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THE DEVELOPMENT OF *COWDRIA RUMINANTIUM* IN NEUTROPHILS*

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ABSTRACT

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The sequential development of *C. ruminantium* (Kwanyanga and Kümm isolates) was followed in caprine leukocyte cultures by light microscopy, direct immunoflourescent microscopy (DFA), indirect immunoflourescent microscopy (IFA) and transmission electron microscopy (TEM). During the febrile response, one to several small cocci, large ring forms or rods were observed in neutrophils in blood smears and cytopreparations of neutrophil fractions using Diff Quik stain, Giemsa stain, DFA and TEM. One to several *C. ruminantium* colonies were seen in up to 35 % of neutrophils maintained *in vitro* for 18 h to 5 days.

The organisms were located in neutrophil phagosomes by TEM and were enveloped by two trilamellar unit membranes. Initially, *C. ruminantium* was tightly enclosed within phagosomes. At 20 h of incubation, organisms were frequently observed undergoing binary fission within enlarged phagosomal vacuoles. At later time periods, neutrophils harboured fully formed colonies (morula) containing numerous organisms. An occasional *C. ruminantium*-infected macrophage (Kümm isolate), and an occasional infected eosinophil (Kümm and Kwanyanga isolate) were found.

INTRODUCTION

Heartwater, an often fatal rickettsial disease of both wild and domestic ruminants, caused by *Cowdria ruminantium*, is widespread in sub-Saharan Africa and has recently been confirmed on 3 Caribbean islands (Barré, Camus, Birnie, Burridge, Uilenberg & Provost, 1984). The potential introduction of heartwater into the United States represents a very serious disease threat to both domestic livestock (cattle, sheep and goats) and wildlife ruminants (deer, antelope, big horn sheep) (Uilenberg, 1982; Logan, Endris, Birnie & Mebus, 1985).

Conflicting reports as to what cell types *C. ruminantium* is associated with in the circulating blood have long been unresolved (Uilenberg, 1983). Heartwater researchers have been puzzled that *C. ruminantium* could be seen in Giemsa-stained endothelial cell smears from the brain and jugular vein but not in leukocytes in blood smears. Jackson & Neitz (1932), have observed ring stages free in the plasma of blood smears and suspicious bodies in mononuclear cells.

Separation of the blood of heartwater infected animals into its various fractions and injection of the fractions into susceptible animals had also failed to resolve which blood cells carry *C. ruminantium*. White blood cell fractions were shown to be infectious (K. E. Weiss, personal communications, in Du Plessis, 1970; Ilemobade, 1976; Ilemobade & Blotkamp, 1978; Uilenberg, 1983); the red blood cell fraction was infectious (Fawi, Karrar, Obeid & Campbell, 1977); both fractions were infectious (Alexander, 1931); as was plasma with thrombocytes (Ramisse & Uilenberg, 1971; Uilenberg, 1983). The methods used differed greatly and many of these studies were not repeated.

Several researchers have tried to propagate *C. ruminantium* in primary cell cultures but without success (Ramisse, 1971, 1972; Uilenberg, 1983). Haig (1955) reported that eggs were infectious 9 days after inoculation, but serial passage of *C. ruminantium* in eggs failed. The source of the organisms and route of inoculation were not reported. Ramisse & Uilenberg (1971) were unable to detect *C. ruminantium* after its inoculation into the yolk sac of embryonated eggs. Also, their attempts at leukocyte culture failed. Ramisse (1971, 1972) was unsuccessful in propagating *C. ruminantium* in primary cells from the ovine foetus (amnion, choroid plexus, skin, spleen, stomach and testes) or adult ovine cells (peritoneal macrophages and spleen). Andreasen (1974) reported that primary cell cultures of *Amblyomma hebraeum* and *Amblyomma variegatum* nymphae infected sheep 9 days after cultures had been inoculated with blood containing *C. ruminantium*. Jongejan, Van Winkelhoff & Uilenberg (1980) were able to maintain infected primary kidney cell cultures derived from *C. ruminantium*-infected goats for 13 days, but saw no colonies in these cultures. Uilenberg (1983) did not find buffy coat cultures derived from *C. ruminantium*-infected goats to be infectious for goats, nor were *C. ruminantium* organisms observed. His negative results led him to believe that circulating macrophages and monocytes were not the cells that carried *C. ruminantium*. Sahu, Dardiri & Wool (1983), on the other hand, suggested that mononuclear cells in buffy coat cultures were infected, but goats were not injected with the cultured material. Logan, Quintero, Whyard & Mebus (1985, a,b) reported the association of 4 isolates of *C. ruminantium* in the blood of goats and buffy coat cultures with granulocytes, in particular neutrophils. More recently, Sahu (1986) confirms that very few mononuclear cells were infected in buffy coat cultures derived from *C. ruminantium*-infected cattle, sheep and goats. He also detected infected neutrophils in buffy coat cultures and reports having seen colonies in blood smears from cattle as early as 24 h following inoculation with *C. ruminantium*.

