

## PATHOGENESIS OF HEARTWATER: I. *COWDRIA RUMINANTIUM* IN THE LYMPH NODES OF DOMESTIC RUMINANTS

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### ABSTRACT

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The intracellular replication of *Cowdria ruminantium* (Cowdry, 1926) is described in reticulum cells of lymph nodes several days prior to the appearance of rickettsial colonies in vascular endothelial cells. This is suggestive of a developmental cycle of the parasite in the lymph nodes. The subinoculation of suspensions of these glands resulted in transmission of the disease.

### INTRODUCTION

*C. ruminantium* (Cowdry, 1926) may be demonstrated in endothelial cells of capillaries in squash smears of the brain and in the endothelium in histological sections of various organs in domestic ruminants suffering from heartwater. The demonstration of this parasite by Cowdry (1925) in an exclusively endothelial position in renal glomeruli and capillaries of the cerebral cortex in sections, provided the earliest method for the laboratory diagnosis of heartwater.

Subsequently Jackson (1931) described a technique for preparing intimal smears of various veins to demonstrate *C. ruminantium* in endothelial cells. This technique remained the method of choice for the diagnosis of heartwater, until Purchase (1945) described the currently used method for demonstrating the parasite in endothelial cells in squash smears of the brain.

The study of heartwater has been hampered by the failure to grow the parasite in artificial media and by lack of knowledge concerning the developmental cycle of the parasite. Closely coupled to this is the question whether the parasite in infective blood is free from the corpuscles and within the field of microscopic visibility. Alexander (1931) hypothesized that the parasite is not evenly distributed in the blood and appears to be attached to the erythrocytes and leucocytes. Evidence has been gained that the infective agent of *C. ruminantium* is associated with the leucocyte fraction of the blood (unpublished data, K. E. Weiss, Veterinary Research Institute, Onderstepoort). Maintaining that they were able to demonstrate single granules of *C. ruminantium* in blood smears of animals with active heartwater, Jackson & Neitz (1932) suggested that this parasite was free in the blood during the parasitaemic stage of the disease. They advanced the theory that rickettsiae introduced naturally or artificially into the blood stream, enter endothelial cells where they develop from a single granule to a large group until the cell ruptures, thus discharging the organisms into the circulation. This view was also held by Cowdry (1926) who claimed that he was able to demonstrate the release of rickettsiae from parasitized endothelial cells. This author emphasized the localization of these organisms exclusively in endothelial cells and their complete innocuousness to cells invaded by them.

While it is believed that *C. ruminantium*, like other rickettsiae, is an intracellular parasite, the probability that, following intravenous inoculation of the organism, a developmental phase occurs in tissues other than vascular endothelium, prompted this study. An attempt was made to trace the localization of the rickettsiae during the incubation period, the acute stages of the disease

and the convalescent period by means of impression smears and sections prepared from various organs. Impression smears were used in addition to sections because of the greater cellular detail and the more accurate study of intracellular events afforded by them.

### MATERIALS AND METHODS

Heartwater was experimentally produced in 12 to 15 month old susceptible Merino sheep by the intravenous administration of 50 ml citrated blood drawn from sheep in the febrile stage of the disease. The strains of *C. ruminantium* used in these studies were those maintained at this Institute for the production of infective blood for immunization and a strain isolated from a goat originating from the Rust de Winter area of the Transvaal. The number of sheep infected, the day on which they were sacrificed and the strains used are indicated in Table 1.

Impression smears on clean microscopic slides were made from the spleen, liver, lungs, bone marrow, kidneys and certain lymph nodes. Those included were the mesenteric, caecal, ruminal, periportal, renal, mediastinal, retropharyngeal, prescapular and popliteal lymph nodes. Hippocampal smears were made in the manner described by Purchase (1945) in each case. The smears were allowed to dry, fixed in methyl alcohol and May Grünwald for 3 and 5 minutes respectively and stained for 50 min in 5 per cent Giemsa in distilled water buffered to pH 6.8. Tissues from the same organs were fixed in 10 per cent buffered formalin, cut and stained with haematoxylin and eosin (HE), and Giemsa according to conventional methods. In addition, sections of lymph nodes were stained by the periodic-acid-Schiff (PAS) method (Pearse, 1961) and Schmorl's lipofuscin method (Pearse, 1961). Similar preparations were made from animals that died from acute heartwater following natural infection (Table 1).

