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Sexuality in piroplasms as revealed by electron microscopy in *Babesia microti*

(syngamy)

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ABSTRACT **Protozoa of the closely related genera *Babesia* and *Theileria* are intraerythrocytic parasites of vertebrates. They have a complex life cycle that includes development in an intermediate vector host, a tick. Whether sexual stages occur in the tick has been a subject of great controversy. The small size of the organism and the complexity of developmental stages in the gut of the tick have prevented a definitive solution of this problem. By means of a simple and straightforward although time-consuming method, it became possible to demonstrate gametes and their sexual fusion in *Babesia microti* developing in the gut of larvae of the tick *Ixodes dammini*. Tick larvae fed on hamsters infected with a human strain of *B. microti* were fixed and processed for electron microscopy. It was found that some of the parasites formed a unique structure shaped like an arrowhead. Because it was suspected that these forms might represent gametes, a search was made for pairs of parasites that were fusing and with each member of the pair emerging from a different erythrocyte. Such a fusing pair could not possibly represent a parasite undergoing division. By study of serial sections such pairs were indeed found. In every case one member of the pair of gametes had an arrowhead structure. This proves sexuality of *B. microti* and makes highly likely its existence in all members of the genera *Babesia* and *Theileria*.**

The life cycle of the piroplasms *Babesia* and *Theileria*, intraerythrocytic parasites of vertebrates, is not yet completely known. Of greatest controversy has been the problem of their sexual reproduction. For decades the opinion prevailed that they reproduce only asexually in the vertebrate host as well as in their vector, the tick (1). During the last several years, light and electron microscopic studies in *Theileria* (2–5) and *Babesia* (6–8) identified developmental stages in the gut of the vector that were similar to the *Strahlenkörper*, bodies with long rays first described and named by Koch at the beginning of this century (9). He suggested that they might represent sexual stages. Recently, unusually shaped forms were detected in thin sections of intraerythrocytic *Babesia microti* (8, 10) and their behavior led to the supposition that they might be "gametocytes," a stage that in *Plasmodium* and other Haemosporidia cannot develop further in the erythrocyte and has to be transferred to the vector where they transform into gametes.

Both of these findings, which were reported almost simultaneously—the rediscovery of Koch's *Strahlenkörper* in *Babesia* and *Theileria* in the gut of the tick, and of gametocytes in intraerythrocytic *B. microti*—needed verification, and extensive electron microscopic studies followed. They resulted in accumulation of much detailed information on the fine structure of developmental stages of *Babesia* and *Theileria* in the vector (11–20). It became increasingly difficult to use the proper terminology for these stages (21) because it was not known whether they are or are not preceded by a sexual process. To fill in the gap some researchers proposed hypothetical life cycles (15, 21). In addition, the finding of sexuality in piroplasms could have important genetic implications, as has been already suggested by some investigators (22).

The major obstacles in finding evidence of sexuality were the complexity and variety of developmental stages in the gut of the vector and the difficulty in distinguishing in thin sections whether an organism is in a state of division or whether fusion of two organisms is taking place. Recently, Mehlhorn *et al.* (17) tried to overcome these difficulties by culturing *Babesia canis in vitro*. However, their work did not bring the desired answer: they still could not distinguish fusion from fission with certainty. In the present study, an approach was used which permitted us to remove this obstacle and solve this difficult problem.

MATERIALS AND METHODS

B. microti is a rodent piroplasm that also infects man, sometimes causing serious and even fatal disease (23). The strain of *B. microti* used in this study was isolated from a human patient in 1978 and named after its donor *B. microti* Lewis. It has been maintained in hamsters by intraperitoneal injection of infected blood every 3–8 weeks. It was transmitted to ticks in July 1979 and was used for this experiment in October 1979. The tick vector *Ixodes dammini* described as a new species by Spielman *et al.* (24) was collected from white-tailed deer (*Odocoileus virginianus*) on Nantucket Island in the years 1975–1977 (25) and maintained in the laboratory by feeding them on uninfected hamsters and rabbits. The ticks were reared from eggs as described (8).

