THE PRESENCE OF ENDOTOXIN ACTIVITY IN CASES OF EXPERIMENTALLY-INDUCED HEARTWATER IN SHEEP

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ABSTRACT

VAN AMSTEL, S. R., OBEREM, P. T., DIDOMENICO, M., KIRKPATRICK, R. D. & MATHEE, JACKIE, 1988. The presence of endotoxin activity in cases of experimentally-induced heartwater in sheep. *Onderstepoort Journal of Veterinary Research*, 55, 217–220 (1988)

The presence of endotoxin was examined in 5 sheep with experimentally-induced heartwater. Two peaks in endotoxin levels were recorded in 4 out of the 5 sheep during the acute stage of the disease. The 1st peak coincided with or occurred shortly after the febrile reaction (over 40 °C). The 2nd peak occurred 3–5 days after the 1st, and in 2 sheep this 2nd elevation in endotoxin levels was associated with severe clinical signs (rapid and laboured breathing, cyanosis and recumbency), and 1 of the 2 sheep died on the day of the 2nd elevation. Both endotoxin peaks were of short duration and levels had decreased in the 24-h follow-up samples.

INTRODUCTION

The discrepancy between the apparently limited morphological cellular changes, including those on an ultrastructural level, and the severity of the effusions found in the lungs and body cavities led to speculation about the involvement of a toxin in the pathogenesis of heartwater (Neitz, 1968; Pienaar, 1970; Camus & Barré, 1982, citing Jackson & Neitz, 1932; Bezuidenhout, 1982; Prozesky & Du Plessis, 1985). Parker & Neva (1954) found that the intravenous injection of lethal and sublethal doses of *Rickettsia prowazekii* into mice produced pathological changes similar to those described for bacterial endotoxins. These changes included focal hepatic necrosis and haemorrhages in various organs, including the intestine.

More recently Amano & Williams (1984) reported on the characterization of lipopolysasccharide (LPS) extracted from Phase 1 *Coxiella burnetii*. They found that the chemical composition of LPS, including the lipid A moiety from *C. burnetii*, differed significantly from that of gram-negative Enterobacteriaceae.

Despite this, LPS extracted with hot phenol-water from Phase 1 *C. burnetii*, showed some biological activities associated with the endotoxic LPS from gram-negative Enterobacteriaceae (Amano & Williams, 1984). On the basis of the foregoing it seems feasible that the causative organism of heartwater (gram-negatively staining rickettsia, *Cowdria ruminantium*) may also contain a biologically active LPS on its outer membrane. Variations in the chemical composition of LPS, including the endotoxically active lipid A, also occur in members of the gram-negative Enterobacteriaceae, with resulting differences in biological activity (Morrison & Ulevitch, 1978).

The biological activities of endotoxin in the body are very diverse and include those changes associated with the pathogenesis of the acute inflammatory reaction. One such reaction of importance is damage to vascular walls with leakage of protein-rich fluid and blood cellular elements. This damage, caused by the direct interaction of endotoxin with endothelial cells, results in such morphological changes as endothelial cell stripping, distortion, vacuolization and absence of endothelial cell nuclei (Morrison & Ulevitch, 1978). In their review on the effects of bacterial endotoxins on host mediation systems, these authors, however, state that other researchers were unable to demonstrate any evidence of endothelial damage in experimentally-induced endotoxaemia in rhesus monkeys, even on an ultrastructural level, despite the presence of fibrin in a variety of tissues.

The concept that large changes in filtraton may result from small changes in the structure of exchanging vessels is discussed by Hurley (1977) and demonstrated by Brigham, Woolverton, Lynn, Blake & Staub (1974) in experimentally-induced *Pseudomonas* bacteremia in sheep. A similar situation seems to be present in heartwater. Prozesky & Du Plessis (1985) found only mild morphological changes in the alveolar endothelial cells in experimentally-induced heartwater in sheep, despite the presence of a severe lung oedema. Single endothelial cells were swollen and necrotic cells were rarely observed. Gaps were seen infrequently between endothelial cells. There seemed to be no correlation between pathological changes and the presence of organisms in the endothelial cells (Pienaar, 1970; Prozesky & Du Plessis, 1985).

A 2nd important inflammatory reaction associated with bacterial endotoxaemia is the activiation of the complement and coagulation cascades.

Endotoxin can initiate both the classic and alternate pathways of complement activation (Morrison & Ulevitch, 1978). Furthermore, it has been shown that complement components may be involved in the lethal effects of endotoxins (Morrison & Ulevitch, 1978). Laboratory measurements of complement components during endotoxaemia show a decline in the levels of these compounds (Morrison & Ulevitch, 1978; McClure, McClure & Johnson, 1981), Du Plessis, Malan & Kowalski (1987) reported constant complement levels in animals with mild reactions during the course of 3 weeks after infection, whereas in the case of calves with severe reactions there was a distinct rise. Depending on the structure of the LPS, striking differences in complement-mediated responses *in vivo* have been recorded (Morrison & Ulevitch, 1978). As the LPS of *C. ruminantium* will most likely be different from that of the endotoxically active Enterobacteriaceae, it is likely that its effects on the complement system might likewise be different.