Sections were also prepared from 1 mm square blocks of the same lymph nodes embedded in Araldite\*. The blocks were fixed in 4 per cent glutaraldehyde in Millonig's buffer (Millonig, 1961), post-fixed in buffered 2 per cent osmium tetroxide, dehydrated and embedded in Araldite (Luft, 1961). Sections, 0.5 to 1  $\mu$  thick, were cut and stained with toluidine blue pyronin (Ito & Winchester, 1963).

In order to control the microscopical findings, 10 ml citrated blood taken on day of slaughter and suspensions of pooled mesenteric and prescapular lymph nodes homogenized in normal saline from five sheep as indicated in Table 1 were each inoculated separately into ten susceptible sheep.

\*CIBA, Ltd., Basle.

## RESULTS

Grossly the lymph nodes, particularly those of the mesentery, were mildly to moderately swollen from the third day after infection onwards. On section an increased fluid content was evident. These changes became more pronounced at the onset of the febrile reaction and were prominent in the terminal stages. Subcapsular petechiae were regular lesions at this stage.

Microscopically various rickettsial developmental stages were demonstrable in Giemsa stained impression smears as well as in sections of mesenteric lymph nodes from 2 to 4 days after infection. In smears vacuoles in the cytoplasm of reticulum cells and macrophages become visible before the suspected intracytoplasmic developmental stages of *C. ruminantium* were seen [Plate 1 (1 and 2)]. The vacuoles which varied in size, were large in reticulum cells and macrophages of the medulla and considerably smaller in those of the cortex. The nuclei of these cells were usually indented or flattened [Plate 1 (1 to 4)]. Usually only one, but occasionally two vacuoles were present in the same cell [Plate 1 (3)]. The vacuole first was clear and then became irregularly opaque and cloudy [Plate 1 (1)]. The first evidence of what was suspected to be the parasite was the appearance of single or multiple corpuscular structures in the vacuoles. At first they occupied a small portion of the vacuoles and were dull, purplish-grey and poorly outlined [Plate 1 (2, 3 and 6)]. These "initial bodies", which varied in size, subsequently divided into a number of smaller granular bodies dark-purple in colour and closely aggregated within the limits of the original "initial bodies" [Plate 1 (4 to 8)]. These granular bodies then appeared to subdivide, decreased in size and increased in number. The final "elementary" granules agglomerated and occupied the entire vacuole [Plate 1 (9 to 12)]. More than one colony of granules occasionally developed in one cell [Plate 1 (12)]. The organisms at this stage in size, shape and staining reaction with Giemsa were indistinguishable from the smallest organisms detectable in endothelial cells of brain capillaries [Plate 1 (15)] or from those present in small groups extracellularly in lymph node smears [Plate 1 (14)]. These smallest groups of extracellular rickettsial organisms detectable in impression smears consisted of two to five organisms in a pale staining eosinophilic matrix. Groups containing larger numbers of organisms were rarely seen.

In  $1\ \mu$  sections stained with toluidine blue the cells harbouring the developmental stages, were located in the medullary cords, the medulla towards the cortico-medullary junction and the cortex.

From 4 days after infection free-lying, spherical, membrane-bounded rickettsial colonies, similar in size and structure to those described intracellularly, were in evidence in both smears [Plate 1 (13)] and sections. In the latter they were demonstrable in medullary sinuses [Plate 2 (18 and 22)]. Round cytoplasmic bodies apparently extruded from cells and containing a vacuole or a vacuole occupied by one or more suspected "initial body" were occasionally seen in smears. These extracellular spherical colonies had to be distinguished from round cytoplasmic bodies consisting of portions of cytoplasm liberated from cells and readily demonstrable in spleen and lymph node smears of normal animals. The colonies were distinctly more granular in appearance and stained metachromatically, whereas the cytoplasmic bodies were pale blue.