For the present work, a large number, 100–150, of tick larvae were deposited on a hamster infected with *B. microti* Lewis with 40–55% parasitemia. Ticks were removed from the hamster at 30 min after the start of feeding and thereafter at 1-hr intervals until repletion (60 hr) and also at 2-hr intervals from 1 hr to 44 hr after repletion. Infected larvae were placed in a drop of 2% or 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and the gut was dissected either *in toto* or in parts, cut into small pieces, transferred into fresh 2% or 4% buffered glutaraldehyde for 1–2 hr in ice and processed for electron microscopy as described (26).

RESULTS

New Organelles Formed. In the gut of *I. dammini* fed for about 60 hr on hamsters infected with *B. microti*, various developmental stages of the parasite were found. Most of the ingested *Babesia* were still inside the erythrocytes, and some of them had acquired new organelles. These were a cytostome and numerous microtubules extending to the outside of the main

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body in bundles of three, forming a long straight tail at the posterior end and two or more arms on the sides. After repletion or, occasionally, about 2 hr before detachment of the feeding larvae from the hamster, a striking structure appeared at the anterior end of the parasite, broad at its base and pointed at the long, protruding end, resembling an arrowhead (Fig. 1). The arrowhead organelle is stiff and dense with a complex inner structure. There is no doubt that the parasites with the arrowhead organelle, a tail, and arms represent Koch's *Strahlenkörper*. The metamorphosis described above takes place while

