intensity in undiluted and diluted sera.

DISCUSSION

The results obtained by administering γ -globulins at the time or during the course of infection by *C. ruminantium* are contradictory in more than one respect. On the one hand, both immune and normal γ -globulin mixed with the infective blood before its administration inhibited the development of clinical disease. On the other hand neither immune nor normal γ -globulin in any way affected the course of clinical heartwater when they were given simultaneously with, but separately from, the infective blood, or 6 days later. Likewise, these substances did not influence the disease when given during the febrile reaction, although it should be noted that the quantities used here were smaller than those given at the same time as the infective blood.

The fragility of *C. ruminantium*, as noted by Alexander (1931), should be considered as a possible cause of the inhibitory effect of γ -globulins when pre-mixed with infective blood. However, the development of heartwater in both control animals inoculated with virulent blood stored at room temperature, indicates that storage for 2 hours did not deprive the rickettsiae of their infectivity. Neither can an immunologically specific reaction have taken place, as both normal and immune γ -globulin produced the same effect.

Although the mechanism of the blocking effect of γ -globulins pre-mixed with infective blood remains to be determined, the conclusion appears justified that γ -globulins obtained from sheep immune to heartwater probably do not specifically influence the pathogenesis of heartwater.

The constant failure to demonstrate specific antibodies against heartwater by means of the indirect fluorescent antibody technique suggests that antibodies detectable by this means do not occur in the serum of animals recovered from heartwater. This technique not only exhibits a high degree of sensitivity in general, but has also given positive results in various other rickettsial infections, when applied either directly (Coons, Snyder, Cheeve & Murray, 1950) or indirectly (Bozeman & Elisberg, 1963).

Based on these preliminary findings, the failure of both immune and normal γ -globulins to influence the course of experimentally induced heartwater and the apparent absence of serum antibodies demonstrable by immuno-fluorescence indicate that in all probability humoral antibodies do not play a role in immunity to heartwater.

SUMMARY

Ammonium sulphate precipitated gamma-globulins obtained from sheep experimentally immunized against *C. ruminantium* failed to protect susceptible sheep against artificial infection by this parasite, irrespective of whether the globulin was administered simultaneously with or six days after the experimental infection. These substances also did not influence the course of the clinical disease when given after the commencement of the febrile reaction.

These results, indicating that serum antibodies probably do not play a significant role in immunity to heartwater, were supported by the absence of humoral antibodies detectable by indirect immuno-fluorescence.

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REFERENCES

- ALEXANDER, R. A., 1931. Heartwater: The present state of our knowledge of the disease. *Rep. vet. Res. Un. S. Afr.*, 17, 89-150.
- BOZEMAN, F. M. & ELISBERG, B. L., 1963. Serological diagnosis of scrub-typhus by indirect immunofluorescence. *Proc. Soc. exp. Biol. Med.*, 112, 568-573.
- COHEN, S. & MCGREGOR, I. A., 1963. Gamma-globulin and acquired immunity to malaria. Immunity to protozoa. Edited by P. C. C. Garnham, A. E. Pierce & I. Roitt. Oxford: Blackwell Scientific Publications.
- COONS, A. H., SNYDER, J. C., CHEEVE, F. S. & MURRAY, E. S., 1950. Localisation of antigen in tissue cells: IV. Antigens of rickettsiae and rump virus. *J. exp. Med.*, 91, 31-37.
- NEITZ, W. O., 1939. The immunity in heartwater. *Onderstepoort J. vet. Res.*, 13, 245-283.
- NEITZ, W. O., ALEXANDER, R. A. & ADELAAR, T. F., 1947. Studies on immunity in heartwater. *Onderstepoort J. vet. Res.*, 21, 243-249.
- PARROT, G., 1937. Les rickettsioses confèrent-elles l'immunité vraie ou la prémunition? *Arch. Institut Pasteur d'Algérie*, 15, 188-213.
- SPREULL, J., 1922. Heartwater. *J. Agric. Un. S. Afr.*, 2, 236-245.