Du Plessis (1985) and Du Plessis, Malan & Kowalski (1987) demonstrated the involvement of conglutinin (K) in the pathogenesis of heartwater. Positive correlation was found between high levels of K and survival in experimentally-induced heartwater in calves. Du Plessis (unpublished data, 1986) found that a decline in K levels during the incubation period of the disease was followed by a rise in immunoconglutinin (IK) levels during the recovery phase. K is a serum protein, found only in ruminants, that reacts with the conglutinogen in fixed complement (C3) (Lachmann, 1967). It is therefore feasible that the presence of conglutinating activity may offer some protection against *C. ruminantium* as it does

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against infection by other virulent pathogens (Lachmann, 1967).

Endotoxaemia in the horse may be associated with various abnormalities in the blood-clotting profile consistent with a laboratory diagnosis of disseminated intravascular coagulation (DIC) (Johnstone, 1986). Such laboratory studies have not been reported on in cases of heartwater. Some macropathological changes associated with DIC have been described in both natural and experimentally-induced cases of heartwater. These include oedema of the lungs and haemorrhages in various organs, including the conjunctiva, lymphnodes, heart, rumen and intestines, the central nervous system, vagina and urinary bladder (Camus & Barré, 1982; Prozesky, 1987). The presence of microthrombi has only once been reported on in experimentally-induced heartwater in mice (Prozesky & Du Plessis, 1985).

Endotoxaemia in the horse is associated with a wide variety of haematological biochemical and haemodynamic changes (Moore, 1981). Typically, these include a primary and precipitous neutropaenia with a subsequent neutrophilia, a thrombocytopaenia and haemoconcentration. Biochemical changes include a hyperglycaemia, lactacidaemia and hyperlipidaemia. The haemodynamic changes observed in endotoxaemia appears to be associated with eicosanoid production (Ward, Fessler & Bottoms, 1986) and include pulmonary hypertension, systemic hypotension and a decrease in stroke volume and cardiac output.

It seemed conceivable that endotoxaemia could play an important role in the pathogenesis of heartwater. A study was therefore undertaken to measure and evaluate the presence of LPS in experimentally-induced heartwater in sheep by means of a quantitative, Limulus amoebocyte lysate (LAL) microassay.

MATERIALS AND METHODS

Experimental animals

Heartwater was induced in each of 5 healthy adult Merino sheep (identified as 3764, 3768, 3702, 3849 and 3705) by the intravenous inoculation of the Ball 3 isolate (Haig, 1952) of *Cowdria ruminantium*, contained in 5 mℓ of blood from an infected sheep. The rectal temperature of each animal was recorded once daily. Clinical disease was considered to have commenced when temperatures above 40 °C were reached. Rectal temperatures above 40 °C were recorded in all 5 experimentally-induced heartwater sheep from 10–16 days post-infection. Blood was collected for endotoxin assay from the 5 experimental heartwater sheep on Day 5 and on Day 2 before experimental infection and then daily from Day 5 until recovery or death. Two sheep (3705 and 3849), suffering from experimentally-induced heartwater, died on Day 16 post-infection, whereas the other 3 were clinically normal by Day 19 post-infection.

All sampling procedures were performed, using strict aseptic techniques. The wool overlying the jugular vein was clipped and shaved and disinfected with povidone iodine[1]. Blood was collected into 10 mℓ pyrogen-free (PF), evacuated, venoject tubes, each containing 200 i.u. of sodium heparin.

Processing of plasma for endotoxin assay

Blood samples were all processed within 30 min of collection. Samples were centrifuged at 200 × g for 10 min to obtain platelet-rich plasma (PRP). The PRP was then transferred to clean pyrogen-free (PF) test tubes and covered with parafilm. Dilution of the PRP was then carried out by adding 10 μℓ to 390 μℓ of PF water in a PF test tube again covered with parafilm. The diluted PRP was then heated to 75 °C for 5 min in a water-bath and centrifuged at 200 × g for 10 min. The supernatant was subsequently decanted into clean, appropriately labelled, PF test-tubes and frozen at −20 °C. All the test tubes used were rendered pyrogen free by heating at 180 °C for 4 h or more.