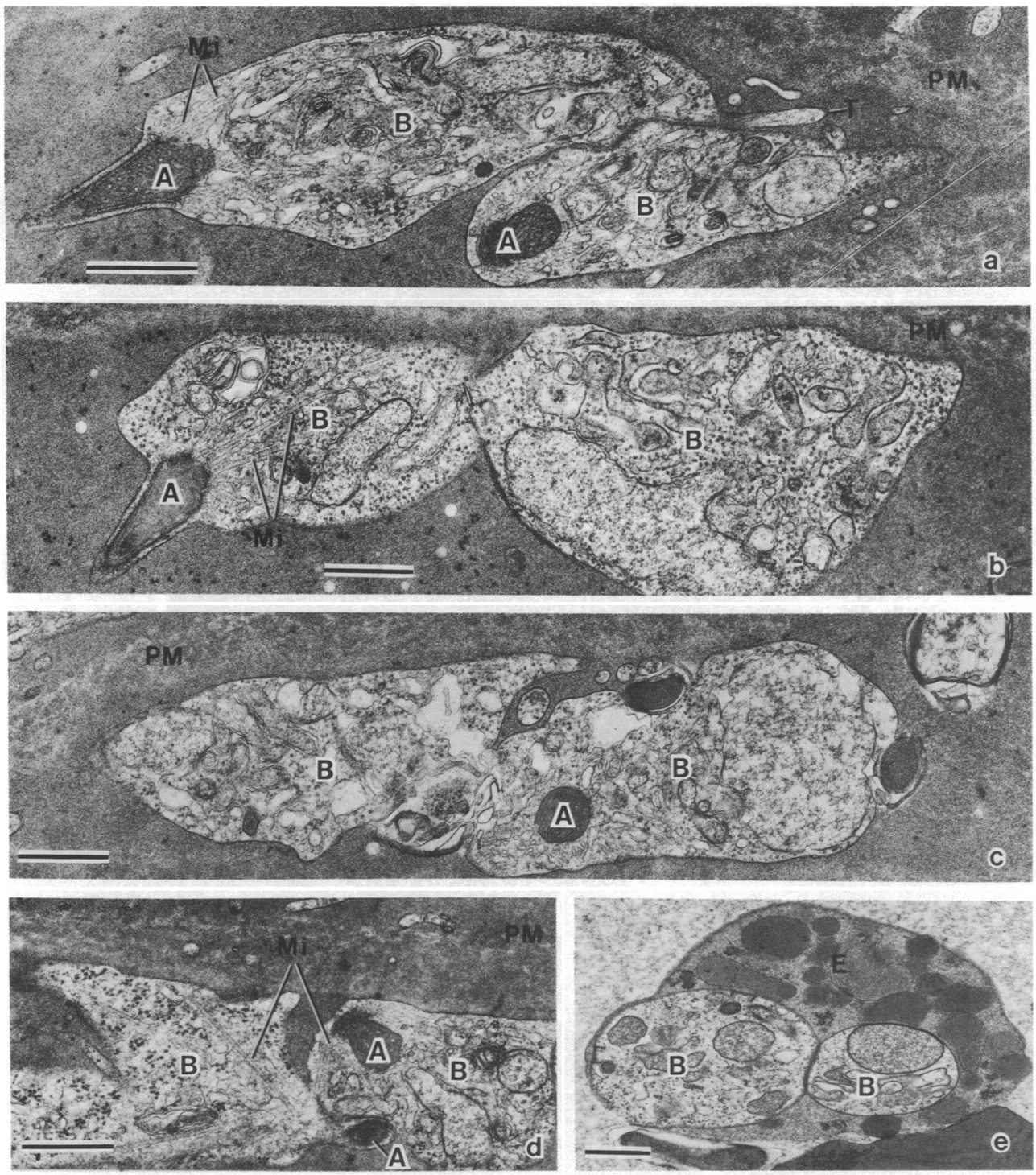

FIG. 1. (*a–d*) *B. microti* in the lumen of the gut of *I. dammini* at 14.5–20 hr after repletion, located close to the peritrophic membrane (PM). (*a*) Two *B. microti* (B), each with an arrowhead organelle (A). In the upper parasite, microtubules (Mi) radiate from the arrowhead. At the end of the body is a short tail (T). (*b*) Two parasites (B) may be in the last stage of division or at the start of fusion. One of them has an arrowhead (A) from which numerous microtubules (Mi) radiate in all directions. (*c*) Two parasites (B) which might be engaged in fusion or division. In one of them is a cross section of an arrowhead (A). (*d*) A pair of parasites (B) might be in the last stage of division or at the start of fusion. Each of them has an arrowhead (A). In the right one, the arrowhead (A) is sectioned at its base; in the left one, the protruding part of the arrowhead (A) is seen in cross section. In both parasites, numerous microtubules (Mi) extend from the arrowhead (A). (*e*) A pair of *B. microti* (B) inside an erythrocyte (E) in the lumen of the gut 1 hr after repletion. It is not known whether they represent fission or fusion. (Bars = 0.5 μm.)

Babesia is still inside the intact or partially lysed erythrocyte (8).

Fusion or Fission? Many parasites were undergoing division, which may start inside the erythrocyte and continue outside it. Often a pair of closely adjacent organisms was found in the lumen of the gut, and it is difficult to know whether they represent a divided or dividing organism or a pair of fusing parasites (Fig. 1 *a–d*). In Fig. 1*a*, both partners possess an arrowhead. Thus, the two parasites most probably represent a divided or dividing organism. This assertion is based on previous findings (26, 27) which will be described in the *Discussion*. The same can be said about Fig. 1*d*. Here two organisms are connected by a narrow strip of cytoplasm and each of the parasites has an arrowhead as seen in cross section. It is as-

