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Studies on the presence of endotoxin activity and inflammatory products (leukotrienes) in cases of experimentally induced heartwater in sheep

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ABSTRACT

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The presence of endotoxin was examined in seven sheep with experimentally induced heartwater. Elevations in endotoxin levels were recorded in one sampling in three of the seven sheep during the acute stage of the disease. The elevations in endotoxin levels were of short duration and decreased in the 24-h follow-up samples. There was no elevation of leukotrienes (B_4, C_4 and D_4) in the blood, or the thoracic or pericardial fluid of any of the sheep.

INTRODUCTION

In a previous study, Van Amstel, Oberem, Didomenico, Kirkpatrick & Matthee (1988) demonstrated the presence of endotoxin activity in the blood of sheep with experimentally induced heartwater. They found two peaks of endotoxin during the course of the disease, one coinciding with the initial temperature reaction and the second associated with severe clinical signs or death. As these changes were not consistent, in that one sheep died despite low levels of endotoxin, it was decided to expand the investigation, using the same assay procedure, namely the quantitative Limulus Amebocyte Lysate (LAL) microassay for the presence of endotoxin.

The involvement of agents associated with inflammation have been speculated upon because of the discrepancy between the apparently limited morphological cellular changes, including those on ultrastructural level, and the severity of the effusions found in the lungs and body cavities (Neitz 1968; Pienaar 1970; Camus & Barré 1982, citing Jackson & Neitz 1932; Prozesky & Du Plessis 1985).

Du Plessis (1985) and Du Plessis, Malan & Kowalski (1987) demonstrated that both complement and the complement-binding protein (conglutinin, unique to ruminants), are possibly involved in the pathogenesis of heartwater. Van Amstel, Gummow & Oberem 1992 found that the use of anti-inflammatory agents

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in experimentally induced heartwater in mice enhanced survival rates in the absence of any specific antibiotic treatment.

Based on the foregoing it was decided to study the possible involvement of the leukotrienes (LT) B_4, C_4 and D_4 in experimentally induced heartwater in sheep.

MATERIALS AND METHODS

Experimental animals

Heartwater was induced in seven healthy adult Merino sheep (identified as 3535, 3499, 3428, 3437, 3459, 3559 and 3438) by the intravenous inoculation of the "Welgevonden" stock of *Cowdria ruminantium*, contained in 5 mℓ of blood from an infected sheep (Du Plessis 1985). The rectal temperature of the animals was recorded twice daily. Clinical disease was considered to have commenced when this reached 40 °C. Rectal temperatures above 40 °C were recorded in all seven sheep from 8–15 days post-infection. Blood was collected for endotoxin assay and LT determinations from the seven experimental sheep on days 12, 6, 5 and 1, before experimental infection, on the day of experimental infection and then daily from day 7 until death, which occurred on days 12, 13 and 15. Three animals died naturally and four more were euthanased because of advanced disease.

All sampling procedures were performed under strict aseptic conditions. The wool overlying the jugular vein was clipped and shaved and disinfected with povidone-iodine (Providone Scrub, Centaur Laboratories, Durban Street, Johannesburg, 2000 South Africa). For the endotoxin assay, blood was collected into 10-mℓ pyrogen-free (PF), evacuated, venoject tubes, each containing 200 IU of sodium heparin. Standard heparinized 10-mℓ venoject tubes were used to collect blood for the LT determination.

Samples of thoracic and pericardial fluid were obtained during post mortem examination. The samples were placed in sterile, evacuated 10-mℓ venoject tubes, identified and stored at –20 °C.

Processing of plasma for endotoxin assay

Blood samples were processed within 30 min of collection. Samples were centrifuged at 200 x *g* for 10 min to obtain platelet-rich plasma (PRP). The PRP was then transferred to clean PF test tubes and covered with parafilm. Dilutions of 1:10 and 1:100 of the PRP was then made by adding PF water in PF test tubes, again covered with parafilm. The diluted PRP was then heated to 100 °C for 10 min in a water bath, and then frozen at –20 °C. All the test tubes used were rendered pyrogen free by heating them at 180 °C for 4 h or more.

Endotoxin assay procedure

The LAL chromogenic assay (QCL-1000, Whittaker Bioproducts Inc., P.O. Box 127, Biggs Ford Road, Walkersville, Maryland, USA 21793) 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 plates in order to limit the amount of endotoxin lost in the assay as a result of adherence to the test tube. The test was run with the use of a series of standard dilutions in water from which a standard curve could be constructed and a linear regression carried out. A correlation coefficient of > 0,98 had to be obtained. For the standard curve 21 Eu/mℓ of *E. coli* endotoxin stock solution was used. All samples were 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, temperature and aseptic techniques was maintained throughout the test.

