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Toxoplasma gondii: differentiation and death of bradyzoites

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Abstract. The living parasites in *Toxoplasma* cysts cannot be eradicated by current therapy and maintain latent infections for many years. Relatively little is known about encysted *Toxoplasma*. We therefore undertook studies using mice infected with the avirulent ME 49 strain of *Toxoplasma*. The bradyzoites in young (12- to 17-day-old) cysts contained the same organelles as did tachyzoites. The bradyzoites of older cysts (4 weeks postinoculation) had differentiated, losing certain organelles and acquiring others. Our major new finding was that in animals inoculated ≥ 4 weeks previously, some bradyzoites were totally disrupted, spilling their contents (perhaps including lytic substances) into the cyst matrix. Many older bradyzoites in the same cysts lacked internal membranes and their viability was questionable, but there were also occasional parasites resembling viable tachyzoites and mature bradyzoites, organisms that might possibly initiate daughter cyst formation after cyst rupture. The life span of an individual bradyzoite may be shorter than formerly appreciated despite the prolonged course of latent infections.

Tissue cysts of *Toxoplasma gondii* are resistant bodies in which the parasites are sequestered during latent infections. The cysts contain hundreds or more of the parasites in slowly dividing forms called bradyzoites and may persist for years without damaging the host (Frenkel 1973). During their encystment, the organisms are protected from circulating antibodies and drugs by the cyst wall. Although current treatments may produce temporary remissions of toxoplasmosis, total eradication of the disease does not presently seem possible (Velimirovic 1984).

Approximately half of the population in the United States exhibits antibodies to *Toxoplasma*, indicating subclinical infection (Krick and Remington 1978). Such quiescent disease is even more prevalent in countries such as France and Brazil (Silveira et al. 1988), in which raw or undercooked meat containing *Toxoplasma* cysts is eaten. Persons with latent disease are at risk of developing toxoplasmosis if they become immunosuppressed (Velimirovic 1984).

The frequency of quiescent *Toxoplasma* infections explains the prevalence of toxoplasmosis in acquired immunodeficiency syndrome (AIDS). Reactivation of latent disease may produce disseminated toxoplasmosis – a common cause of death in AIDS and the leading cause of cerebral lesions that produce such devastating symptoms as seizures, mental confusion, and paralysis (Velimirovic 1984). Recently, ocular toxoplasmosis has been found to be a complication of AIDS that leads to blindness (Holland et al. 1988). Because of the severity of these infections, it is imperative that more be learned about *Toxoplasma* cysts and the factors that control their infectivity.

During a preliminary investigation of *Toxoplasma* cysts, we detected great variability in the structure of the enclosed bradyzoites. Furthermore, all of the cysts that we observed were extracellular, whereas Ferguson and Hutchison (1987), who used a different strain of *Toxoplasma*, found only intracellular cysts. These observations suggested that further structural studies of cysts might be fruitful. We therefore undertook such studies using outbred Swiss Webster mice inoculated with an avirulent strain (ME 49) of *Toxoplasma* with the goal of analyzing the development and differentiation of cysts in vivo.

Materials and methods

Maintenance of Toxoplasma stocks

The avirulent ME 49 cyst-forming strain of *Toxoplasma gondii* was obtained from Dr. J. Remington, Palo Alto Medical Research Foundation (Palo Alto, Calif.). Stocks were maintained by intraperitoneal inoculation of Swiss Webster mice (Harlan Sprague Dawley, Inc., Madison, Wis.) with 10–100 cysts obtained from the brains of infected mice. The cysts were suspended in Hanks' bal-

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anced salt solution (HBSS) containing 10 μg streptomycin/ml and 10 units penicillin/ml.

Production of cysts

The brain or half-brain from each infected mouse was homogenized with a mortar and pestle in 1 ml HBSS containing streptomycin and penicillin as noted above. The total number of cysts in 0.05 ml was counted by light microscopy using a 10X objective, and the approximate number of cysts per brain was estimated. To maintain stocks, approximately 10 cysts were inoculated intraperitoneally into normal Swiss Webster mice weighing 20–25 g. In an attempt to produce greater numbers of cysts for experimental purposes, approximately 25–100 cysts of this avirulent strain were inoculated into similar mice. Consequently, the inocula used in these studies contained 10–100 cysts. The maximal inoculum (100 cysts) was well tolerated. A few animals developed slight fever and other mild symptoms of illness. Following treatment for 2–3 days with sulfadiazine (4 mg/ml) in the drinking water, these symptoms subsided and recovery was complete. Treated animals exhibited fewer cysts than did untreated mice and were excluded from the study.

Preparation of specimens

Infected mice were killed at intervals of 12 days to 15 months after inoculation. Mice under deep pentobarbital anesthesia were exsanguinated and immediately perfused with HBSS, then with a mixture of 1% formaldehyde with 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer containing $CaCl_2$, pH 7.4 (PG; Karnovsky 1965). The brains were removed. To obtain the best specimens for electron microscopy, one half of each brain was used for cyst counts; if cysts were numerous, the other half was embedded for morphological studies by light and electron microscopy. In one instance at 12 days after inoculation when cysts were scarce, cysts were isolated under light microscopy from homogenized brain by pipet, dropped into fixative, prepared as noted below, and embedded in a pellet. The number of cysts examined by electron microscopy at various times postinoculation is shown in Table 1.