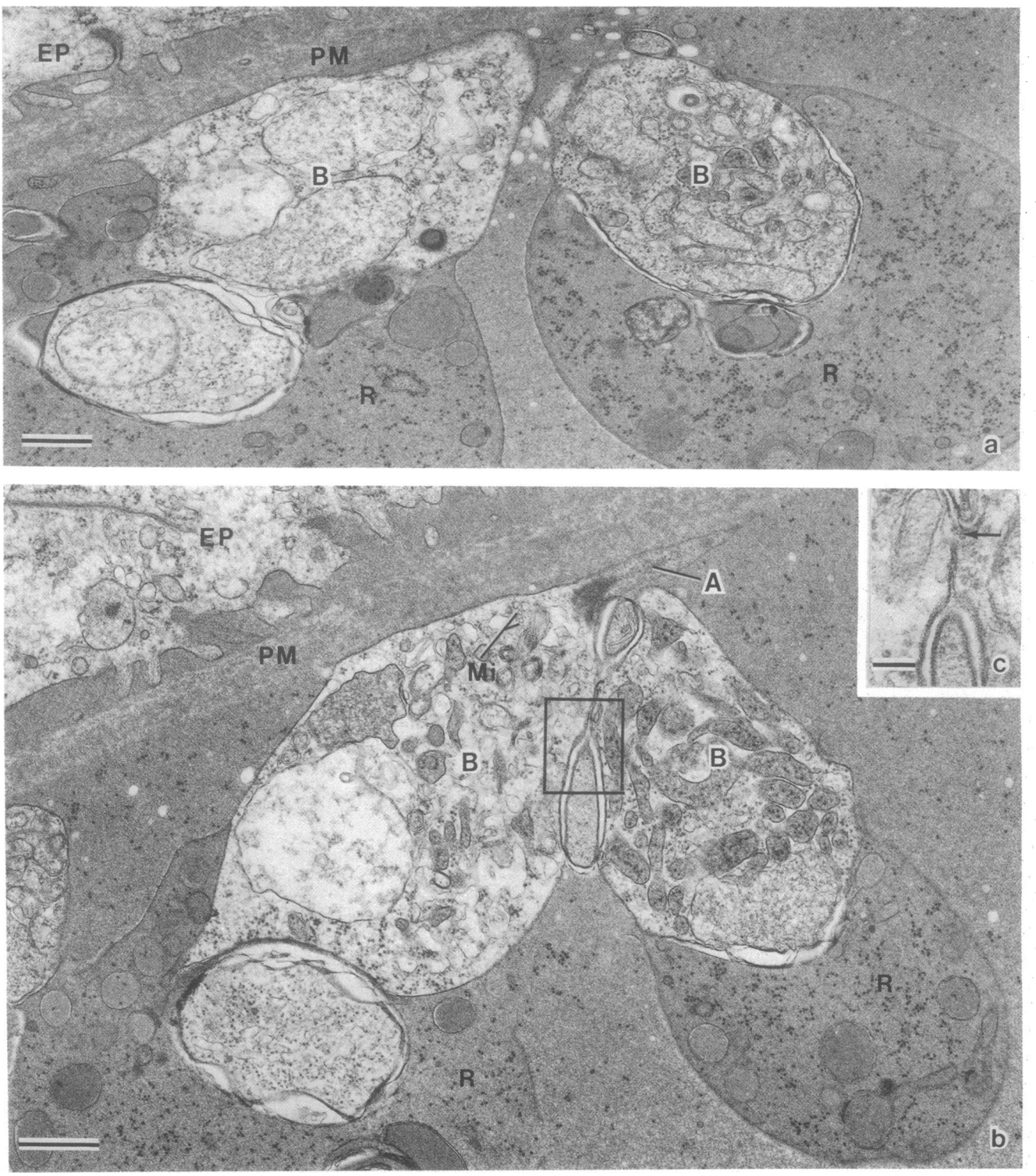

FIG. 2. *B. microti* in the lumen of *I. dammini* gut at 14.5–16 hr after repletion. (*a*) Two *Babesia* (B) on the way out of two neighboring reticulocytes (R). The parasite at the left is located close to the peritrophic membrane (PM); behind it is an epithelial cell (EP) of the gut wall. (*b*) Serial section relative to *a*, showing the two *Babesia* (B) touching each other. The parasite at the left has an arrowhead (A) and several microtubules (Mi) in cross section. (*c*) Serial section of outlined area in *b* at higher magnification to show that fusion between the two organisms (at arrow) has already occurred. (Bars = 0.5 μm in *a* and *b* and 0.1 μm in *c*.)

sumed that they are in a state of division. In Fig. 1 *b* and *c*, only one partner has an arrowhead. The possibility still remains that the missing arrowhead in the other partner may be present in another thin section of this organism. Therefore, no definite conclusion can be drawn. Fig. 1 *b* and *c* may represent fusion as well as fission. The same applies to Fig. 1*e*.

Fusion Confirmed. It seemed that the only way to determine that fusion takes place between two parasites was to search for *Babesia* emerging from two neighboring erythrocytes and to see whether they may be in the process of merging. Figs. 2 and 3 demonstrate clearly such an event. In Fig. 2*a*, a *Babesia* is moving out from each of the two reticulocytes. In Fig. 2*b*, a serial section relative to Fig. 2*a*, the two *Babesia* are closely adjacent and are touching each other at several points. One of the parasites has an arrowhead organelle sectioned tangentially. Fusion between the cytoplasms of the two *Babesia* is clearly demonstrated in another serial section (Fig. 2*c*) in which higher magnification shows the area where the two parasites are in close contact. More advanced stages of merging of two *Babesia* are represented in serial sections of Fig. 3 where small pseudopods of two parasites are protruding from two neighboring reticulocytes (Fig. 3*a*). A serial section through the same reticulocytes (Fig. 3*b*) shows that the two *Babesia* have already fused, forming a bridge between the two host cells. A part of an arrowhead extending to the outside of the main body of the parasite can be seen in cross section. Another example of fusion between two *Babesia* is seen in Fig. 3*c*. Here, two reticulocytes are connected by one *Babesia*, leaving no doubt that the single parasite derives from two merging or merged parasites, each moving out from a neighboring reticulocyte. Fig. 3*d*, a serial section relative to Fig. 3*c*, shows that the parasite on the left side is equipped with an arrowhead.