From previous work with *Ehrlichia* tribe rickettsiae (Gribble, 1969; Nyindo, Ristic, Huxsoll & Smith, 1971), one would expect to readily find *C. ruminantium* in blood or in buffy coat cultures. The purpose of these studies was to identify which cells carry *C. ruminantium* in the blood and to determine if this cell could be propagated *in vitro*. Such cells could be used to infect a secondary cell line, or to develop a diagnostic test to detect antibodies specific for *C. ruminantium* in the sera of animals (Logan *et al.*, 1985 a,b).

MATERIALS AND METHODS

Animals

Three Nubian goats ranging in age from 1–5 years were housed in animal rooms at 22 °C and given free

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choice of pelleted alfalfa feed, water and salt-mineral blocks.

Cowdria ruminantium *source*

Amblyomma hebraeum nymphae infected as larvae with the Kwanyanga isolate were provided by P. K. I. MacKenzie[1]. *A. hebraeum* (Spesbona isolate) nymphae, infected as larvae with the Kümm isolate, were provided by J. L. du Plessis[2].

Method of infection

Goat 1 was inoculated intravenously with 4 mℓ of a mouse liver homogenate (*C. ruminantium*, Kwanyanga isolate, 54th passage in Swiss mice) (MacKenzie & Van Rooyen, 1981). A second Kwanyanga isolate infection was initiated in Goat 2 by intravenous inoculation of 10 mℓ of a blood stabilate collected in sodium EDTA and maintained in liquid nitrogen for 1 071 days, as previously described (Logan *et al.*, 1985a). Goat 3 was infected with the Kümm isolate by tick feeding (15 adult female and 15 adult male *A. hebraeum*), as previously described (Logan *et al.*, 1985a).

Collection of blood

Venous blood was collected during the febrile response. Sodium heparin or ACD (8 g citric acid, 22 g sodium citrate, 24,5 g glucose per ℓ of distilled H_2O) were used as anticoagulants. Blood that was collected without anticoagulant was placed in 100 mℓ serum vials and defibrinated with glass beads (Hess & De Tray, 1960).

Separation of leukocytes

Goat 1

Sixty mℓ of heparinized blood from Goat 1 was mixed with an equal volume of RPMI medium with L-glutamine[3] supplemented at the time of culture with an additional 1 mM L-glutamine, 20 mM HEPES buffer, pH 7,3[3] 10 % foetal bovine sera[4] overlayed on Ficoll-Paque[5] and centrifuged for 30 min at 700 × g. The mononuclear fraction was collected according to the protocol of Liggitt (1983). In addition, the fraction at the interface (granulocytes) above the packed red cells was collected. These cells were centrifuged at 200 × g for 10 min and the red cells were lysed in 0,2 % NaCl followed by 1,2 % NaCl, as described by Carlson & Kaneko (1973).

Defibrinated blood was centrifuged for 30 min at 850 × g, the buffy coat was collected, and red cells were lysed. The cells were centrifuged at 200 × g and washed twice with RPMI medium. The cell count was adjusted to 1×10^6, using a medium consisting of one part RPMI with 10 % foetal bovine serum, one part filtered plasma from the infected goat, and one part filtered conditioned medium from a 4-day L-929 cell culture[6]. Eight, 4 chamber Lab-Tek tissue chamber slides[7] were seeded with defibrinated blood and leukocytes collected from Ficol-Paque. The remaining cell suspension was placed in 25 cm^2 tissue culture flasks[8].

Goat 3

Ninety mℓ of defibrinated blood and 120 mℓ of heparinized blood were collected from Goat 3. Leukocytes were separated from the blood as described above except that Gey's solution was used for red cell lysis (Mishell & Shiigi, 1980). One half of the final cell suspensions was saved for electron microscopy and the remaining cells were resuspended at 1×10^6 cells/mℓ in 3 parts RPMI with 10 % FBS and 10 % homologous-infected goat plasma and one part conditioned medium from L929 cells. Approximately 0,2 mℓ of cell suspension was placed in 8, 4-chamber Lab-Tek tissue culture slides and 6 mℓ in 25 cm^2 tissue culture flasks.

Goat 2

One hundred and eighty mℓ of blood was collected from Goat 2 in ACD. The goat was subsequently treated by intramuscular injection of 20 mg/kg oxytetracycline[9]. Neutrophils were collected from the blood, using a modification of the protocol (courtesy of David Slauson, Cornell University) of Carlson & Kaneko (1973). The blood was divided into 35 mℓ aliquots in 50 mℓ polypropylene plastic centrifuge tubes[10] and centrifuged for 15 min at 700 × g. The plasma, buffy coat and upper surface layer of the red cells were discarded. The red cells in the remaining fraction were lysed by the addition of 20 mℓ of cold distilled H_2O. After gentle mixing for 30 s, 10 mℓ of cold 2,7 % NaCl was added, mixed, and the volume brought to 50 mℓ with cold Hanks Balanced Salt Solution (HBSS) lacking calcium and magnesium, pH 7,2[11]. The cell suspension was centrifuged for 10 min at 200 × g and the supernatant discarded. The hypotonic lysis was repeated once, the cells were washed in HBSS and re-suspended in 90 mℓ of RPMI supplemented with 15 % conditioned L-929 cell medium, 10 % FBS, HEPES buffer, pH 7,3, and 1-glutamine. The cell suspension was placed in 75 cm^2 tissue culture flasks[8]. The cells were incubated at 37 °C in a humidified atmosphere of 3 % CO_2-97 % air.