The primary developmental stages described were most numerous in the lymph nodes of the mesentery, caecum and fore-stomachs. The P-shaped mesenteric

node closest to the ileo-caecal junction regularly revealed the greatest number of affected cells in artificial and natural cases in sheep and goats. The superficial lymph nodes were less markedly and the periportal, renal and retropharyngeal nodes rarely affected.

Other tissues belonging to the reticulo-endothelial system were not examined extensively. Those examined were the spleen, bone marrow, intestinal Peyer's patches and Kupffer cells of the liver. Some highly suspicious intracytoplasmic bodies were detected in the spleen and Kupffer cells.

From 5 days after infection pigment granules were detectable in intracytoplasmic and free rickettsial colonies [Plate 1 (13) and Plate 2 (18 and 22)]. Groups of these pigment granules independent of the rickettsial developmental stages were seen in the cytoplasm of reticulum cells and macrophages and as free-lying granules and were numerous in smears from both infected and normal mesenteric nodes. These pigment granules stained dark-blue with Giemsa and positively to the Schmorl's and PAS methods and were suspected to be lipofuscin. Except for the tinctorial differences, some of these granules closely resembled rickettsiae in size and shape. Those in developing rickettsial colonies increased in size later during the course of the disease.

Another feature associated with infection by *C. ruminantium* both in smears and sections of lymph nodes was the presence of very large reticulum cells, the cytoplasm of which was densely packed with rickettsia-like granules morphologically and tinctorially similar to the intra- and extracellular rickettsiae [Plate 2 (21, 23 and 24)]. The rickettsia-like granules in these exceptionally large cells consisted of numerous minute groups of two to five rickettsia-like granules in a pale eosinophilic matrix [Plate 2 (21)]. These cells were present from the 4th day after infection and were observed in one animal on the 44th and in another on the 64th day after infection. The granules were seldom uniform in colour as some of them stained a dark blue and others a greenish-blue with Giemsa, particularly during the later stages of the disease. From the 5th post-infection day all the granules of all these large cells progressively became denser and of a darker colour than rickettsial organisms and stained Schmorl and PAS positive.

Confined to the 3rd day after infection and closely resembling the very large reticulum cells just described, were similar cells of the same size containing granules which were, however, not as numerous. These cells also contained the small groups of rickettsia-like granules which were sparsely distributed throughout the cytoplasm, and appeared to be forerunners of the more abundant granulated reticulum cells.

In HE sections these large granulated reticulum cells occurred singly or in small clusters [Plate 2 (24)] in the region of the cortico-medullary junction as well as in the cortex. The cytoplasm of these cells was vesicular or faintly granular and distinctly granular in Giemsa stained sections [Plate 2 (23)]. These large granule bearing cells were absent in smears and sections from non-infected control animals.

From the 4th post-infection day macrophages containing phagocytosed granular material of varying size and shape and of indefinite morphology appeared in increasing numbers. In many cases these granules closely resembled the small extracellular groups of two to five rickettsiae [Plate 1 (14)], but were less distinct. In addition to phagocytosed granules suspected to be rickettsial organisms, lipofuscin and other pigments occasionally were present in the same macrophage. In sections these

