

THE PATHOGENESIS OF HEARTWATER

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ABSTRACT

DU PLESSIS, J. L., MALAN, LETITIA & KOWALSKI, Z. E., 1987. The pathogenesis of heartwater. *Onderstepoort Journal of Veterinary Research*, 54, 313-318 (1987)

Hypotheses on the pathogenesis of heartwater, which have been published so far, are briefly reviewed. Attempts were made at counteracting the effects of vaso-active substances released by mast cells by treating mice infected with *Cowdria ruminantium* with antagonists to histamin and serotonin on one hand, and with mast cell stabilizers on the other, but were not successful. Preliminary findings suggest that a hypersensitive type of reaction, triggered by the release of pharmacologically active substances, may possibly be basic to the pathogenesis of heartwater. Complement, and the products of arachidonic acid metabolism, possibly play a role in the release of the vaso-active substances.

INTRODUCTION

The pathogenesis of heartwater (HW) is one of several of the very important aspects of the disease that is poorly understood. There seems to be general agreement that increased permeability of smaller blood vessels plays an important role in the pathogenesis of the disease (Clark, 1962; Owen, Littlejohn, Kruger & Erasmus, 1973; Du Plessis, 1975; Prozesky & Du Plessis, 1984). Opinions differ, however, on how the increased permeability is caused. Most researchers ascribe the vascular defect to a toxin (Steck, 1928; Pienaar, Basson & Van der Merwe, 1966; Neitz, 1968; Pienaar, 1970; Ilemobade, 1976), but such a toxin has not been demonstrated and no experimental evidence supports this theory. Although vascular lesions in the brain were at first ascribed to the direct necrotizing effect of the HW agent (Pienaar *et al.*, 1966), Pienaar (1970) subsequently conceded that cytopathic effects were rarely seen in brain endothelial cells that were themselves parasitized by *Cowdria ruminantium*, and he consequently favoured the toxin hypotheses. The absence of a direct link between the presence of colonies of the HW agent and cytopathic changes of the cells harbouring them was later confirmed by Prozesky & Du Plessis (1985).

Another hypothesis is that vaso-active substances liberated by mast cells are responsible for the increased permeability (Du Plessis, 1975). The findings advanced in support of this hypothesis were the decrease in the number of perceptible mast cells in the peritoneal cavity and spleen of mice infected with the Küm m strain of *C. ruminantium* and light as well as electron microscopical changes in the liver and spleen of the mice that were consistent with the effects of pharmacologically active substances released by mast cells. Degranulation of mast cells was confirmed by electron microscopy and it was shown that the mast cells do not undergo necrosis after degranulation but become activated, evidenced by the presence of numerous ribosomes, rough endoplasmic reticulum and mitochondria. Further observations (J. L. du Plessis, unpublished data, 1976) suggested that the mast cells become degranulated upon contact with the HW agent and that the degranulation is therefore not necessarily IgE-antibody mediated.

Subsequent attempts to demonstrate a rise in blood levels of serotonin or histamin in mice and sheep with fatal HW were unsuccessful. It was then decided to ascertain whether mast cell products play a role in the pathogenesis of the disease by treating animals, infected with a lethal dose of *C. ruminantium*, with histamin and serotonin antagonists.

Using calves as experimental animals, 2 further lines of thought were followed. Since certain aspects of clinical HW are reminiscent of a hypersensitive type of reaction, and since complement (C') plays an important role

in immune-complex mediated hypersensitivity (Tizard, 1977), levels of C' in relation to the presence of immunoglobulin (IK), autoantibodies in response to C' bound to immune-complex (Lachmann, 1967), were determined during the course of infection. Secondly, the experimental animals were used for a preliminary study on the role possibly played by the products of arachidonic acid metabolism in the pathogenesis of the disease, since these substances are known to be vaso-active and play a role as the slow reacting substance of anaphylaxis, a proposed mediator of hypersensitivity reactions (Dahlen, Björk, Hedqvist, Arfors, Hammerström, Lindgren & Samuelsson, 1981).