Monitoring the cultures

Following leukocyte separation, the final cell suspension was monitored by cytocentrifugation[12] and stained either with Diff-Quick[13], Giemsa[14] or DFA. At approximately 24 h intervals, cells were examined after Diff-Quick, or Giemsa staining. Cytocentrifuge preparations were periodically monitored with DFA and IFA (Logan, Whyard, Quintero & Mebus, unpublished data, 1986).

Electron microscopy

On the 4th day of culture a pool of cells from Goat 1 was sedimented at 200 × g and fixed overnight in Karnovsky fixative (pH 7,2) (Karnovsky, 1965). The cells were washed in cold 0,1 M cacodylate buffer (pH 7) with 0,12 M CaCl. Samples were post-fixed with 1 % osmium tetroxide, dehydrated in a graded series of ethanols and propylene oxide and embedded in Effapoxy resin[15] by a rapid preparation procedure (Bencosme & Tsutsumi, 1970). Ultra-thin sections were stained with saturated aqueous uranyl acetate and lead citrate and were examined by electron microscopy[16].

Leukocytes from Goat 3 were fixed immediately following separation from the blood, and leukocytes from culture were fixed at 20 h, 42 h and 60 h. The fixative used was 2,5 % paraformaldehyde, 5 % glutaraldehyde and 0,03 % trinitrophenol in 0,1 M cacodylate buffer (pH 7,4) (Rikihisa, Perry & Cordes, 1985). The cells were washed in 0,2 M cacodylate buffer (pH 7,4) and the samples were processed as described above.

[1] Coopers, South African (Pty) Ltd.
[2] Veterinary Research Institute, Onderstepoort
[3] Flow Laboratories, McLean, VA
[4] Hyclone Laboratories, Logan, VT
[5] Pharmacia Fine Chemicals, Piscataway, N.J.
[6] L-929 cells courtesy Walter Reed Army Medical Research Institute
[7] Niles Scientific, Naperville, IL
[8] Costar, Cambridge, MA
[9] Liquamycin LA-200, Pfizer, New York, NY
[10] Falcon Becton Dickinson Labware, Oxnard, CA
[11] Sigma Chemical Co. St. Louis, MO
[12] Cytospin, Shandon Southern Instruments, Inc. Sewickley, PA
[13] American Scientific Products, Edison, NJ
[14] Harleco, American Scientific Products, Edison, NJ
[15] Ernest F. Fullam Inc., Schenectady, NY
[16] Model EM 201, Philips Electronic Inc., The Netherlands