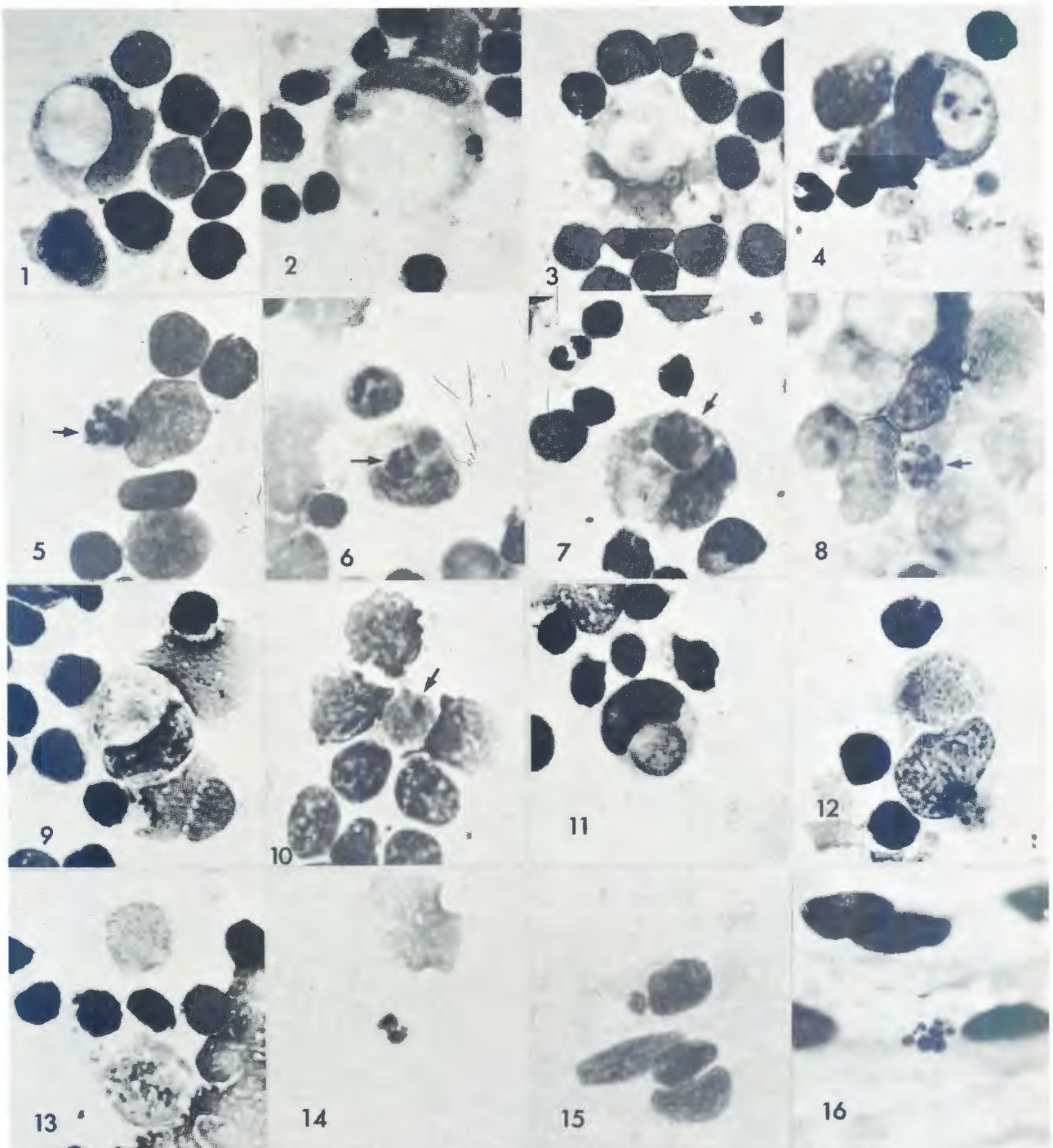


PLATE 1 *C. ruminantium* in lymph node impression (1 to 14) and brain squash smears (15 and 16), stained with Giemsa  $\times 1200$ . 1 to 3. Vacuoles in reticulum cells, with early initial bodies in 2 and 3. 4 to 8. "Initial bodies", some in the process of subdivision. 10. Agglomeration of elementary bodies with one initial body still visible. 11 and 12. Intracytoplasmic rickettsial colonies prior to extrusion. 13. Two extracellular rickettsial colonies. 14. Two groups of four to six organisms resulting from disintegration of extracellular colonies. 15 and 16. Colonies of *C. ruminantium* in brain endothelial cells. Note the resemblance of the organisms in 14 to those in the brain endothelial cell on first day of the febrile reaction (15) and their dissimilarity to organisms of different size and shape in the fatal natural case shown in 16.