Specimens for electron microscopy. After dissection, fixation of the perfused brain tissue was continued in PG for 4–5 h at room temperature. Specimens were postfixed overnight in 1% osmium tetroxide in Palade's buffer (pH 7.6) at 4° C. Some of the specimens perfused with PG were stored in 1.5% glutaraldehyde in sodium cacodylate-HCl buffer (pH 7.4) plus 1% sucrose at 4° C for 1–2 days and were postfixed in 2% osmium tetroxide in the same buffer in the absence of sucrose for 2 h at 4° C. All specimens were then stained en bloc with aqueous buffered uranyl acetate for 1 h (Farquhar and Palade 1965), dehydrated in ethanol and propylene oxide, and embedded in Epox 812. Thin sections were stained with aqueous uranyl acetate and Reynolds' lead citrate and then examined with a Siemens Elmiskop 1A or a Zeiss EM 10CR electron microscope.

Specimens for light microscopy. Thick sections (0.25–0.5 μm) of Epox 812-embedded specimens were stained with 1% toluidine blue containing 1% borax. Areas containing cysts were selected for thin sectioning and examination by electron microscopy.

Table 1. Number of *Toxoplasma gondii* cysts examined by electron microscopy at various times after the inoculation of mice with avirulent strain ME 49

Time p.i.	Cysts inoculated (*n*)	Mice (*n*)	Cysts examined (*n*)	Cysts in brain (*n*)
12 days	25	1	1	600
17 days	100	2	6	1800 each
1 month	100	1	3	5200
3 months	50	1	8	2200
6 months	50	1	4	4400
15 months	10	1	2	300

p.i., Postinoculation

Results

Ultrastructural examination of cysts (Fig. 1) confirmed and extended the results of previous studies (Chobotar and Scholtyseck 1982; Ferguson and Hutchison 1987; Matsubayashi and Akao 1963; Mehlhorn and Frenkel 1980; Scholtyseck et al. 1974; Senaud 1969; van der Zypen and Piekarski 1967). Our major new finding was that bradyzoites of the ME 49 strain quickly differentiated, reached maturity and had begun to degenerate by 4 weeks after inoculation.

We observed that bradyzoites underwent continuous changes through several stages that can arbitrarily be designated as follows: (1) young or tachyzoite-like parasites, (2) mature bradyzoites, (3) degenerating bradyzoites, and (4) disintegrated bradyzoites. The entire sequence occurred within 4 weeks postinoculation, much more rapidly than had formerly been appreciated. Each stage is described and illustrated below.

In the youngest cysts, which were examined at 12 days after inoculation, most of the bradyzoites (Fig. 2) closely resembled tachyzoites as described by Ferguson and Hutchison (1987). The unit membranes of the inner membrane complex (IMC) were intact and the rhoptries were prominent. Cytoskeletal structures such as the conoid and microtubules appeared normal. The Golgi complex, mitochondria, and endoplasmic reticulum (ER) were well preserved and occupied their usual locations, but micronemes and amylopectin granules were few or absent (Fig. 2).

In animals that had been inoculated for longer periods ($\geq$1 month) before specimen retrieval, mature, fully differentiated bradyzoites were seen in which micronemes and amylopectin granules were numerous and other intact organelles (rhoptries, mitochondria, ER, IMC, and dense bodies) were present (Figs. 3, 4).

By as early as 1 month after inoculation as well as in older specimens, many of the bradyzoites had degenerated and were vastly different in structure (Fig. 5). Rhoptries and mitochondria were less commonly seen. The anterior two-thirds of the parasites were filled with large numbers of micronemes, amylopectin granules, and dense bodies. Moreover, in these bradyzoites the membranes of most organelles had disappeared (Fig. 5). Only vestiges of the IMC and nuclear envelope were found, and mere remnants of the Golgi complex and ER were occasionally seen. The micronemes, albeit numerous and displaying typical size and shape, lacked a limiting membrane.

In some cysts examined at 1–3 months after inoculation, an occasional bradyzoite was totally disrupted and could be identified only by residues of cytoskeletal or-