MATERIALS AND METHODS

Treatment of infected mice with serotonin/histamin antagonists

Conventional outbred Swiss white mice were infected with 0.2 ml of Küm m strain-infected mouse liver and spleen homogenate representing a dose of 200 LD₅₀ per mouse. At this dosage level control untreated mice showed clinical signs of a ruffled haircoat and dyspnoea 10 days after infection. They were subsequently treated parenterally with the serotonin and histamin antagonists, cyproheptadine, promethazine HCl and methysergide at various intervals after infection, and at different dosage levels, as indicated in Table 2. To stabilize the mast cells of a further 6 groups of 10 mice each, they were given hydroxyzine 2 HCl at 2 dosage levels in their drinking water for 28 days before being infected, and for 10 days thereafter. The preliminary determination of the approximate daily intake of water by each mouse assured the dosage levels indicated in Table 2. In 4 of these 6 groups cimetidine hydrochloride was used in combination with hydroxyzine 2 HCl for the last 14 days before infection and for 10 days thereafter.

Infection of calves

Twelve Bonsmara bull calves varying in age from 14-180 days were inoculated intravenously with 5 ml of sheep blood infected with the Germishuys strain of *C. ruminantium*. This strain was isolated from a sheep suffering from natural HW. It is highly pathogenic to cattle and sheep but non-pathogenic to mice (J. L. du Plessis, unpublished data, 1985). Early morning temperatures were recorded and the reaction indices of the calves calculated as previously described (Du Plessis, 1985). The calves were not treated. Heparinized and non-heparinized samples of blood were collected on the day of infection and thereafter at 5-7 day intervals for 20 days after infection. These were subjected to the following assays.

Assay of complement

C' levels were determined by allowing the sheep red blood cell-amboceptor system, used in the complement fixation test, to react with appropriate dilutions of the calf sera within 4 h after the collection of the blood.

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TABLE 1 Volumes of reagents in complement assay

| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|-----------------------|-----|-----|-----|-----|------|-----|------|------|------|-----|-----|
| Serum dilution | 1/1 | 1/2 | 1/3 | 1/4 | 1/5 | 1/6 | 1/8 | 1/10 | 1/12 | — | — |
| Serum, ml | 1,2 | 0,6 | 0,4 | 0,3 | 0,24 | 0,2 | 0,15 | 0,12 | 0,1 | — | — |
| Veronal buffer, ml | — | 0,6 | 0,8 | 0,9 | 0,96 | 1,0 | 1,05 | 1,08 | 1,1 | 1,2 | 0,6 |
| Haemolytic system, ml | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 | 0,6 |
| G-pig C', ml | — | — | — | — | — | — | — | — | — | — | 0,6 |

TABLE 2 Kümm strain infected mice treated with histamin and serotonin antagonists

| Drugs | Antagonist to | Dosage level $\mu\text{g/g/day}$ | Days before and after infection that treatment was commenced | | No. of mice treated/no. that survived |
|---------------------------------------------------------------|-------------------------|----------------------------------|--------------------------------------------------------------|----------|---------------------------------------|
| | | | Before | After | |
| Cyproheptadine | Histamin and serotonin | 3 | 0 | 9 | 9/0 |
| Promethazine HCl | Histamin | | 0 | 10 | 6/0 |
| Methysergide | Serotonin | 100 | 0 | 7 | 6/0 |
| Methysergide | Serotonin | 100 | 0 | 9 | 6/0 |
| Hydroxyzine 2 HCl | Mast cell degranulation | 1 & 0,5 ⁽¹⁾ | 28 | 10 | 20/0 |
| Hydroxyzine 2 HCl ⁽²⁾ and cimetidine hydrochloride | Mast cell degranulation | 1 & 0,5 1 & 0,5 | 28 14 | 10 10 | 40/0 |