Figs. 2 *a–c* and 3 *a–c* supply good evidence that fusion between two *Babesia* takes place, leaving no doubt of a sexual process in *B. microti*. The two fusing organisms represent gametes. The gametes differ in their fine structure. The major difference is the presence of an arrowhead in one of the fusing gametes.

DISCUSSION

Two parasites, one with an arrowhead organelle and each coming out from a neighboring reticulocyte, have been caught in

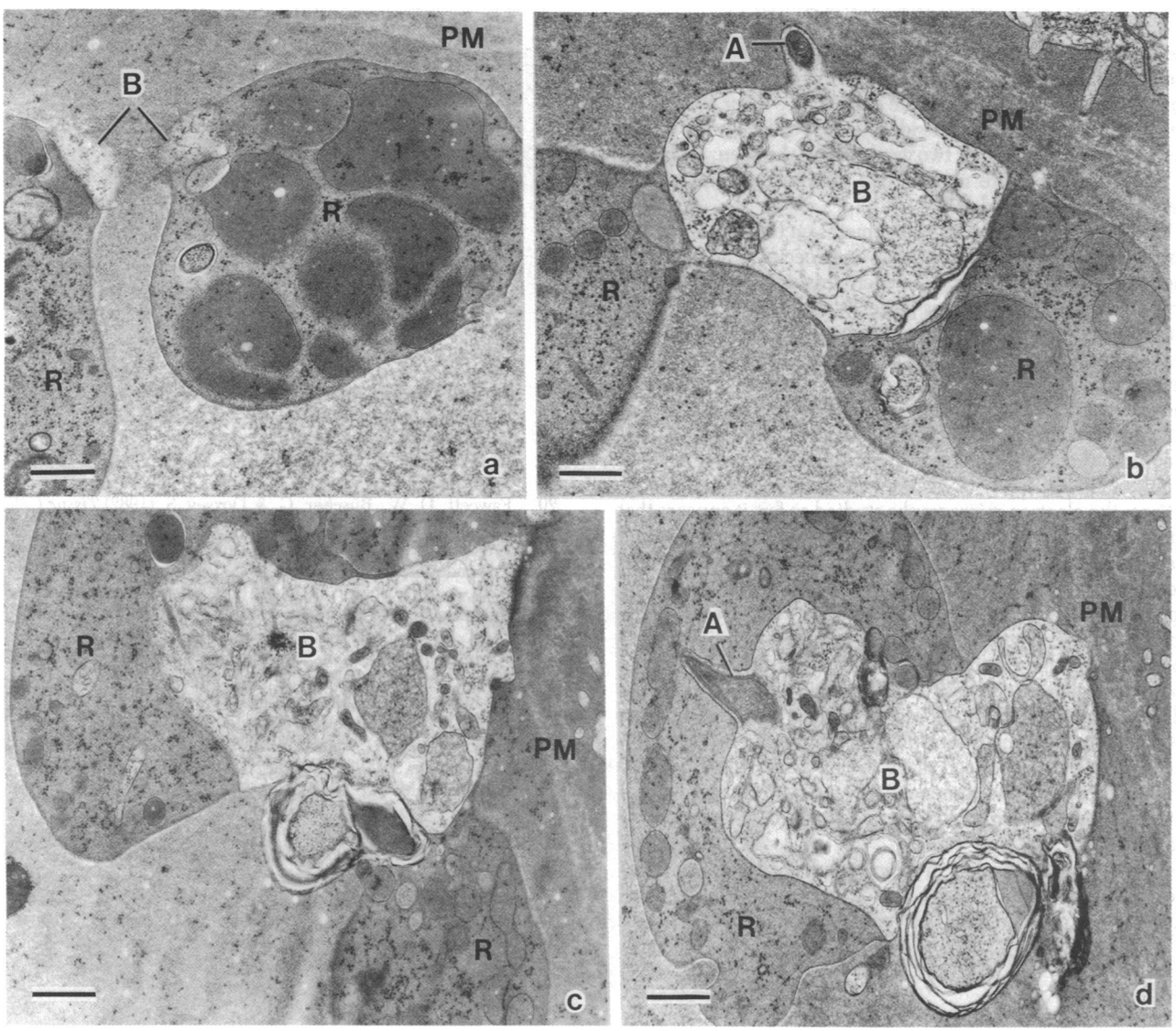

FIG. 3. *B. microti* at 14.5–16 hr after repletion in the gut of the vector located near the peritrophic membrane (PM). (*a*) From two neighboring reticulocytes (R), small pseudopods of *Babesia* (B) are protruding. (*b*) Serial section through the same reticulocytes (R) shows that the two *Babesia* (B) have already fused. Note cross section of the distal part of an arrowhead (A). (*c*) Two reticulocytes (R) connected by a *Babesia* (B). (*d*) A serial section relative to *c* shows that the part of *Babesia* on the left side has an arrowhead (A). (Bars = 1.0 μm.)

the act of fusion, providing proof of sexuality in *B. microti*. This demonstrates syngamy in a piroplasm. Our previous tentative assumption that gametes and gametocytes are present in *B. microti* (8) has now been confirmed. The sequence of sexual events in *B. microti*, leading to the formation of zygotes, is now clear and can be summarized briefly as follows.

Gametes are derived from gametocytes which in *B. microti* are formed in the intraerythrocytic stage, at which two types of trophozoites are found (10). The majority of trophozoites differentiate into and reproduce asexually as merozoites (28, 29). A small percentage of trophozoites, however, do not differentiate or reproduce but rather increase in size and acquire peculiar shapes by twisting and folding as if trying to accommodate their growing bodies in the limited space of the host erythrocyte (10). The number of these unusually shaped parasites increases with the age of the infection (8) and they remain in the peripheral blood until they are sucked in by a tick. They represent gametocytes (8, 29). In the gut of the vector *I. dammini* these gametocytes undergo a complete metamorphosis (8), acquiring new structures, the most prominent being the arrowhead organelle. A process similar to exflagellation in Haemosporidia (30) takes place, and several gametes (the number of which is not yet known) are formed either inside or outside the erythrocyte or partially inside and outside the host cell. Sometimes a gametocyte with three arrowheads, each marking a presumptive gamete, leaves the erythrocyte (8) or several gametes are found inside or outside the host cell but only one of them has an arrowhead; or two gametes may be seen, each with an arrowhead, or there may be two gametes but only one of them has an arrowhead.