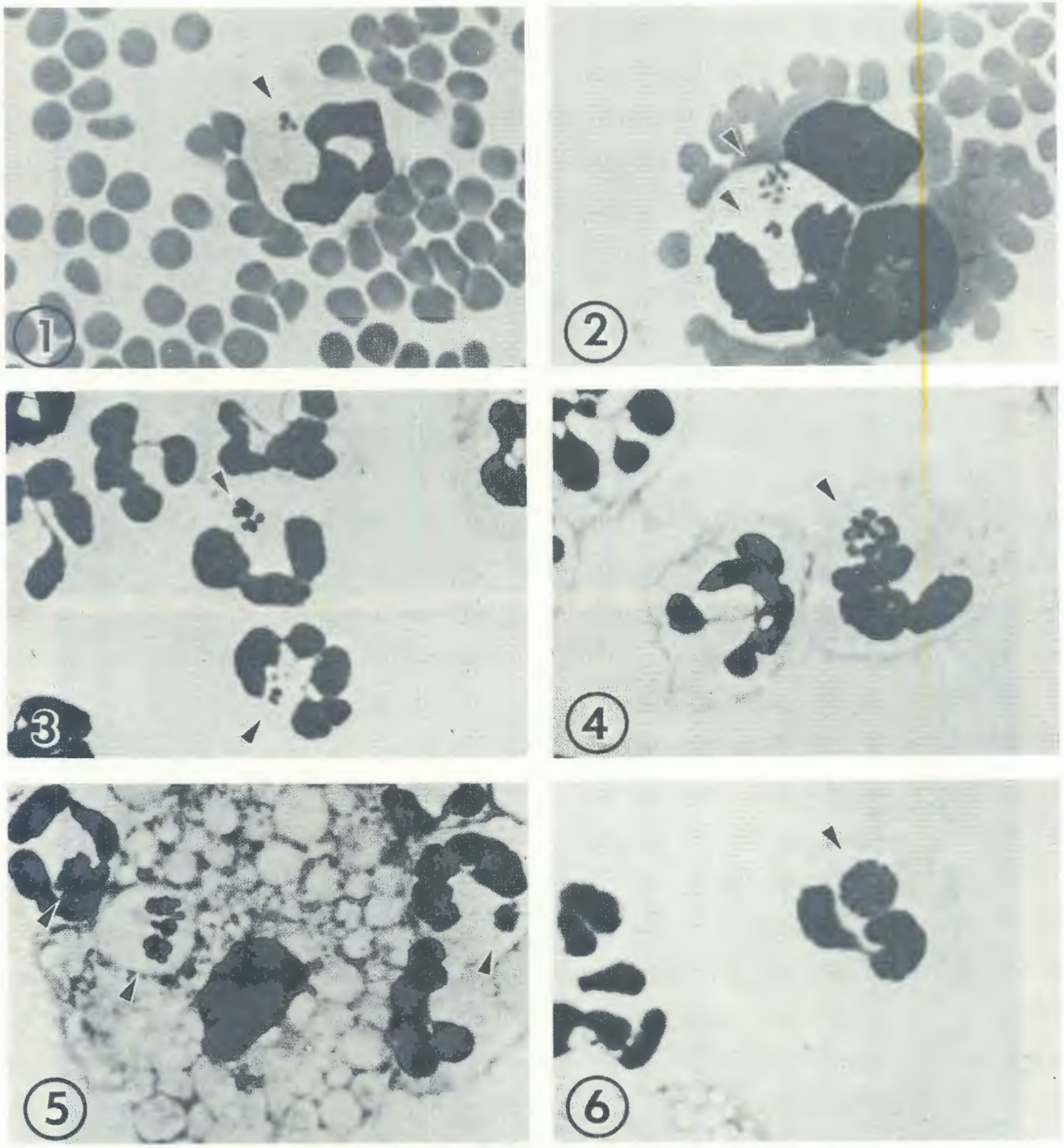


FIG. 1 *C. ruminantium*-infected neutrophil in a blood smear. Arrow indicates 3 organisms. Goat 3, Kümm isolate. Diff-Quik × 2000

FIG. 2 *C. ruminantium*-infected neutrophil in a blood smear. Arrows indicate 2 colonies. Goat 2, Kwanyanga isolate. Diff-Quik × 2000

FIG. 3 *C. ruminantium*-infected neutrophils maintained in culture for 18 h. Arrows indicate colonies. Goat 3, Kümm isolate. Diff-Quik × 2000

FIG. 4 *C. ruminantium*-infected neutrophil maintained in culture for 60 h. Arrows indicate large ring form organisms. Goat 3, Kümm isolate. Diff-Quik × 2000

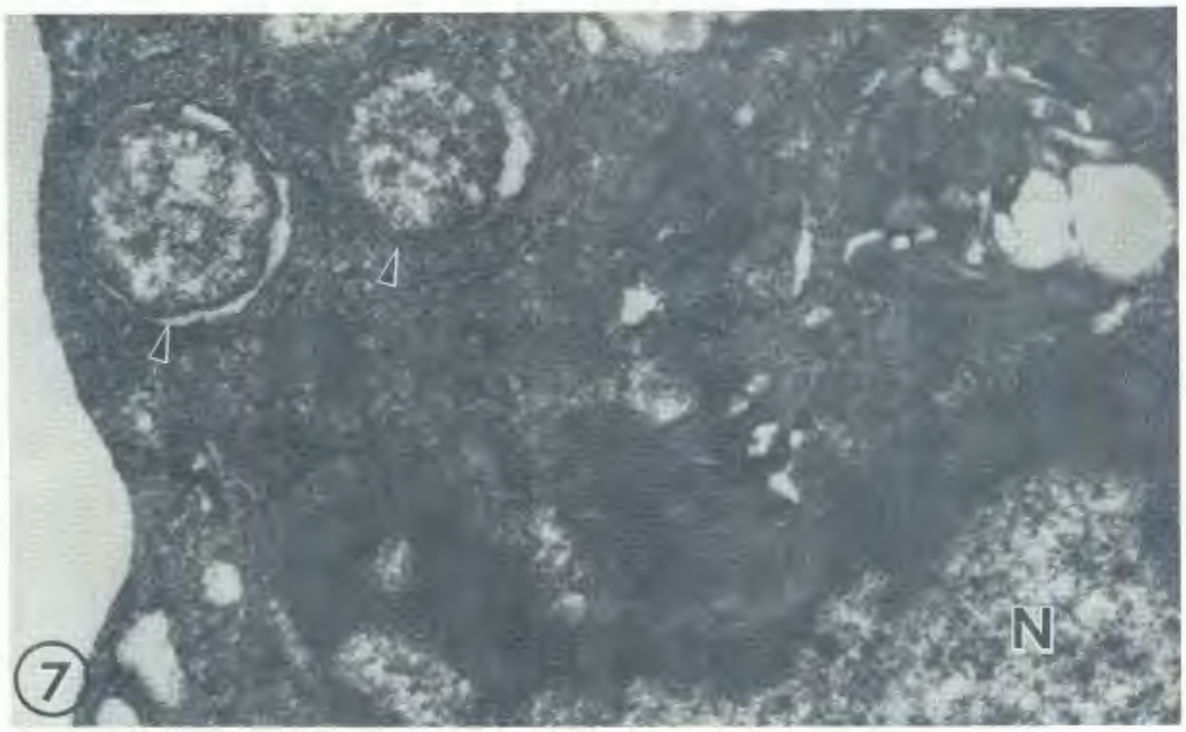
FIG. 5 *C. ruminantium*-infected neutrophils and a macrophage with a large vacuole containing a colony in culture for 60 h. Arrows indicate colonies. Goat 3, Kümm isolate. Diff-Quik × 2000