⁽¹⁾ Dosage level to each of 2 groups of 10 mice

⁽²⁾ Hydroxyzine 2 HCl for 14 days and in combination with cimetidine hydrochloride for another 14 days before infection

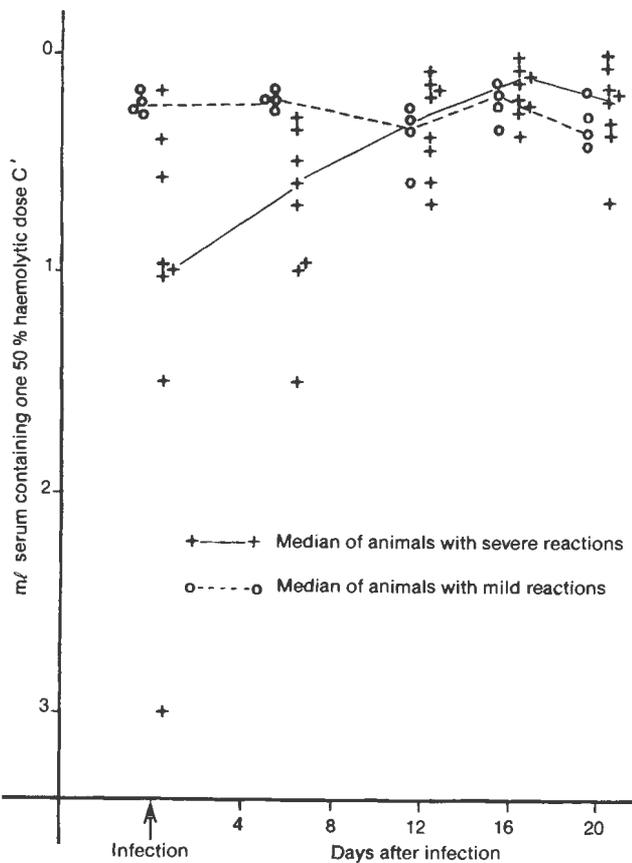


FIG. 1 Complement levels of calves infected with *C. ruminantium*

Preliminary tests had shown that there was a noticeable drop in the C' level of calf sera measured 6–8 h after collection compared with the levels recorded within 1–4 h after collection.

The assay was performed as follows:

One volume (0,6 ml) of haemolytic system, consisting of equal volumes of 3% sheep red blood cell suspension and appropriately diluted haemolysin in veronal buffer

was added to 2 volumes (1,2 ml) of calf serum diluted in veronal buffer in 9 tubes, as shown in Table 1. Tube 10 containing veronal buffer and haemolytic system and tube 11 containing haemolytic system, veronal buffer and excess guinea pig C' served as 0 and 100 % haemolysis controls.

The tubes were incubated in a water-bath at 37 °C for 30 min. After 3 ml of veronal buffer has been added to each tube to obtain a sufficiently large volume to permit of a spectrophotometric reading, the tubes were centrifuged at 2 000 r.p.m. for 5 min. The supernatant fluids were then decanted into clean tubes. The optical density (OD) was read using a photometer set at 541 nm wavelength. The supernatant from tube 10 was used as a blank to zero the photometer and the reading of the supernatant from tube 11 to obtain the target OD representing 100 % haemolysis. The OD of each tube was recorded and the percentage haemolysis calculated for each tube by the formula:

$$\% \text{ haemolysis (Y)} = \frac{\text{OD} \times 100}{\text{Target OD}}$$

The dilutions of calf sera giving the greatest number of end points between 10 and 90 % haemolysis were selected and the value of the expression $\frac{Y}{100-Y}$ calculated for these end points, where Y = % haemolysis. Using log/log graph paper with the vertical axis representing the dose of serum, and the horizontal axis the value of $\frac{Y}{100-Y}$, the

values of $\frac{Y}{100-Y}$ were plotted on the graph paper. These

points must fall on a straight line. Where a line joining the plotted points crosses the vertical '1' line, the 50 % haemolytic dose (C'H₅₀) of the serum and, therefore, the volume of calf serum containing one C'H₅₀ C' is indicated (Alton, 1977).