Endotoxin assay procedure

The LAL chromogenic assay[2] was used according to the manufacturer's directions. Processed plasma samples were thawed at room temperature and vortexed for 30–60 seconds. They were then dispensed into the wells of the microtitre plate in order to limit the amount of endotoxin lost in the assay as a result of adherence to the test-tube. Results were plotted against a water standard curve, using a 21 Eu/mℓ *E. coli* endotoxin stock solution (0111:B4). All the samples were tested in triplicate and the absorbency read at 405 nm within 30 min of the addition of 100 μℓ of a 25 % acetic acid solution to stop the reaction. Strict adherence to time and temperature and aseptic techniques were applied throughout the test. A water blank was also run to blank all values. The results were expressed in endotoxin units/mℓ as the endotoxin in the samples was not necessarily the same as that of the standard endotoxin stock solution used in the test (Harris, Stone & Stuart, 1983).

RESULTS

The rectal temperatures and consentrations for endotoxin recorded in the 5 sheep suffering from experimentally-induced heartwater are shown in Fig. 1–5.
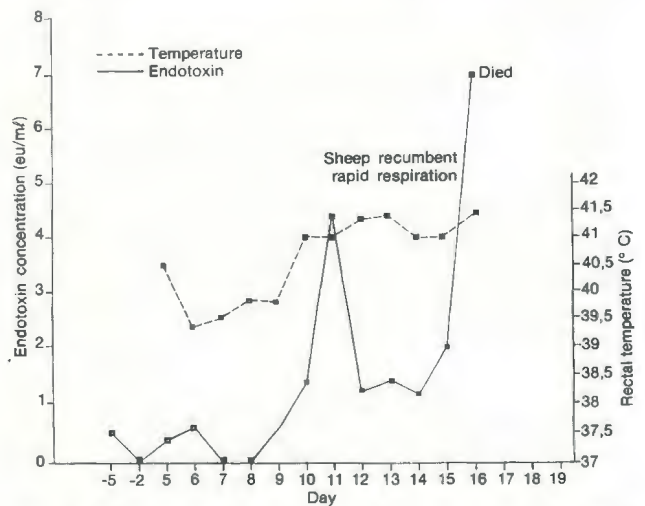

FIG. 1 Results of endotoxin and temperature recordings obtained from sheep 3705

From Fig. 1–5 it is evident that endotoxin concentrations showed similar patterns in all 5 sheep with experimentally-induced heartwater. An initial peak was recorded in endotoxin concentration which either coincided with the febrile reaction (3768, 3764 and 3849) or occurred shortly after (3705 and 3702). In all cases this elevation in endotoxin levels had disappeared after 24 h. A 2nd peak in endotoxin levels occurred 3–5 days after

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[1] Provodine scrub, Centaur Laboratories, Durban Street, Johannesburg, 0002

[2] QCL-1000, Whittaker Bioproducts Inc., P.O. Box 127, Biggs Ford Road, Walkersville, Maryland, USA 21793
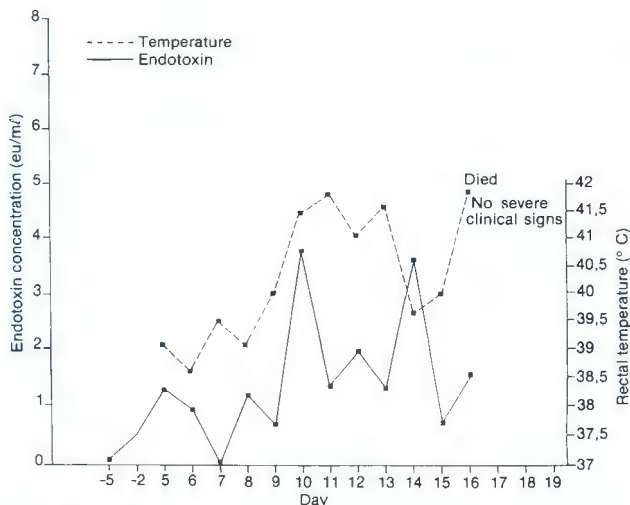
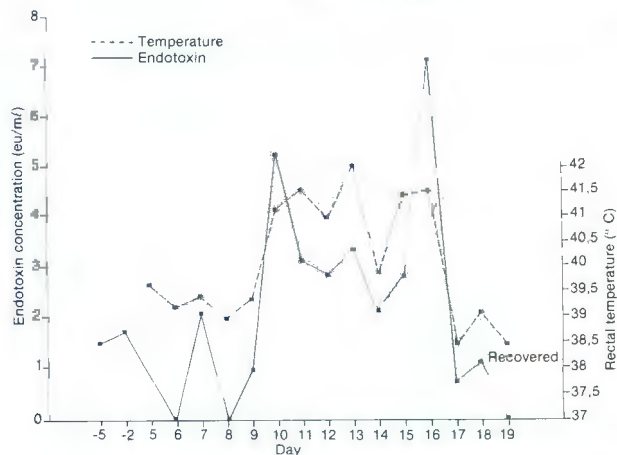FIG. 2 Results of endotoxin and temperature recordings obtained from sheep 3849