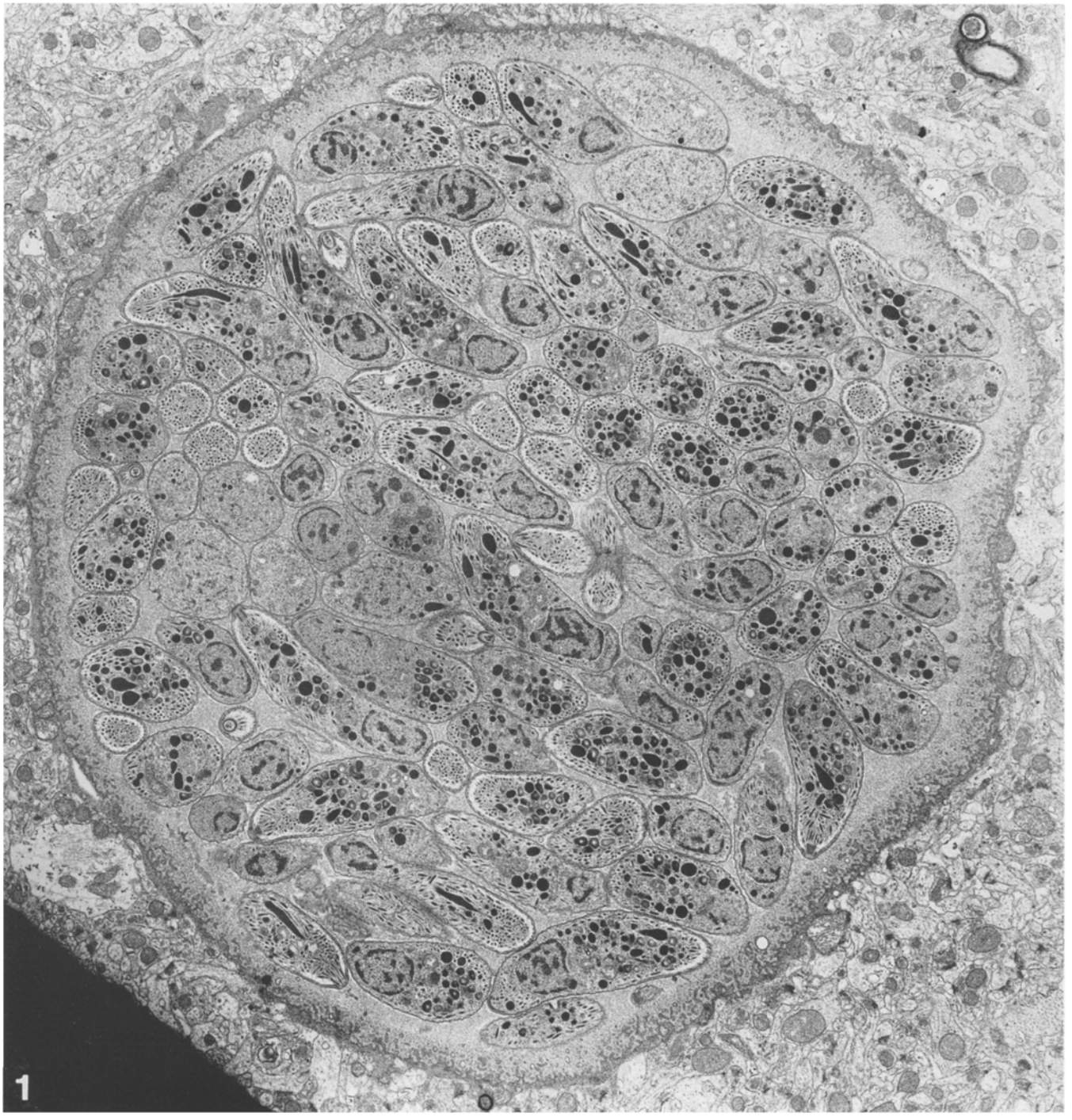
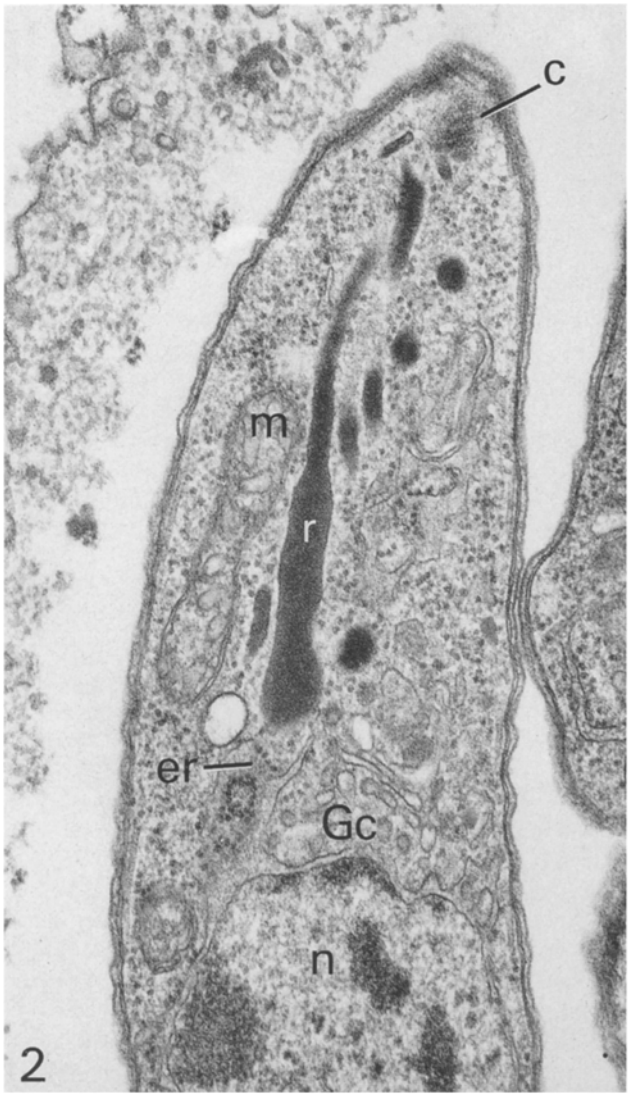
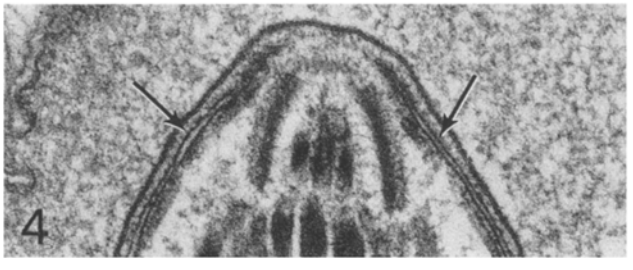