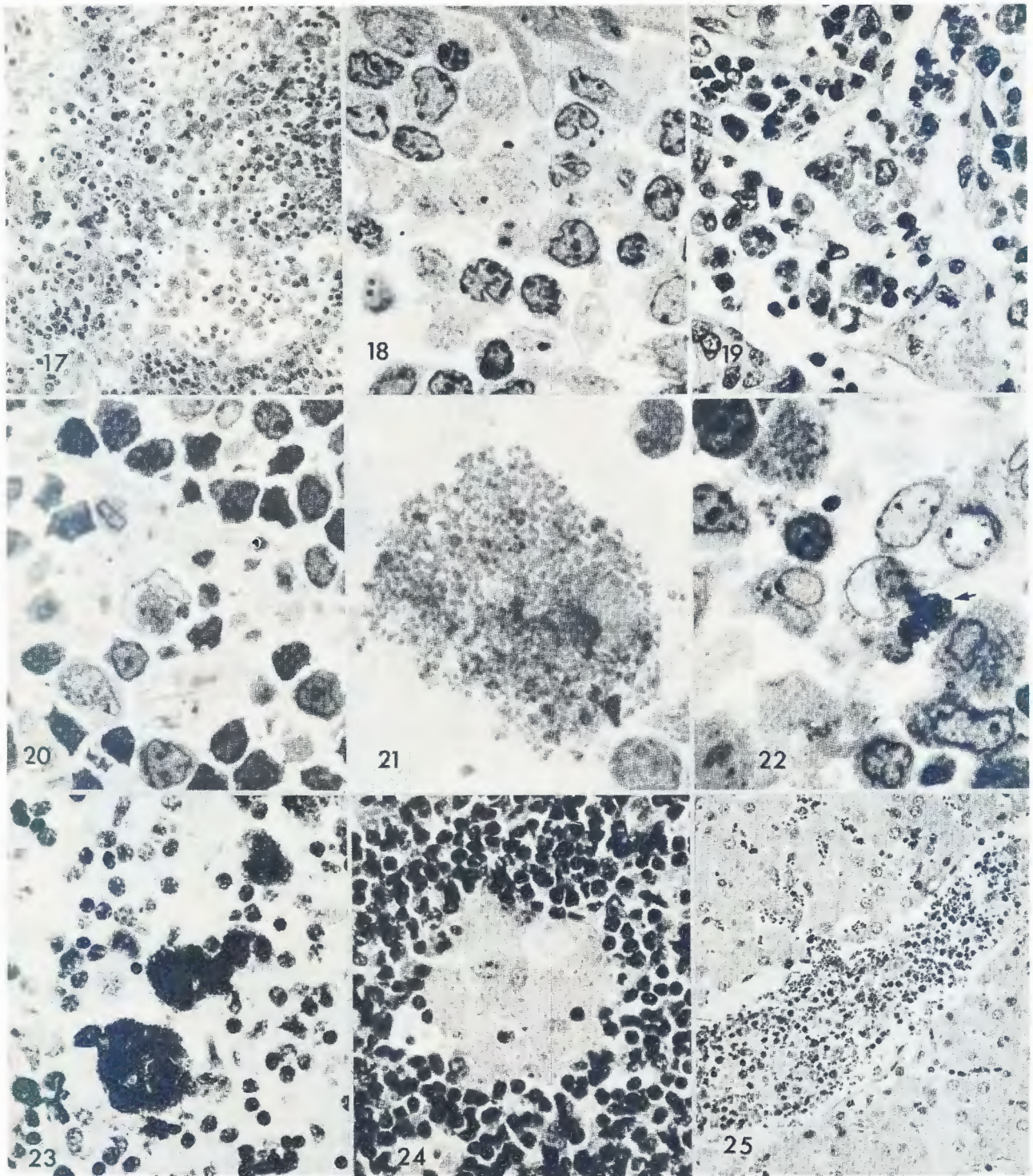


PLATE 2 Photomicrographs of lymph nodes (17 to 20 and 22 to 24) and liver (25) sections and an impression smear of a lymph node (21). Numbers 17, 18, 20 and 22 are Araldite sections stained with toluidine blue, Numbers 19, 24 and 25 are paraffin sections stained with HE, Number 23 is a paraffin section stained with Giemsa and Number 21 is from an impression smear stained with Giemsa. 17. Sinus histiocytosis,  $\times 200$ . 18. Macrophages and several extracellular colonies in medullary sinus,  $\times 1200$ . 19. Erythrophagocytosis in renal node,  $\times 500$ . 20. Intracellular colony and initial body in process of sub-division in same cell,  $\times 1200$ . 21. Large granulated reticulum cell,  $\times 1200$ . 22. Macrophages, extracellular (top left) and intracellular (bottom right) rickettsial colonies and pigment (arrow) in medullary sinus,  $\times 1200$ . 24. Islet of large granulated reticulum cells,  $\times 500$ . 25. Leucocytosis of portal vessel,  $\times 200$ .

actively phagocytosing macrophages occupied the medullary sinuses and constituted the predominating cell type here.

From the 4th post-infection day the reaction of the lymph nodes to the presence of the parasite was characterised by a widening and hypercellularity of the medullary sinuses. Activated reticulo-endothelial cells of the sinuses were prominent and largely responsible for the increase in the number of cells [Plate 2 (17)]. Macrophages with inflated nuclei undergoing necrosis and cellular and nuclear debris became more abundant as the course approached the onset of the febrile reaction. Erythrophagocytosis [Plate 2 (19)] in the medullary sinuses of most lymph nodes except those of the mesentery was seen during the febrile reaction but never before that.

In brain smears the earliest typical rickettsial organisms were observed in the cytoplasm of endothelial cells on the seventh post-infection day in one of two sheep sacrificed on this day. The febrile reaction commenced on this day in this particular animal. A few small rickettsial colonies consisting of granules of the same size, shape and colour as those observed free and intracellularly in lymph nodes, were seen in the cytoplasm of some endothelial cells [Plate 1 (15)]. Rickettsial colonies were never detected in the endothelial cells before the commencement of the fever. Towards the terminal stages of the febrile reaction marked pleomorphism of the organisms in the brain was evident. The number and size of rickettsial colonies and the size of the organisms varied considerably, but, in general, there was an increase in the number and size of colonies as well as in the size of the individual organisms as the febrile reaction progressed. Colonies with medium to large organisms were