The first test, carried out on 1:10 dilutions, indicated severe inhibition as only 20 % of a known amount of endotoxin added to the samples was recovered. The correlation coefficient of the standard curve was 0,99 (limit > 0,98). In an attempt to remove this inhibition, some samples of both the 1:10 and 1:100 dilutions were heat-treated once more and spiked with endotoxin. The LAL test was rerun on these samples. Results still indicated inhibition at the 1:10 dilution, but the 1:100 dilutions gave very good spiked recoveries of almost 100 %. Based on these results the 1:100 dilutions unspiked with endotoxin, but heat-treated twice, were used for the final testing. The test was run in duplicate.

The normal range of the LAL (QCL-1000) test is 0,1–1,0 Eu/mℓ. Therefore, at a 1:100 dilution, results are detectable in the range 10–100 Eu/mℓ. Results outside the range of the test are indicated as > 100 or < 10. The tests in this case had correlation coefficients of 0,98 and 0,99, respectively.

Processing of plasma for LT determinations

Blood samples were processed within 30 min of collection. Samples were centrifuged at 200 x *g* for 10 min to obtain PRP. The PRP was then transferred to clean pyrogen-free test tubes and stored at –20 °C.

Leukotriene assay procedure

Plasma and thoracic and pericardial fluid samples were thawed at room temperature. An initial extraction procedure was carried out on each of the samples using 20 mℓ ethyl acetate, following which the

ethyl-acetate phase was removed and evaporated under nitrogen (N_2). The sample was then taken up in $CHC\ell_3$:MeOH, (Chloroform:Methanol) made up in a 2:1 ratio. Such processed samples were filtered through a 0,22-μ filter and separated by means of high-phase liquid chromatography (HPLC) by the method and under the conditions described by Fallon, Booth & Bell (1987).

RESULTS

Concentrations of endotoxin recorded in the seven sheep suffering from experimentally induced heartwater are shown in Table 1.

From Table 1 it is evident that endotoxin concentrations increased in three of the seven sheep on day 10 post-infection. This increase in endotoxin concentration occurred 2 d after the temperature reaction of 40 °C. Early-morning rectal temperatures on day 10 are shown in Table 2. The sheep with increases in endotoxin concentrations also seemed to have higher respiratory rates on that day, compared with other sheep (see Table 2). Increases in endotoxin levels shown by sheep 3499 on day –5 and sheep 3428 on day 9 were very small and were not associated with changes in their respiratory rates.

Concentrations of LT B_4, C_4 and D_4 in the blood, and the thoracic and pericardial fluids, are shown in Tables 3–6.

From Tables 3–5 it is clear that no increases in LT B_4, C_4, and D_4 occurred in the blood during the course of the disease. Levels of LT found in the thoracic and pericardial fluid were generally lower (B_4) or approximately the same (C_4 and D_4) as those found in the blood.

TABLE 1 Results of endotoxin obtained from 7 sheep with experimentally induced heartwater

Day from infection	Endotoxin concentration (EU/mℓ)						
	Sheep number						
	3535	3499	3428	3437	3459	3559	3438
–12	< 10	< 10	< 10	< 10	< 10	< 10	< 10
–6	< 10	< 10	< 10	< 10	< 10	< 10	< 10
–5	< 10	14	< 10	< 10	< 10	< 10	< 10
–1	< 10	< 10	< 10	< 10	< 10	< 10	< 10
0	< 10	< 10	< 10	< 10	< 10	< 10	< 10
7	< 10	< 10	< 10	< 10	< 10	< 10	< 10
8	< 10	< 10	< 10	< 10	< 10	< 10	< 10
9	< 10	< 10	11	< 10	< 10	< 10	< 10
10	< 10	< 10	< 10	32	89	> 100	< 10
11	< 10	< 10	< 10	< 10	< 10	< 10	< 10
12	< 10	< 10	< 10	< 10	< 10	< 10	< 10
13	< 10	< 10	ND[a]	< 10	< 10	< 10	< 10

[a] ND = Not done

TABLE 2 Endotoxin concentrations, rectal temperatures, and heart and respiratory rates of seven sheep on day 10

Sheep number	Endotoxin concentr. (Eu/mℓ)	Heart rate/ min	Respiratory rate/ min	Rectal temperature (°C)
3535	< 10	80	72	41,0
3499	< 10	72	60	41,0
3428	< 10	72	64	41,0
3437	32	72	82	41,0
3459	89	74	80	41,0
3559	> 100	74	80	41,1
3438	< 10	74	60	41,3

DISCUSSION

The results of this study seem to differ somewhat from those reported earlier by Van Amstel *et al.* (1988). Only one peak of endotoxin was recorded in three of the seven sheep with experimentally induced heartwater. This occurred 2 d after the temperature reaction of 40 °C. In this case elevations of endotoxin seem to have been associated also with more severe clinical signs, specifically increases in respiratory rates.

In this study all sheep either died naturally or were euthanased on humane grounds due to severe clinical disease. One of the sheep died on day 12 and five on day 13 post-infection, whereas sheep 3559 died on day 15. Samples taken on days 12 and 13 did not show any increases in endotoxin concentrations in the blood of any of the sheep.