Fig. 1. A cyst containing hundreds of bradyzoites from a mouse that had been inoculated 3 months previously with *Toxoplasma* strain ME 49. ×6000

ganelles – the conoid and microtubules (Figs. 6, 7). In some cases the microtubular skeleton outlined the shape of the preexisting organism (Fig. 8). Amylopectin granules released from such bradyzoites were often swollen to several times their normal size (Fig. 6). The numerous micronemes had completely disappeared. Cysts containing disrupted bradyzoites also showed many degenerating bradyzoites.

Many cysts contained a mixture of intact and degenerating parasites (Fig. 9). The degenerating organisms were slightly denser than those that appeared viable, and at higher magnification the fragmentation of membranes and mitochondria was obvious (Fig. 10). The younger parasites, which sometimes lay immediately adjacent to degenerating ones (Fig. 9), contained normal internal membranes (Fig. 9, insets) and a full complement of organelles, including rhoptries, mitochondria, Golgi complex, ER, and IMC. Such organisms appeared to be fully viable, suggesting that the bradyzoites of a single cyst were at different stages of maturation, presumably





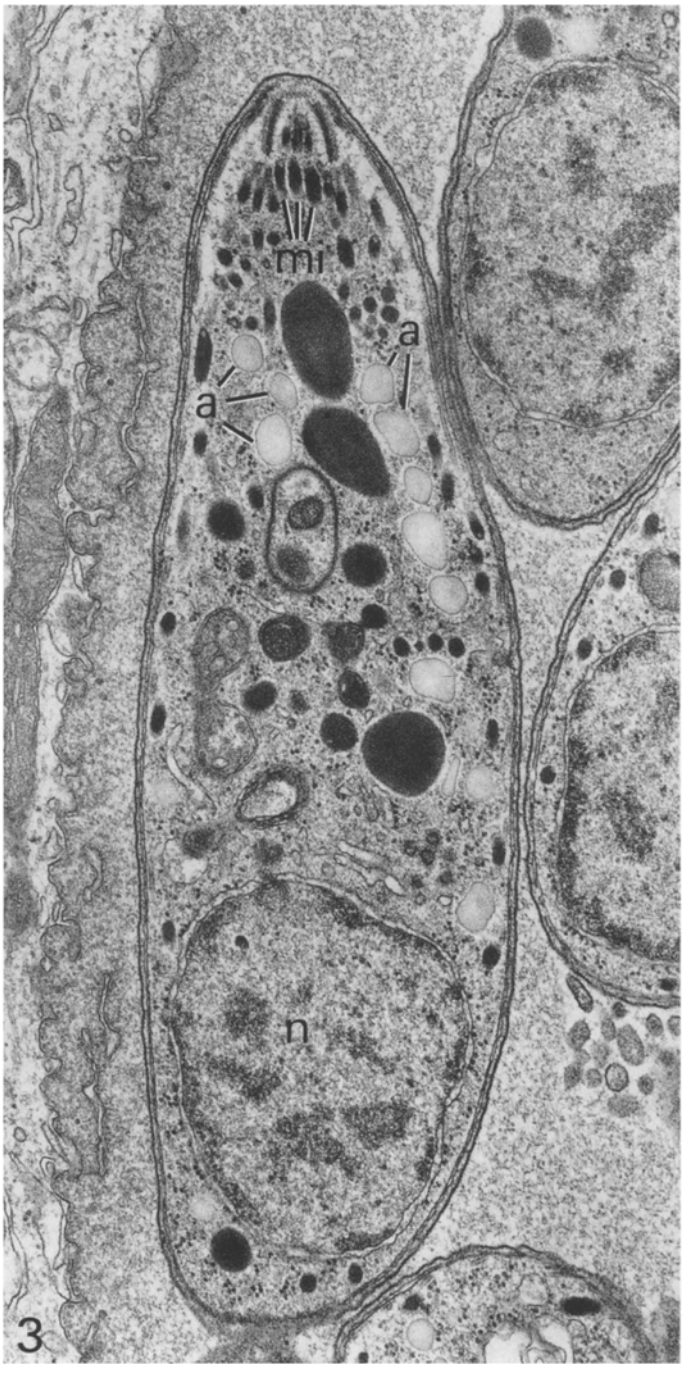


Fig. 2. Typical bradyzoite in a cyst isolated from the brain of a mouse that had been inoculated 12 days previously with *Toxoplasma* strain ME 49. The encysted parasite closely resembles a tachyzoite. The cyst matrix is unusually lucent, possibly because of damage inflicted during isolation from the brain. *c*, Conoid; *er*, endoplasmic reticulum; *Gc*, Golgi complex; *m*, mitochondrion; *n*, nucleus; *r*, rhoptry. × 51 000. **Fig. 3.