Assay of conglutinin and immunoconglutinin

Levels of conglutinin were determined as previously described (Du Plessis, 1985). After the assay for conglutinin was completed, 0,02 ml of 0,02 M Na₂ EDTA in

TABLE 3 Levels of complement (C'), conglutinin (K) and immunoconglutinin (IK) in the sera of calves reacting to *C. ruminantium*

| Calf No. | Age in days at infection | Febrile reaction | | | Reaction index | Complement C' H ₅₀ /ml serum | | Reciprocal of K and IK titres | | |
|----------|--------------------------|------------------|------------------|---------------|----------------|-----------------------------------------|-------------------------------|-------------------------------|----|----------|
| | | Day of onset | Duration in days | Max. temp. °C | | Day of infection | Highest level after infection | Day of infection | K | IK |
| 1 | 14 | 13 | 11 | 40,2 | 10 | 0,15 | 20 | 20 | 20 | 10 |
| 2 | 25 | 15 | 7 | 40,9 | 6,7 | 0,23 | 10 | 10 | 10 | 10 |
| 3 | 73 | — | — | — | 0 | 0,3 | 0,16 (5) ⁽¹⁾ | 10 | 10 | 40 |
| 4 | 73 | 11 | 7 | 41,3 | 21,6 | 3 | 0,25 (11) | 20 | 10 | 40 (11) |
| 5 | 94 | 14 | 8 | 40,8 | 10,9 | 0,3 | 0,2 (14) | 40 | 40 | 80 (18) |
| 6 | 101 | 13 | 6 | 41 | 20,6 | 1 | 0,13 (14) | 20 | 10 | 80 (18) |
| 7 | 122 | 10 | 8 | 41 | 32,6 | 1 ⁽²⁾ | 0,25 (14) | 40 | 20 | 80 (19) |
| 8 | 129 | 13 | 6 | 41,6 | 32,5 | 0,4 ⁽²⁾ | 0,2 (15) | 40 | 20 | 20 (12) |
| 9 | 164 | 12 | 11 | 40,3 | 24 | 0,15 | 0,15 (13) | 10 | 10 | 160 (12) |
| 10 | 171 | 12 | 9 | 41,2 | 20,6 | 0,6 | 0,1 (16) | 160 | 20 | 80 (16) |
| 11 | 180 | 17 | 12 | 40,1 | 29,5 | 1 | 0,15 (13) | 40 | 10 | 80 (20) |
| 12 | 180 | 12 | 8 | 40,8 | 35,3 | 1,5 ⁽²⁾ | 0,4 (16) | 320 | 80 | 160 (19) |

⁽¹⁾ 0,16 (5) = 0,16 ml serum of calf 1 contained one 50% haemolytic dose of C' on day 5 after infection

⁽²⁾ Calves 7, 8 and 12 died from the infection

veronal buffer was added to each reaction tube of the conglutinin assay. Tubes were gently shaken. The added Na₂ EDTA disrupted any agglutination caused by conglutinin but did not disrupt any agglutination caused by IK. The titre of the IK assay was the reciprocal of the highest dilution of calf serum at which agglutination persisted (Lachmann, 1967).

Assay of products of arachidonic acid metabolism

Cyclo-oxygenase and terminal prostaglandin-(PG-) and thromboxane (TX)B₂-forming activity was assayed in the heparinized blood according to a method employed in the testing of milk, with the exception that 5 ml blood instead of 5 ml milk were used (Z. E. Kowalski, unpublished data, 1986).