Merging or syngamy between gametes on the way out of the host cell indicates that fertilization may occur at a very early stage of gamete formation. However, this does not mean that such early fusion always takes place. What most probably happens is that this process often occurs between gametes outside the host cell. In such instances, it is difficult to know whether the two gametes are in a final stage of division or in an initial stage of fusion as described and shown in Fig. 1 *a–d*. It is also not known whether syngamy may occur between gametes derived from the same gametocyte, but here again, we might run into the same difficulty of distinguishing division from fusion.

From the electron micrographs studied so far, it appears that *B. microti* has two types of gametes: one with an arrowhead and one without it. Syngamy occurs between the two different types and they should be considered as anisogametes. The resulting zygote never has more than one arrowhead. By means of the arrowhead, the zygote is able to penetrate a solidified peritrophic membrane through which even small particles such as ribosomes are unable to pass (26). It then invades the epithelial cell of the gut wall (27). A *Babesia* without an arrowhead cannot pass the barrier of the peritrophic membrane or enter the epithelial cell. The arrowhead plays an important role in preparing the way out of the lumen of the gut. The remaining parasites without an arrowhead are doomed to destruction by digestion.

The finding of sexuality in *B. microti* has an important implication. There is now every reason to believe that a sexual process similar to that in *B. microti* occurs in all other species of *Babesia* and *Theileria*. In all species of both genera, organisms with an arrowhead have been found in the gut of the vector. It should be added that, since the rediscovery of Koch's *Strahlenkörper*, the organisms with an arrowhead organelle were considered by all investigators of piroplasms as possible candidates of sexuality. However, no one had been able to provide proof of it. The lack of clear-cut evidence compelled some investigators to use such terms as "supposed sexual stages" (7) or "supposed gametes and syngamy" (17). Now the evidence has been provided and the long-lasting dispute as to sexuality in piroplasms has been finally solved.

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