** A more highly differentiated bradyzoite from a mouse that had been inoculated 6 months previously. The number of micronemes (*mi*) and amylopectin granules (*a*) has increased, and the nucleus (*n*) is in its typical posterior position. All organelles appear intact. × 34 000. **Fig. 4.** Higher magnification of Fig. 3, showing the two membranes of the inner membrane complex (*arrows*). × 68 000

because of a difference in the time of bradyzoite replication.

Just as the bradyzoites appeared to be of different ages, the cysts themselves evidently differed in age. For example, in a single animal, some cysts contained primarily mature bradyzoites and others displayed predominantly degenerated bradyzoites, suggesting a longer period of development. This implies that there were differences in the times of formation of the cysts. This observation will be studied further in a subsequent investigation.

The thickness of the cyst wall was judged by the distance between the outermost bradyzoites and the external limiting membrane of the cyst. The average thickness

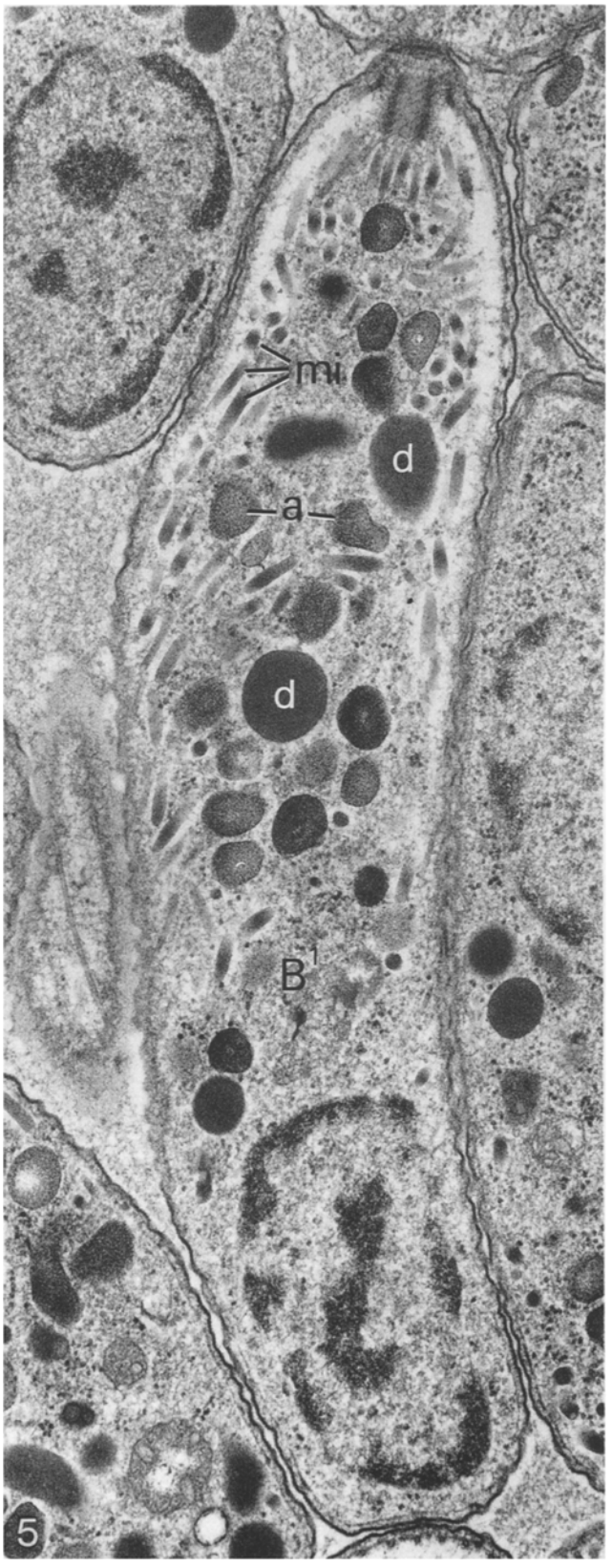


Fig. 5. Longitudinal section of a degenerating bradyzoite (B^1) from a 3-month-old cyst. Micronemes (*mi*), amylopectin granules (*a*), and dense bodies (*d*) are numerous, but the Golgi complex, endoplasmic reticulum, and nuclear envelope are absent. ×37000

varied from 150 to 1000 nm. The apparent thickness of the cyst wall varied with the orientation of the section so that it was difficult to derive absolute values from a small sample in the absence of serial sections. The thickness did not correlate with the time postinoculation but varied even in a single animal. No defects in any of the cyst walls were observed. None of the cysts was found in a host cell.