The discrepancy between the results of this study and those described earlier by Van Amstel *et al.* (1988) may be related to the source of the endotoxin. In this regard three possibilities exist, namely exogenous endotoxin, endotoxin derived from gram-negative enterobacteria due to an increase in capillary permeability, or endotoxin derived from *C. ruminantium*.

Contamination of blood samples with exogenous endotoxin cannot be excluded but is unlikely, since a strict aseptic technique was adhered to in both these studies.

If the endotoxin is derived from the initiating cause, it seems feasible that there should be a more consistent pattern in the release of endotoxin during the life cycle of *C. ruminantium*, particularly in this study as the course and severity of the disease were so similar in all seven sheep.

It seems more likely that the endotoxin found in both studies may have originated from gram-negative

TABLE 3 LTB_4 in plasma (p mol/mℓ)

Animal identification	Days from infection												
	–13	–6	–5	–1	0	+6	+7	+8	+9	+10	+11	+12	+13
3535	12	15	18	11	24	29	16	17	21	14	9	10	15
3499	16	19	17	12	18	19	20	23	15	11	17	22	13
3428	19	12	22	26	19	15	22	17	18	11	15	14	–
3437	11	17	18	21	13	6	11	18	22	22	19	16	17
3459	22	15	13	9	16	15	17	16	23	22	27	12	16
3559	8	15	16	14	13	11	28	16	21	15	13	14	22
3438	7	18	19	17	13	12	5	20	16	10	12	14	8

TABLE 4 LTC_4 in plasma (p mol/mℓ)

Animal identification	Days from infection												
	–13	–6	–5	–1	0	+6	+7	+8	+9	+10	+11	+12	+13
3535	2	2	2	1	0	3	2	3	0	1	1	2	4
3499	3	3	1	2	1	4	2	0	2	3	0	2	2
3428	0	2	3	2	2	2	1	3	1	3	1	2	–
3437	2	1	2	1	2	1	0	1	2	1	1	2	0
3459	2	1	1	3	3	2	1	2	2	1	2	1	2
3559	1	1	2	2	2	1	1	0	2	1	1	1	0
3438	1	1	2	1	1	1	1	0	3	1	2	2	1

TABLE 5 LTD_4 in plasma (p mol/mℓ)

Animal identification	Days from infection												
	–13	–6	–5	–1	0	+6	+7	+8	+9	+10	+11	+12	+13
3535	3	1	3	2	1	4	2	3	1	2	1	3	4
3499	2	1	2	1	2	3	2	1	1	2	1	4	3
3428	1	0	2	3	2	1	3	4	1	3	2	1	–
3437	1	2	3	0	1	2	1	2	3	0	0	3	1
3459	1	1	2	4	2	2	0	2	2	0	1	2	3
3559	2	2	1	2	2	0	2	1	3	0	2	2	1
3438	2	0	2	1	1	0	0	1	2	2	3	2	2

gut bacteria because of the vascular-permeability defect which develops during the acute stage of the disease. Variations in the extent of the increase in capillary permeability, degree of intestinal peristalsis, rate of endotoxin release and elimination from the blood could explain the discrepancy in the findings between this and the earlier study (Van Amstel *et al.* 1988). The similarities in the findings of these studies should, however, also be emphasized. In both it was found that large increases in the levels of endotoxin coincided with or occurred during the acute febrile stage of the disease and were accompanied by increases in the severity of clinical signs including recumbency, depression and increased respiratory rates.

It therefore seems that although endotoxin may play some aggravating role in the clinical course of the disease, it is unlikely to be a key player in the basic pathogenesis of the disease, namely the severe increase in capillary permeability, as was also demonstrated by severe effusions into body cavities found on post mortem examination in these seven sheep.

The leukotrienes are potent inflammatory mediators and are generated via arachidonic acid from phospholipid membranes in response to cellular injury

TABLE 6 LT in thoracic and pericardial fluid (p mol/10 mℓ)

Animal identification	Fluid LTs					
	Thoracic			Pericardial		
	B4	C4	D4	B4	C4	D4
3438	36	8	10	11	4	3
3559	23	3	10	7	2	1
3437	26	5	9	17	5	6
3428	29	9	11	22	1	9
3559	18	6	3	17	4	5
3499	17	2	5	11	5	3

caused by endotoxin or poor tissue perfusion (Bottoms & Adams 1992).

Some of the pathophysiologic effects ascribed to leukotrienes are similar to changes observed with heartwater. These include a decrease in cardiac function and an increase in vascular permeability with plasma-water leakage and oedema (Bottoms & Adams 1992).

Detecting and quantitating *in vivo* production of leukotrienes is difficult, because of a brief biological half-life and low plasma concentrations. Attempts to detect and quantitate leukotrienes in blood and lung lymph have yielded variable results, although analysis of pulmonary-oedema fluid from humans with acute respiratory-distress syndrome yielded high concentrations of leukotriene D_4 (Bottoms & Adams 1992).

Although this study yielded negative results with regard to the elevation in leukotriene concentrations in the blood and the thoracic and pericardial fluid in experimentally induced heartwater, more work needs to be done in this regard.

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