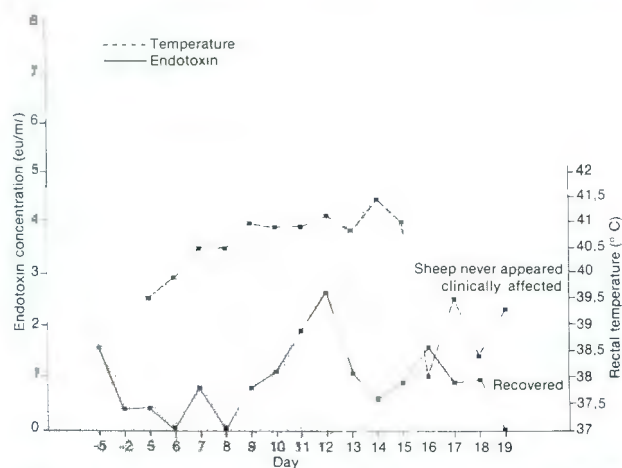FIG. 4 Results of endotoxin and temperature recordings obtained from sheep 3768

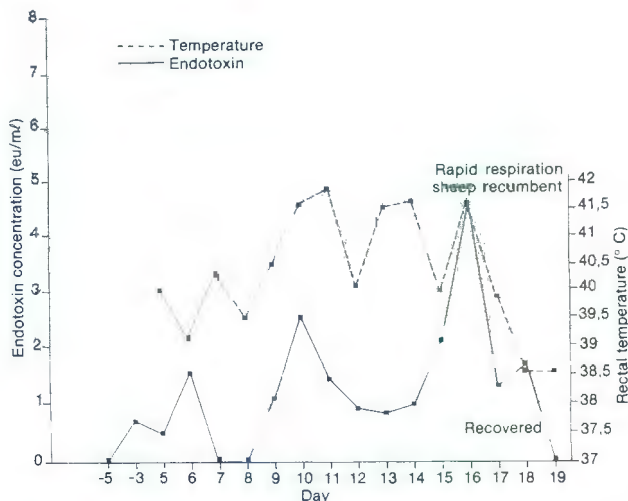FIG. 3 Results of endotoxin and temperature recordings obtained from sheep 3702

FIG. 5 Results of endotoxin and temperature recordings obtained from sheep 3764

the 1st (except in Sheep 3702 which never appeared clinically affected throughout the trial). In 2 sheep, (3764 & 3705), this 2nd rise in endotoxin levels was accompanied by severe clinical signs (laboured and rapid breathing, cyanosis and recumbency). One of these sheep (3705) died on the same day. This 2nd peak in endotoxin levels had also disappeared after 24 h. In the 3 sheep that recovered, endoxin concentrations were very low at the time of clinical recovery.

DISCUSSION

From the results it seems possible that endotoxin may play a role in the pathogenesis of heartwater. Van Amstel, Reyers, Guthrie, Oberem & Bertschinger (1988) have shown that protein leakage and thus capilary permeability also coincide with the onset of the febrile reaction. It is therefore feasible that the initial endotoxin peak as shown in these results could directly or indirectly play a role in the development of this increase in capillary permeability. The sudden drop in endotoxin levels can be explained by a non-antibody-mediated tolerance to endotoxin, which can be induced in hours and is probably due to increased clearance or degradation of endotoxin and to a diminished ability of leucocytes to keep responding to endotoxin (Smith, 1986). The cascade of events triggered by endotoxin

which is responsible for the increase in capillary permeability may continue, however (Smith, 1986).

As in these cases 2 peak increases in endotoxin levels have also been observed in experimentally-induced endotoxaemia in horses through carbohydrate overload (Sprouse, Garner & Green, 1987). These authors found the 2nd peak occurred in 45 % of the experimental animals, with an average of 16 h between the 2 peaks. They suggested that the foregoing strongly supports the involvement of the Schwartzman reaction. In this study, however, there was a 3–5 day period between the 2 endotoxin peaks.

The source of the endotoxin in this study is not known. It is possible that with the increase in capillary permeablility the integrity of the intestinal mucosal barrier to endotoxin may be broken down and that both peaks may represent gram-negative bacterial endotoxin. It should be noted that 2 sheep (3764 & 3705) showed clinical signs (rapid and laboured breathing and cyanosis) commonly associated with endotoxaemia from gram-negative bacterial origin (Moore, 1981).

In conclusion, the results of this study seem to indicate that endotoxin may be involved in the pathophysiology of heartwater, but the characterization of the endotoxin and its exact role in the pathogenesis of the disease needs to be elucidated.

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