FIG. 6 *C. ruminantium* colony (morula) within a neutrophil maintained in culture 72 h. Arrow indicates colony. Goat 2 (Kwanyanga). Diff-Quik × 2000

RESULTS

Animals

Goat 1 developed a fever of 40 °C on the 8th day post-inoculation (dpi); blood was collected on the 10th dpi when the temperature was 41,1 °C. The animal recovered without treatment by 15 dpi. Goat 3 developed a fever (40,8 °C) 22 dpi following the first placement of male ticks on the goat and died later that day. Leukocyte cultures and brain smears were positive for *C. ruminantium*. Goat 2 delivered 2 kids on 12 dpi. The following day its temperature was 40,8 °C and on 14 dpi it was 41,4 °C. At this time blood was collected for culture and the animal was treated once with oxytetracycline.



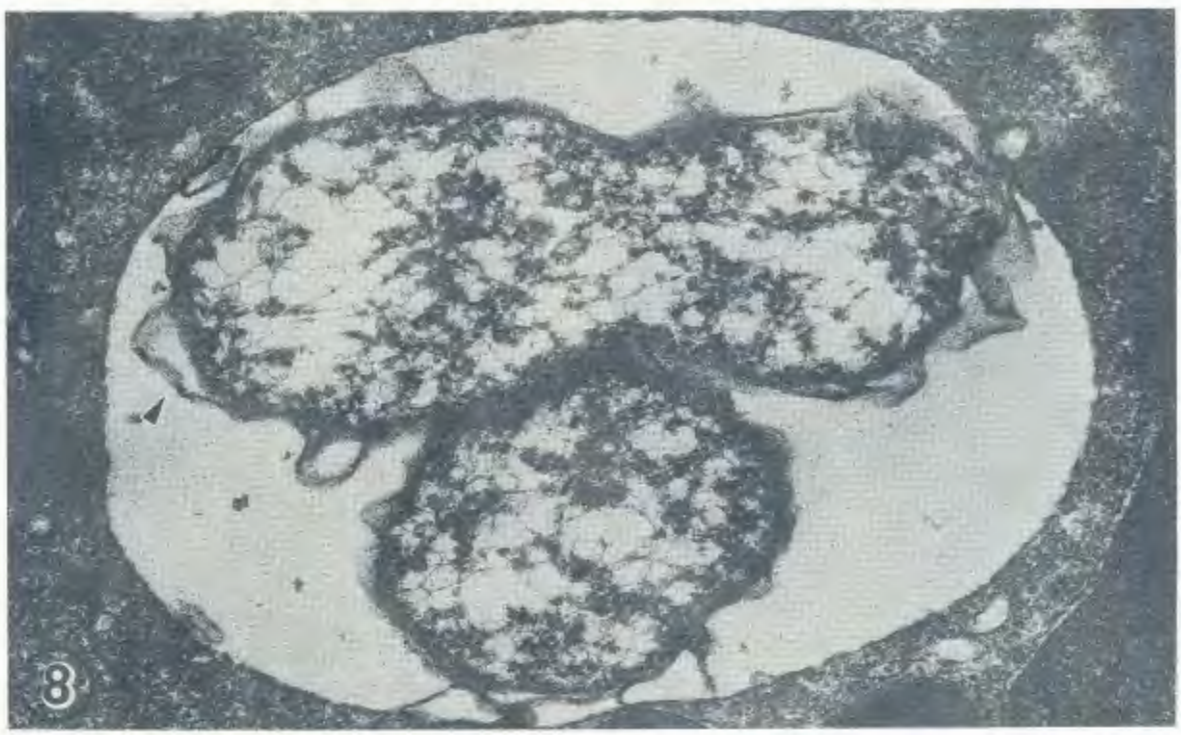


FIG. 7 *C. ruminantium* within phagosomes in the cytoplasm of a neutrophil at time of blood collection. Arrow—two organisms, N=nucleus. Goat 3, Kümm isolate. × 38 000

FIG. 8 *C. ruminantium* within a phagosome of a neutrophil after 20 h in culture. The organism on top is dividing. Arrow indicates rippled outer membrane. Goat 3, Kümm isolate. × 60 000