Effects of calf sera on the infectivity of *C. ruminantium* in mice

To determine the *in vitro* effect of C' in the sera of the calves on the infectivity of the HW agent, 2 ml of freshly collected serum were divided into 2 aliquots. One aliquot was inactivated for 30 min in a water-bath at 56 °C, and 0,5 ml of Kümm strain-infected mouse liver and spleen homogenate, representing 2 000 LD₅₀ doses, added to each aliquot. A control tube containing the same infective dose and one ml of buffered lactose peptone were included. The tubes were incubated at 37 °C for 30 min and 0,2 ml per mouse inoculated intraperitoneally into groups of 5 mice per serum sample.

RESULTS

Treatment of infected mice with serotonin/histamin antagonists

All the drugs listed in Table 2 were ineffective in prolonging the course of the infection, or in preventing the eventual death of the mice. It must be noted, however, that the histamin and serotonin antagonists were administered only 1-3 days before the onset of clinical signs, the manifestation of what may probably already be an advanced stage in the pathogenesis of the disease.

Complement levels in reacting calves

The C' levels of the 12 calves recorded on the day when they were infected appeared to be related to the severity of the reaction that was elicited (Table 3). A volume of 0,4 ml of serum or more containing one C' H₅₀ was recorded in 7 out of the 8 calves that had reaction indices above 20 (severe reactions), whereas in the case of all 4 calves with reaction indices of 10,9 or less, volumes of 0,3 ml of serum or less contained one C' H₅₀.

It can be seen from Fig. 1 and Table 3 that there was a rise in the levels of C' during the febrile reaction of the 8 calves that had severe reactions.

In vitro effect of calf sera on the infectivity of the HW agent to mice

The mortality score of mice inoculated with calf sera to determine the *in vitro* effect of C' on the HW agent is given in Table 4. It is evident that all, or most of the mice injected with the HW agent in the presence of the sera of calves 1, 2, 3 and 8 with higher levels of C', survived, but not one injected with the sera of calves 4 and 7 survived. Significantly lower levels of C' were recorded in the sera of these 2 calves. None of the mice inoculated with the HW agent in the presence of the inactivated sera survived. It would seem therefore that bovine C' inhibits the infectivity of the Kümm strain of *C. ruminantium*.

Conglutinin and immunoconglutinin

The conglutinin and IK titres recorded in the sera of the calves are given in Table 3. Substantial levels of IK were recorded in the sera of all except the 2 youngest