Discussion

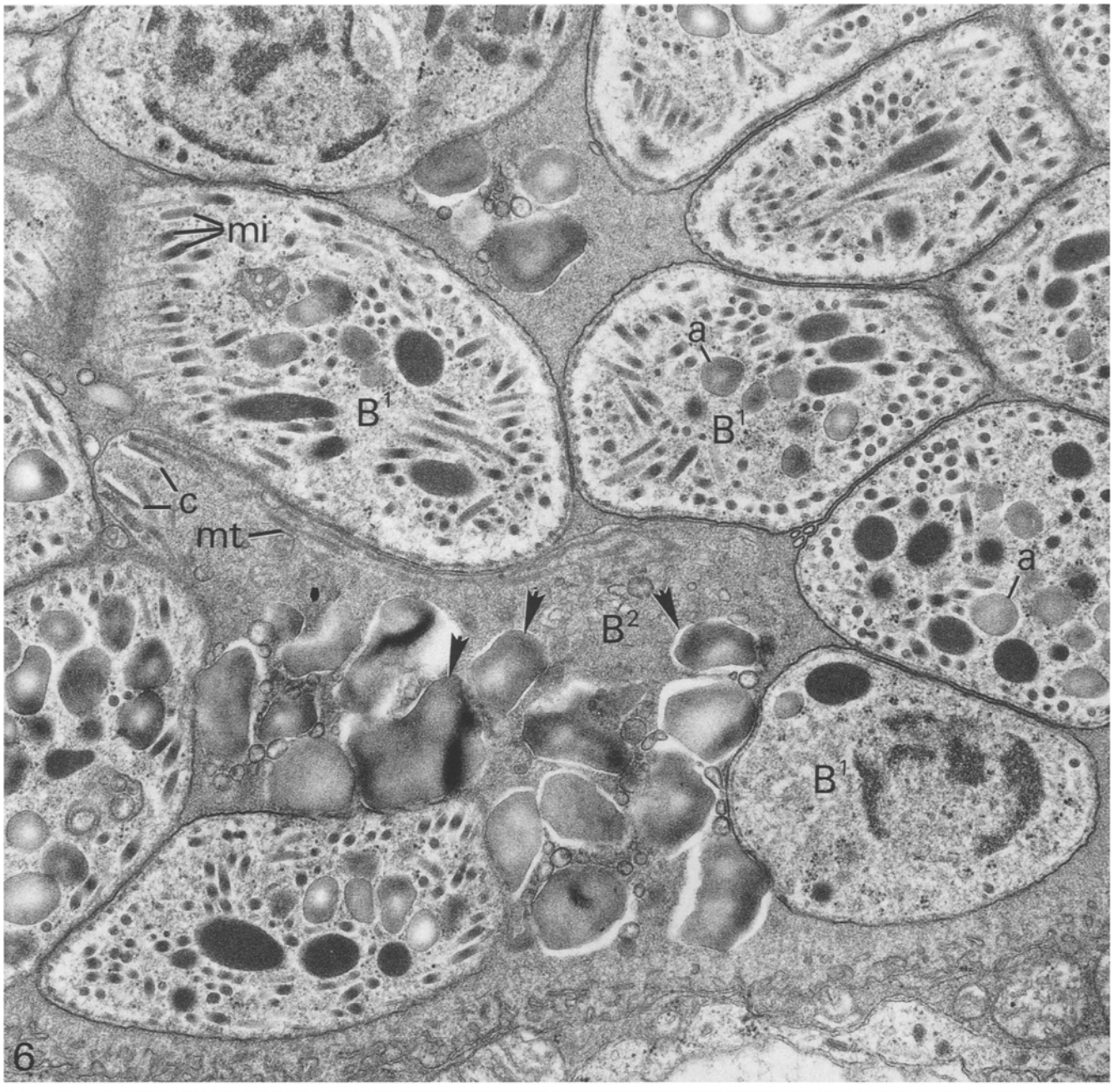
Degeneration of bradyzoites

In the present study we found clear evidence that many encysted bradyzoites degenerate and may even fragment completely at as early as 4 weeks after the onset of infection. The first sign of bradyzoite degeneration is the loss of internal membranes so that some organelles such as the Golgi complex and ER disappear completely, whereas others such as the micronemes persist in the cytoplasm without a limiting membrane. Eventually, even the plasmalemma itself is disrupted and the remaining cytoplasmic contents are spilled into the cyst matrix. Although the amylopectin granules swell and for a certain time mark the location of parasite disruption, it may be significant that naked micronemes lacking a membrane are not seen after the parasites have ruptured; they are apparently quickly dissolved in the cyst matrix.