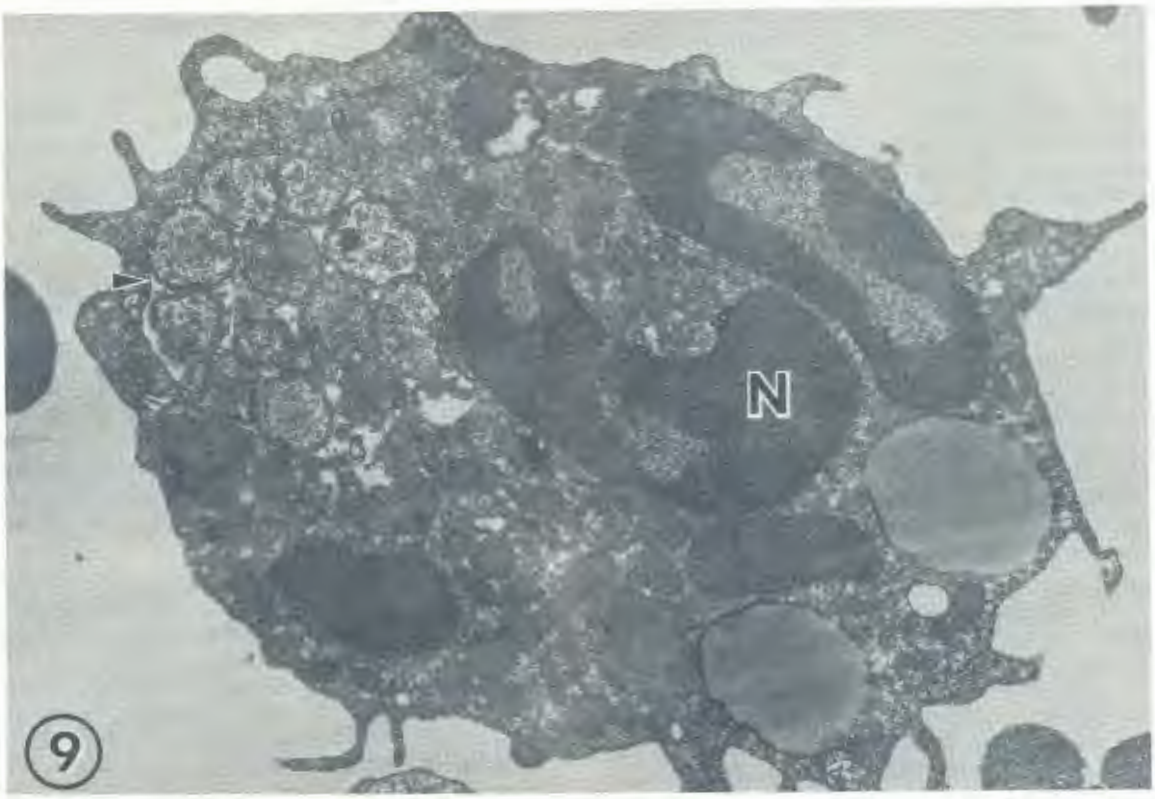
Collection of leukocytes

Leukocyte cultures collected from Ficoll-Paque, defibrinated blood (Goats 1, 2), and red cell fractions (Goat 3), had *C. ruminantium* colonies in neutrophils.

Monitoring of cultures by light microscopy

The superior fixation and staining with Diff-Quik stain made detection of organisms in blood smears and cytopreparations much easier than with Giemsa stain. The use of cytocentrifugation to monitor leukocyte cultures provided superior cell morphology and more clearly outlined colonies than smears made from leukocyte fractions. Likewise, the Lab-Tek system provided a simple method for directly examining cell monolayers.

C. ruminantium organisms were detected in circulating neutrophils, as shown for the Kümm isolate (Fig. 1) and the Kwanyanga isolate (Fig. 2). However, organisms were not easily found and were more frequently seen in the collection of neutrophils along the feathered edge of blood smears. Many colonies were light-pink with Giemsa stain and hard to detect. The organisms ranged from red to dark-purple with Diff-Quik stain. The