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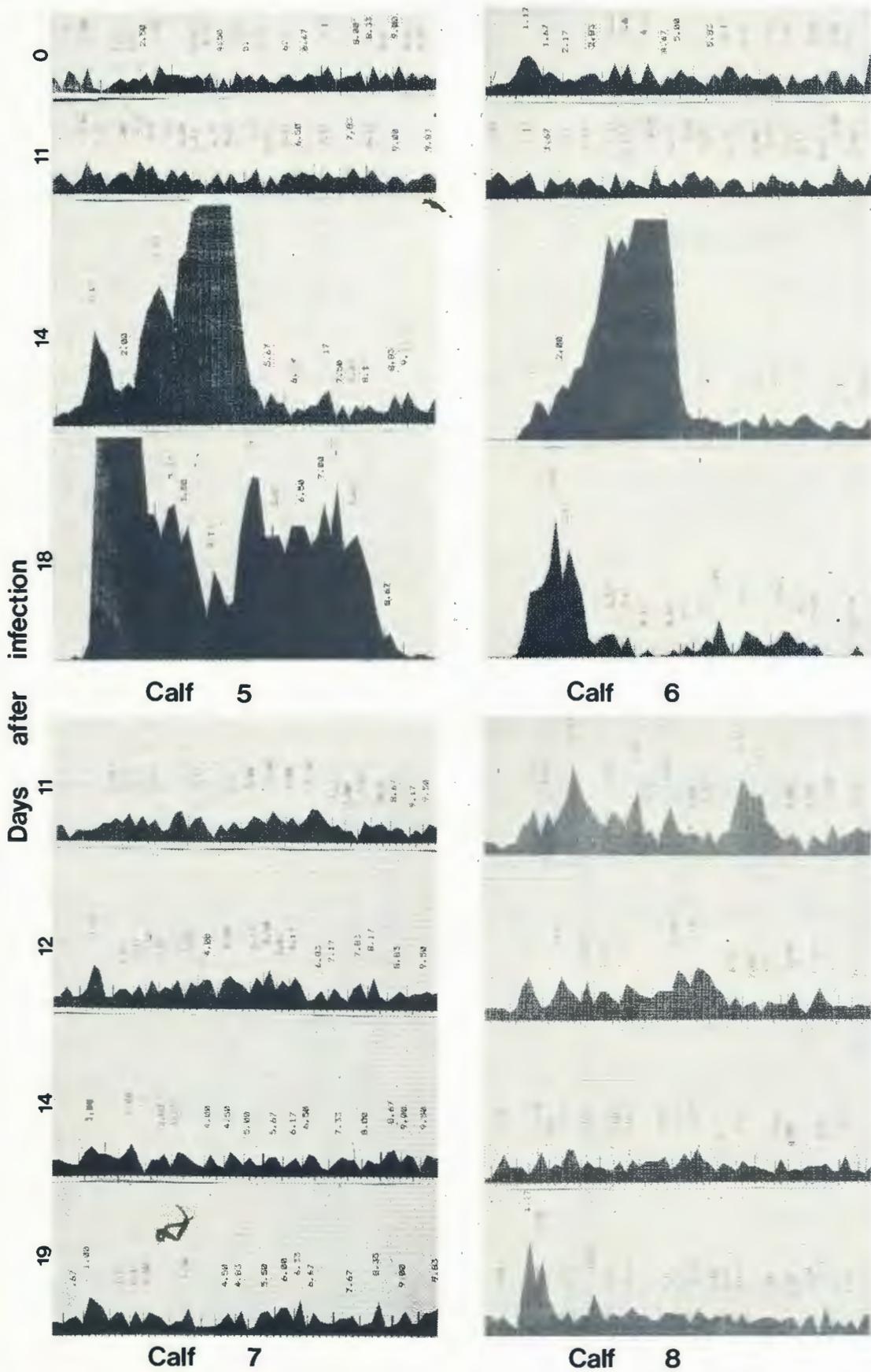


FIG. 2 Chromatograms of the cyclo-oxygenase and terminal prostaglandins—and thromboxane A_2 -forming activity of radiolabelled arachidonic acid in the blood of calves reacting to heartwater

TABLE 4 Effects of calf sera before and after inactivation at 56 °C on the infectivity of *C. ruminantium* to mice

| Calf No. | ml serum containing one C'H ₅₀ | No. of mice that survived | |
|-------------------------------|-------------------------------------------|---------------------------|--------------------------|
| | | Before serum inactivation | After serum inactivation |
| 1 | 0,15 | 3 | 0 |
| 2 | 0,23 | 4 | 0 |
| 3 | 0,3 | 5 | 0 |
| 4 | 3 | 0 | 0 |
| 7 | 1 | 0 | 0 |
| 8 | 0,4 | 3 | 0 |
| Control infective inoculation | | 0 | — |

calves at 11–20 days after infection. There was no correlation between the severity of the reactions shown by the calves and the titres of IK recorded.

Products of arachidonic acid metabolism

The profiles of cyclo-oxygenase and terminal PG- and TXB₂-activity obtained in the heparinized blood of 4 calves collected on the day of infection, and at intervals thereafter, are shown in Fig. 2. It can be seen that the elution pattern between calves that survived (calves 5 and 6) differed from those that died (calves 7 and 8). There was also a distinct difference between the patterns on the days when the animals were infected, and those observed 14 and 18 days afterwards, in the case of the 2 surviving calves.