The micronemes have long been believed to be secretory organelles akin to the rhoptries (Chobotar and Scholtyseck 1982). There is now good evidence that the rhoptries contain lytic substances (Norrby 1971; Schwartzman 1986) that are released at the moment of host cell penetration (Nichols et al. 1983), apparently facilitating parasite entry by producing a localized rupture of the host cell plasmalemma. The function of the micronemes remains to be proven. If, as presumed, their content is lytic like that of the rhoptries, the release of such substances within the cyst may play a significant role in the dissolution of the cyst wall.

Technical considerations

The outer membrane of *Toxoplasma* is tough; it resists deformation during isolation and is not readily penetrated by fixatives. In our previous studies of *Toxoplasma* (Nichols and O'Connor 1981), we found that the two membranes that limit the saccules of the IMC were not well preserved unless the parasites had been thoroughly postfixed in osmium tetroxide. Osmium is a heavy metal that specifically binds to membranes and enhances membrane definition because of its electron density. We were particularly concerned that all stages of specimen preparation be adequate and that internal structures be faithfully preserved because of the significance of our observation that bradyzoites degenerate as early as at 4 weeks after inoculation, more rapidly than had previously been thought. We were reassured that our techniques were



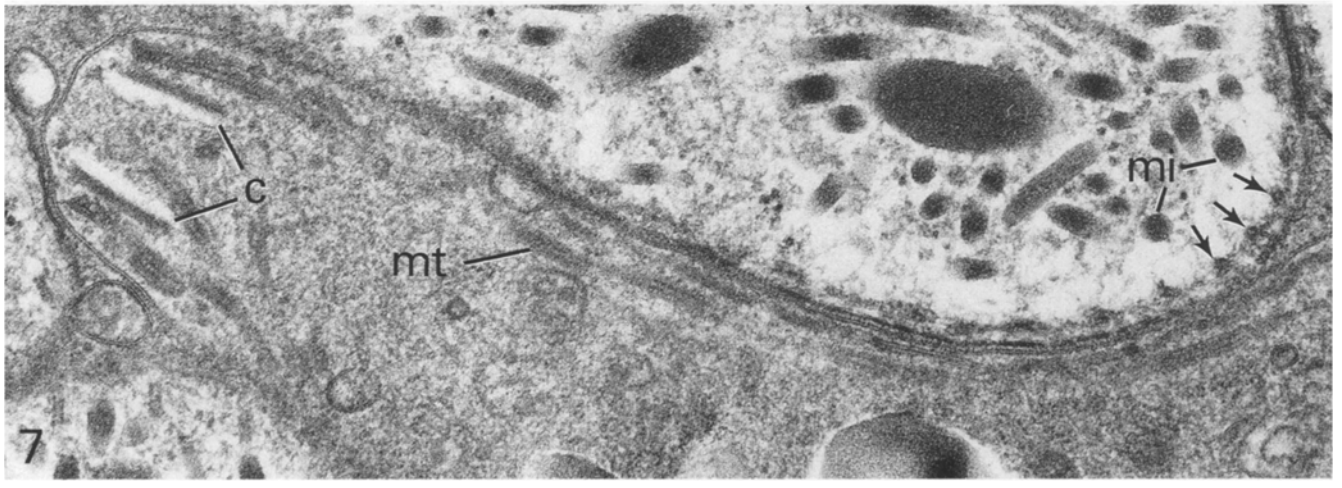


Fig. 6. A disrupted bradyzoite (B^2) amid several degenerating ones (B^1) from a 3-month-old cyst. The released amylopectin granules (*arrowheads*) have swollen to many times their normal size (*a*). The microtubules (*mt*) and conoid (*c*) of the ruptured organism are relatively intact, but other organelles have vesiculated. × 28000.

Fig. 7. Higher magnification of a part of Fig. 6, showing the conoid (*c*) and microtubules (*mt*) more clearly. Note also that the inner membrane complex of the adjacent parasite has disappeared, but the microtubules remain in place (*arrows*). The micronemes (*mi*) display no limiting membranes. × 68000