usually quite small [Plate 1 (16)] and consistently inferior in size to those consisting of the smallest rickettsiae which often parasitized considerable lengths of capillaries. In some cases both small, intermediate and large colonies and organisms occurred in the same case, while in others either small or larger organisms in small or larger colonies predominated. Similar variation was observed in brain smears of natural fatal cases. The pleomorphism was also evidenced by the shape of the rickettsiae, which varied from round, oval or ringform to horseshoe-shaped organisms. This variation in shape was seen only in case of medium and large organisms and only at the end of the febrile reaction.

As shown in Table 1 citrated blood and lymph node suspensions, from two sheep sacrificed 7 and another sacrificed 4 days after infection, which were inoculated separately into six heartwater susceptible sheep, produced heartwater in each of the six animals. The diagnosis was confirmed by the examination of brain smears which were positive in each case. A similar attempt 44 and 64 days after recovery from clinical experimental heartwater did not result in transmission.

No differences in the results obtained were noted between the two different strains of *C. ruminantium* used, or between the artificially infected sheep and the naturally infected cattle and goats.

#### DISCUSSION

While it has been accepted until now that *C. ruminantium* exclusively parasitizes vascular endothelial cells, their intra- and extracellular existence in lymph nodes in artificially and naturally infected animals has been observed microscopically in this study. Their presence in lymph nodes has been proved by subinoculation of suspensions.

Equally significant is the fact that *C. ruminantium* can be demonstrated in lymph nodes several days prior to their appearance in brain endothelial cells. This finding is incompatible with the hitherto accepted view advanced by Cowdry (1926) and Jackson & Neitz (1932) that the multiplication of these organisms takes place only in vascular endothelium. This study suggests that *C. ruminantium* initially replicates in reticulum cells of lymph nodes from which they are probably eventually released into the efferent lymph and from there into the blood stream. Only then do the organisms apparently enter vascular endothelial cells throughout the body where they undergo multiplication, presumably by binary fission. This view is supported by the fact that blood and lymph nodes are capable of transmitting the infection at least 1 to 3 days before brain capillaries are parasitized. It is therefore believed that primary development and replication take place in lymph nodes with consequent metastasis to vascular endothelial cells where further multiplication takes place.