A quantitative assessment was not attempted, as the assay had been developed for milk and not blood. However, peaks that correspond with the retention times of those of radiolabelled standards were obtained. Due to co-elution of other radioactive substances, most probably lipoxigenase products (leukotrienes) of radiolabelled arachidonic acid, there was no clear resolution of the peaks and quantitation was not possible. It must be noted that the marked differences between the profiles recorded on days 14 and 18 in the case of calves 5 and 6, and those of calves 7 and 8, can in all probability be ascribed to this phenomenon.

DISCUSSION

One of the alternatives to a toxin as a possible cause of increased vascular permeability, the cardinal lesion in the pathogenesis of HW, is the release of vaso-active substances by mast cells (Du Plessis, 1975).

The unsuccessful attempts in the present study to prevent the fatal outcome of mice infected with the Kümm strain of *C. ruminantium* by treating them with histamin and serotonin antagonists, or by stabilizing their mast cells to prevent degranulation, do not support this hypothesis. The mere fact that an antagonist to a pharmacologically active substance does not counteract its deleterious effects does not, however, rule out altogether the involvement of such a substance. Furthermore, the fact that the histamin and serotonin antagonists were only administered late during the incubation period when the pathogenesis of the disease had probably already reached an advanced stage, should also be borne in mind. Until the decrease in the number of perceptible mast cells in the peritoneal cavity and spleen, and some specific ultrastructural changes in the liver and lung of mice infected with the Kümm (Du Plessis, 1975) and the Welgevonden (Prozesky & Du Plessis, 1985) strains of *C. ruminantium* can be satisfactorily explained, the involvement of mast cell mediators in the pathogenesis of HW cannot be discounted.

The experiments reported in the present study to investigate the role of C' in the pathogenesis of HW must be seen as preliminary and deal with only some of the aspects of the highly complex C' system. The levels of C' recorded on the day the calves were infected with the

HW agent appeared to be related to the severity of the reactions subsequently shown by them. C' levels in the pre-infection serum of the calves that had mild to moderate reactions were higher than those of the animals that showed severe febrile reactions accompanied by clinical signs and death in the case of 3 of them. Furthermore, there was very little fluctuation in the C' levels of the animals with mild reactions over the course of 3 weeks after infection, whereas in the case of all the calves with severe reactions, there was a distinct rise. The C' system therefore appears to be activated during the reaction that occurs during infection with *C. ruminantium*.

The inhibitory effect of C' on the infectivity of the HW agent as manifested in an *in vitro* test is relevant to the role played by this serum component in the pathogenesis of the disease. The fact that the sera failed to inhibit the infectivity of *C. ruminantium* after being inactivated at 56 °C, suggests that C' is the serum factor responsible for the loss of infectivity of the HW agent. The *in vitro* bactericidal effect of C' is well known (Roitt, 1977) and it is therefore possible that the level of C' in the serum of a calf at the time of infection influences the subsequent course and severity of the reaction.

During and towards the end of the febrile reaction in all except the 2 youngest calves there was a substantial rise in the levels of IK, auto-antibodies to C' fixed to immune-complexes. This is proof of the formation and presence of immune-complexes. Although, on one hand, there was no difference between the IK titres of the animals with mild and those with severe reactions, and, on the other, there is as yet no histopathological evidence of immune-complex formation in HW, the involvement of an immune-complex type of hypersensitivity reaction in the pathogenesis of HW cannot be excluded and deserves further investigation.

The pathway of pathogenesis in HW remains vague, but the preliminary evidence that products of arachidonic acid metabolism, C' and immune complexes may be involved, possibly suggests that a hypersensitive type of reaction triggered by the release of pharmacologically active substances is at its basis. Furthermore, although the involvement of a toxin cannot as yet be ruled out altogether, available evidence, although preliminary at this stage, rather suggests that in addition to the role played by C', future research should be directed at the different mechanisms of release of vaso-active substances and the action of the products of arachidonic acid metabolism.

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