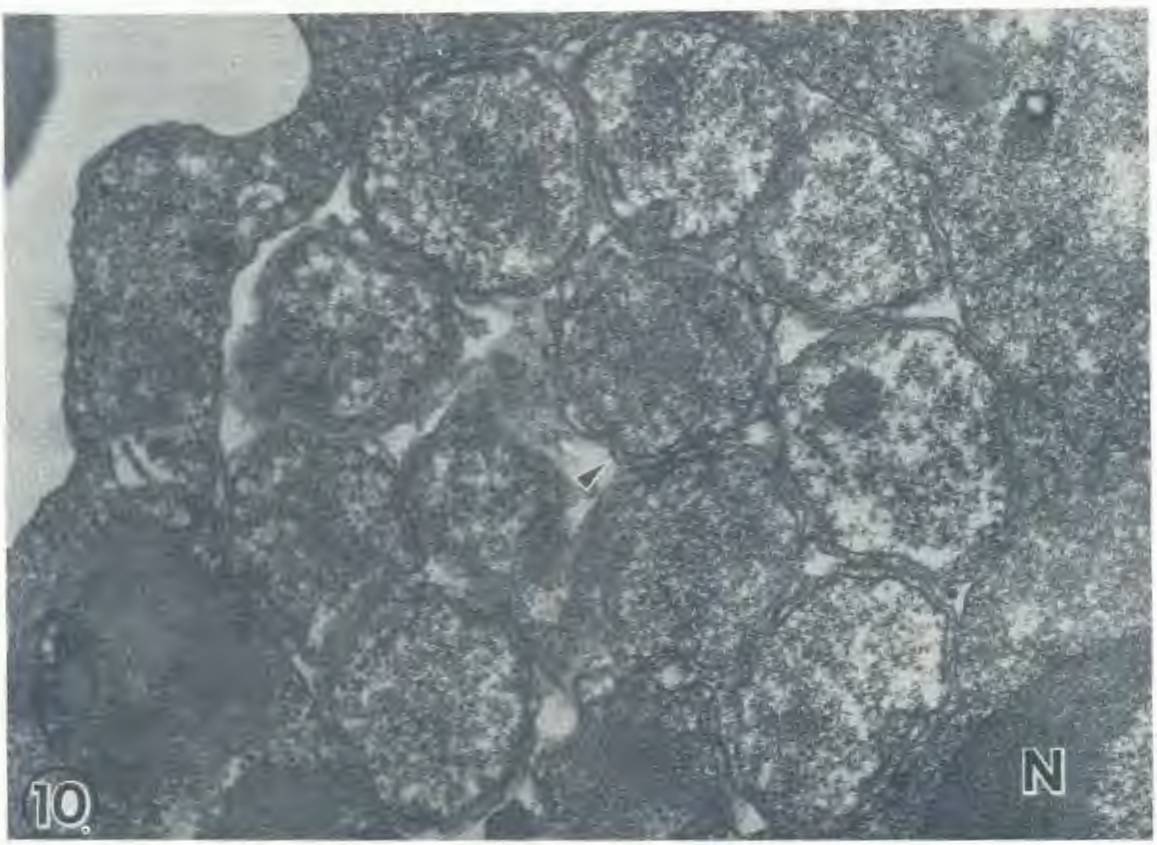


FIG. 9 Neutrophils with a fully formed colony within a phagosome after 96 h in culture. Arrow—phagosomal membrane. Goat 1, Kwanyanga isolate. × 20 000

FIG. 10 Same colony as Fig. 9 with clearly indicated (arrow) double unit membranes. Goat 1, Kwanyanga isolate. × 51 300

organisms were pleomorphic (cocci, short rods and ring forms). The larger forms (Fig. 4) often stained poorly (red) and were called ring forms. Infected neutrophils often had only 1 detectable organism, but sometimes small colonies were detected (Fig. 2). Fully formed colonies (morula) were found easily in blood smears from Goat 2. Detection of *C. ruminantium* in blood smears from febrile animals, however, is generally not easy and can be unrewarding in cases where the number of infected neutrophils is low (5 %). In blood smears, organisms were detected exclusively in neutrophils.

Leukocyte fractions consisted of lymphocytes, monocytes, neutrophils and eosinophils. Individual organisms were more easily and frequently found in neutrophils in these preparations. Although no differential cell counts were made in these studies, fewer eosinophils were observed in leukocyte fractions in *C. ruminantium*-infected animals than in leukocyte fractions collected from uninfected animals.

Often 18–24 h in culture, colonies were detected in neutrophils (Fig. 3). After 2–3 days fully formed colonies in the form of morula were easily detected in neutrophils. Approximately 35 % of the neutrophils from Goat 3 had 1 to several morula (Fig. 4, 5, 6).

Both supernatant cells and monolayers from Lab-Tek slides were examined for each time period by light microscopy. After 24–48 h incubations mononuclear cells had attached to the glass slides and formed monolayers. The supernatant fluid collected from these cultures had both macrophages and neutrophils. After 60 h, an occasional infected macrophage was detected in the supernatant of cultures from animals inoculated with the Kümm isolate (Fig. 5). At this time the neutrophils has begun to degenerate and disintegrate, and some free organisms and colonies were seen in cytopreparations. At this time, macrophages were large vacuolated cells, and many had adherent neutrophils. The macrophages appeared to be quite activated as was indicated by pronounced vacuolation with phagocytized cellular debris. Colonies were observed in a very small percentage of macrophages in cytopreparations or within the monolayer between 60 h and 8 days of culture. Organisms were not observed after 8 days in macrophages. Monolayers of macrophages were confluent and appeared healthy during the 21 days of culture. No colony was detected over a similar time period in macrophages from Goat 1 or 2 inoculated with the Kwanyanga isolate. An occasional eosinophil was observed to contain a colony. No morphological differences between the 2 isolates could be detected by light microscopy.

By light microscopy, *C. ruminantium* colonies or individual organisms were rarely seen in neutrophils in the feathered edge of blood smears. Examination of blood smears with DFA was unsatisfactory. *C. ruminantium* was detected in cytopreparations of leukocyte fractions at the time of separation by DFA. After culture, colonies were larger and easier to identify in neutrophils by DFA and IFA.