Some knowledge was gained on the mode and sequence of rickettsial replication in lymph nodes. It would appear that following the phagocytosis of infectious rickettsiae by reticulum cells, vacuoles develop in the cytoplasm of these cells. Vacuole formation is apparently a regular feature of intra-cellular growth of rickettsiae of other species and has been described in guinea-pig testis (Linsz, Stelzner & Urbach, 1969) and monolayer cultures of chick embryo entodermal cells (Weiss & Pietryck, 1956) infected with *Coxiella burnetii* (Derrick, 1939) and in the case of *Rickettsia prowazekii* (Rocha-Lima, 1916) cultured in L cells (Kordová, 1964).

How each "initial body" subsequently develops into a clump of rickettsial granules could not be determined. The agglomeration of several clumps of simultaneously developing "initial bodies" in a cell appears to produce an endoplasmic rickettsia-like colony.

There is a close similarity in size and morphology between the endoplasmic colonies and free rickettsial colonies, both of which are demonstrable in medullary sinuses and the former also in the cortical parenchyme and medullary cords. It may therefore be deduced that, subsequent to intracellular growth, the colonies of organisms are extruded *in toto* from host cells which probably have undergone necrosis.

Pigment granules, closely associated with and similar in size and shape to rickettsia-like organisms but staining dark-blue with Giemsa, appear in both intra- and extracellular rickettsial colonies. They are indistinguishable from the granules which finally occupy the cytoplasm of large granulated reticulum cells and which histochemically have been proved to be lipofuscin.

The close association between lipofuscin granules and the suspected rickettsiae leads one to suspect that the formation of this pigment may be related to the organisms which, through the action of lysosomes, undergo degradation within phagosomes of the host cell. Novikoff (1961) postulated that lipofuscin granules may be considered as altered lysosomes in which oxidised cephalin and other materials have accumulated. If rickettsiae were subject to the detrimental action of lysosomes, their survival would depend on whether the endoplasmic colony is extruded in time to avoid the destructive action of lysosomes. The timely extrusion of infectious colonies is probably facilitated by necrosis of host cells, which explains the presence in lymph node sinuses of necrotic macrophages and cellular debris. This and other factors which determine the balance between survival of the host cell with its organelles re-

TABLE 1 Results of transmission attempts to susceptible sheep and occurrence of *C. ruminantium* in lymph nodes and brain squabs smears of animals experimentally and naturally infected in relation to interval between infection and slaughter

Animal	Interval between infection and slaughter (days)	Period of febrile reaction before slaughter (days)	Transmission attempts to susceptible sheep		Strains of <i>C. ruminantium</i>	Suspected rickettsial developmental stages in lymph nodes				Brain smears	
			Blood	Lymph nodes		Vacuolar reticulum cells	Intra-cellular rickettsiae	Extra-cellular rickettsiae	Large granulated reticulum cells	Colonies	Organisms
Experimental sheep	S1	3	+	+	Rust de Winter	+	+	—	—	—	—
	2	4	+	+	Rust de Winter	+	+	—	—	—	—
	3	4	+	+	Onderstepoort	+	+	—	—	—	—
	4	5	+	+	Rust de Winter	+	+	+	—	—	—
	5	7	+	+	Rust de Winter	+	+	+	+	+	Small
	6	7	+	+	Onderstepoort	+	+	+	+	+	Small
	7	10	+	+	Rust de Winter	+	+	+	+	+	Small
	8	13	+	+	Rust de Winter	+	+	+	+	+	Medium sized
	9	14	+	+	Rust de Winter	+	+	+	+	+	Small
	10	14	+	+	Rust de Winter	+	+	+	+	+	Small
	11	16	+	+	Onderstepoort	+	+	+	+	+	Small
	12	18	+	+	Rust de Winter	+	+	+	+	+	Small
	13	44	Neg	Neg	Rust de Winter	+	+	+	+	+	Small & large
	14	64	Neg	Neg	Onderstepoort	+	+	+	+	+	Small & large
Natural cases											
Bovine B1						+	+	+	+	Numerous, small	Large
B2						+	+	+	+	Numerous	Small & medium sized
Caprine C1						+	+	+	+	Numerous	Medium to large
C2						+	+	+	+	Numerous small & large	Small to large