Electron microscopy

The development of *C. ruminantium* (Kwanyanga and Kümm strains) in neutrophils was observed by transmission electron microscopy (TEM). Both methods of fixation for TEM gave excellent morphological detail. Organisms were found in phagosomes of neutrophils. Each organism was surrounded by double unit membranes of equal thickness (Fig. 10). At the time of blood collection, organisms were tightly enclosed within phagosomes of neutrophils (Fig. 7). Following 20 h of culture organisms were undergoing binary fission (Fig. 8). They elongated into rod forms and the outer unit membrane was rippled. At later time periods with the Kümm isolate (42 h, 60 h, not shown) colonies were morphologically similar to those seen in the Kwanyanga-infected neutrophils (96 h) (Fig. 9, 10). These colonies (morulas) were fully formed and had many organisms within phagosomes.

Discussion

The failure of many workers to demonstrate *C. ruminantium* in neutrophils in primary cultures may be due to a number of factors. In the current work, frequent monitoring of cultures was critical. The examination of supernatant cells as well as adherent cells was the key to our success.

Carlson & Kaneko (1973) found that bovine buffy coats were almost exclusively mononuclear cells. The neutrophils were located within the red cell fraction when blood was centrifuged for 15 min at 1000 × g. This may explain why Fawi *et al.* (1977) found the red cell fraction to be infectious in their *C. ruminantium* studies. Ilemobade & Blotkamp (1978) stated that they collected both the mononuclear and granulocytic fractions from blood separated on Ficoll-Hypaque gradients and these pooled cells were infectious; the red cell fractions were not.

The 3 techniques used to collect leukocytes presented here were selected to demonstrate that a variety of methods can be used to detect *C. ruminantium* in neutrophils. Although it has not been reported here, we have used the Ficoll-Paque and defibrinated blood buffy coat technique on several other occasions with goats to demonstrate infected neutrophils. Colonies have been detected in caprine neutrophils from 4 isolates of *C. ruminantium* (Logan *et al.*, 1985 b,c). The first detection of *C. ruminantium* in neutrophils was in a 5 day culture of mononuclear cells collected on Ficoll-Paque. The fraction had a small number of neutrophils, a few of which had colonies. The neutrophils containing colonies were in the supernatant medium and were collected by cytocentrifugation. Colonies were not observed in mononuclear cells, collected on Ficoll-Paque on previous occasions, from *C. ruminantium* infected goats.

The largest numbers of *C. ruminantium*-infected neutrophils can be collected from the red cell fraction by the methods of Carlson & Kaneko (1973). The cells collected in this manner do not survive in culture as long as neutrophils collected on Ficoll-Paque or from defibrinated blood buffy coats because of the twice repeated hypotonic lysis employed. However, collecting neutrophils from the red blood cell fraction is now routinely used in our laboratory for antigen production. Large numbers of neutrophils can be collected from a relatively small amount of blood; cells do not have to be held longer than 2–3 days; the method is simple, inexpensive, and requires very few reagents.

Neutrophils do not characteristically attach to glass or plastic as do mononuclear cells such as macrophages; they are free in the supernatant medium (Wardley, Lawman & Hamilton, 1980; Liggitt, 1983). They are an end stage cell; and bovine neutrophils have a transit time in blood of only 6,4–7,5 h (Vincent, Chanana, Cronkite & Joel, 1974). They do not divide in circulation and thus cannot be maintained in culture for more than a few days (Cline, 1975).

Typically the fluid is changed on buffy coat cultures after a few hours or up to 24 h to eliminate free floating cells (Wardley *et al.*, 1980). Most neutrophils are removed by this process. Following media changes, often 4–5 days elapse before cultures are observed again; by this time, many of the neutrophils have degenerated and colonies have been released or have broken out of cells and are free in the medium. It is probable that, at this stage, some colonies are taken up by stimulated

macrophages. Although occasionally a colony was detected in a macrophage between 60 h and 8 days, we saw no evidence that these organisms were replicating in macrophages. Instead, the monolayers became more confluent and the inclusion bodies that were suspected to be colonies disappeared. Macrophages handled in this manner are stimulated and are probably actually killing *C. ruminantium*.

The concept of the marginal pool of neutrophils sequestered in sites such as small capillaries in the lung, coupled with the newly emerging understanding of the role of neutrophils in increasing microvascular permeability in the lung, should be considered by workers interested in *C. ruminantium* (Boggs, 1975; Flick, Perel & Staub, 1980). The close association of neutrophils with endothelial cells and *C. ruminantium* in circulating neutrophils may help to explain the pulmonary oedema seen with *C. ruminantium*.

In the past, the performance of serological tests for *C. ruminantium* had been limited because of the lack of a suitable antigen. Du Plessis (1981), however, has successfully used mouse peritoneal macrophages infected with the Kümm isolate as an antigen source for the IFA test. The Kümm isolate is not typical of other isolates of *C. ruminantium* and is unique in that it has been detected in the mouse peritoneal macrophages.

Primary neutrophil cultures provide a rapid simple method of obtaining large amounts of antigen from any *C. ruminantium* isolate. Antigen preparations from infected neutrophils can be used for serologically comparing different isolates of *C. ruminantium*. Propagation of *C. ruminantium* in neutrophils may provide an alternate method to infect secondary cell lines and expedite antigen purification.

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