sponsible for the breakdown of ingested microbes and the development and extrusion of rickettsiae may yet prove to be important in the pathogenesis of the disease.

The origin of large granulated reticulum cells may possibly be explained by the failure of developing rickettsiae to escape from parasitized reticulum cells, subsequent multiplication of the organisms and hypertrophy of the cell. Subject to the action of lysosomes the organisms are degraded and presumably replaced by lipofuscin granules. Although these cells persist for months after the clinical disease and their cytoplasm retains their dense granular structure, it is doubtful if any organisms survive, as suspensions of lymph nodes containing these cells from two recovered sheep failed to produce heartwater when inoculated in susceptible animals 44 and 64 days after infection. Although these large lipofuscin containing cells may regularly be demonstrated in the lymph nodes of sheep suffering and recovered from heartwater, they may not always be related to this disease, as cells which have some resemblance to them occur in the nodes of sheep dying from other causes.

In heartwater leucocytosis is the characteristic histopathological lesion described by Steck (1928) in sections of the liver, kidneys and brain. The presence in blood vessels and sinusoids [Plate 2 (25)] of macrophages which are indistinguishable from those seen in lymph node sinuses, suggests that the cells chiefly responsible for the leucocytosis may originate from lymph nodes reacting to developing *C. ruminantium*. To prove this it would be necessary to mark these cells. However, their activation in the presence of growing rickettsiae strongly points to the lymphadenitis as the source of the leucocytosis rather than the infiltration of macrophages into the lymph nodes and blood from another organ. There is evidence that the activation and proliferation of the sinus and littoral histiocytes is elicited by the intracytoplasmic rickettsial growth on the one hand and by the phagocytosis of liberated organisms by these cells on the other.

The histopathology of the lymph nodes in heartwater is characterised by sinus histiocytosis, effacement of the cortical lymph follicles and the presence of large granulated reticulum cells singly or in islets in the region of the cortico-medullary junction and cortex. Although all lymph nodes are affected to some extent during the course of the disease, differences in the degree and nature of their involvement exist at different stages. Proliferating endoplasmic and extracellular rickettsia-like colonies are particularly prevalent in the mesenteric nodes and somewhat rare in the superficial nodes. The occurrence of large granulated reticulum cells coincides with that of proliferating organisms. If *C. ruminantium* has in fact a predilection for the mesenteric nodes because the afferent lymph supply of these glands favours intracellular conditions for this parasite, it might be of value to study the biochemical conditions existing in these glands before attempts are made to grow this organism artificially.

The extreme pleomorphism of rickettsiae in brain endothelial cells is a salient feature of heartwater. The predominance early in the febrile reaction of small to large colonies with organisms equal in size to those en-

countered in lymph node cells and their definite trend to enlarge and adopt ring-, bacillary and cocobacillary forms towards the end of the febrile reaction and at the time of death may point to multiplication at this site as well.

#### SUMMARY

Intra- and extracellular structures which are considered to be developmental stages of *C. ruminantium* are described in the lymph nodes of ruminants infected with this parasite. These organisms occur in the lymph nodes several days before typical colonies of *C. ruminantium* can be demonstrated in endothelial cells of the brain.

Subinoculation of lymph gland suspensions obtained from infected sheep before the onset of the febrile reaction resulted in transmission of the disease and proved the presence of the organisms in these organs.

It is thought that the replication of the organisms in lymph node reticulum cells and macrophages starts by the appearance of one or several "initial bodies" in vacuoles in these cells. These bodies subdivide to form intracytoplasmic rickettsia-like colonies which are eventually extruded from cells undergoing necrosis.

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