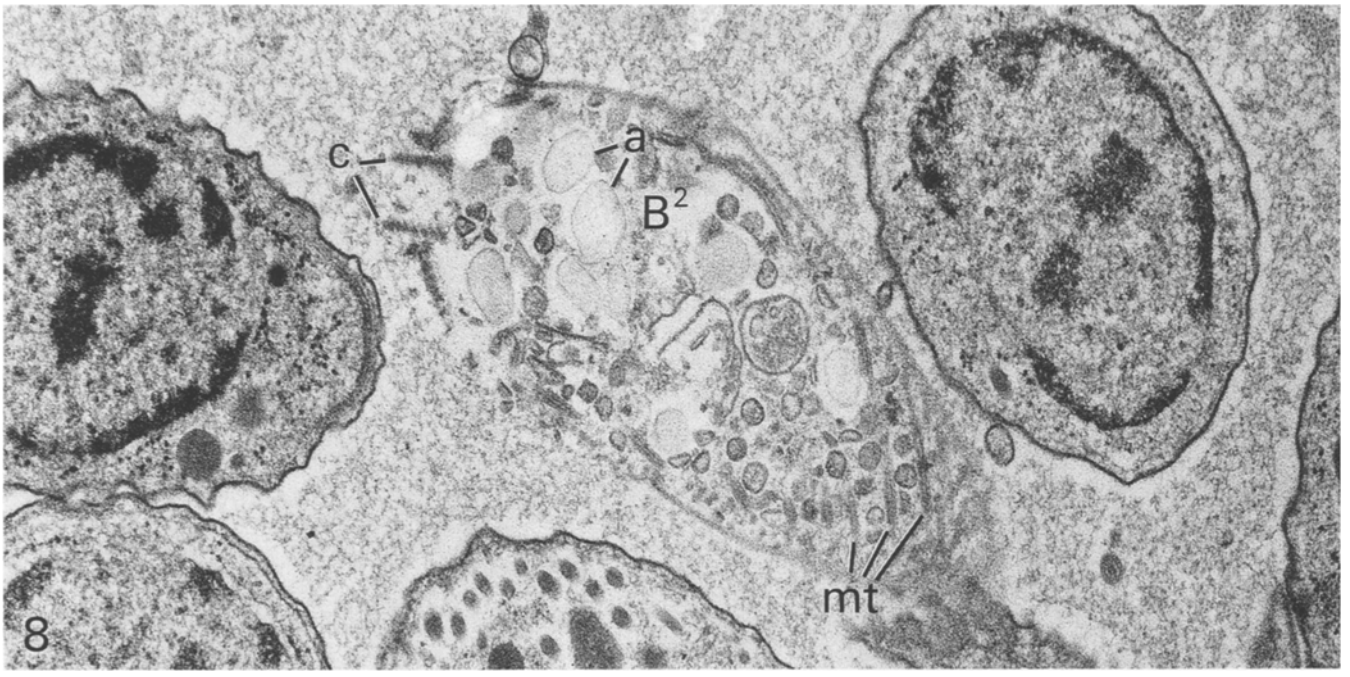


Fig. 8. Another disrupted bradyzoite (B^2), in this case from a 15-month-old cyst, showing the same features noted in Fig. 6. The remaining conoid (*c*) and microtubules (*mt*) outline the body of the former parasite. *a*, Amylopectin granules. ×42000

adequate when we observed a striking difference in the structure of bradyzoites within a single cyst.

Cyst development

Cyst formation is initiated by organisms that have replicated within a parasitophorous vacuole, perhaps in response to some signal that occurs at the onset of the immune response. The few organisms in the host cell begin to form a cyst wall by modification of the parasitophorous vacuole (Matsubayashi and Akao 1963) and then continue to divide until the cyst contains hundreds or more organisms (Frenkel 1988). It is thus clear that there may be considerable differences in the ages of the organisms in a single cyst, depending on the time at which they replicated. It is therefore not surprising that a few younger parasites may remain intact at the same time at which older organisms are beginning to deteriorate.

Strain-specific differences

It is important to note that our results apply to a specific strain of *Toxoplasma* in a specific strain of mouse. The virulence of the parasites and the resistance of the hosts differ in other strains and are therefore important variables that affect the outcome of *Toxoplasma* infections (Frenkel 1973). It should be noted in this respect that the results of Ferguson and Hutchison's (1987) thorough studies using SRRA strain *Toxoplasma* and STR outbred mice differed in several ways from those obtained in the present study. All of the cysts observed by the former investigators were inside neurons, which were identified by their synapses. In contrast, we found no cysts inside host cells. In addition, Ferguson and Hutchison did not report the degeneration of bradyzoites inside cysts. Whether bradyzoite degeneration is affected by the location of the organisms in a host cell is not known. It may be that in the experiments by the former authors, bradyzoites received continuous nutrition from intact host cells and consequently retained their viability, whereas in the present study, death of the host cells resulted in the death of the encysted parasites. On the other hand, it is possible that degeneration of bradyzoites occurred but was not observed by Ferguson and Hutchison. However, these investigators did note that in older cysts, the bradyzoites were not tightly packed but were scattered about in a lucent matrix, suggesting the possibility that some of the organisms had disappeared. The differences between their observations and the present findings suggest that there may be functional variations among strains of *Toxoplasma*.

Cyst rupture

In a study of Ferguson et al. (1989), it was clearly demonstrated by electron microscopy that cysts break down in vivo. These authors demonstrated the infiltration of mononuclear leukocytes into a broken cyst and the phagocytosis of parasites by macrophages, documenting the effectiveness of cellular immunity in the brain of an asymptomatic immunocompetent mouse. The disruption of cysts is also inferred from the observation of satellite lesions around old scars in the retina; such lesions are believed to result from the dispersion of *Toxoplasma* organisms following cyst rupture (O'Connor 1974).

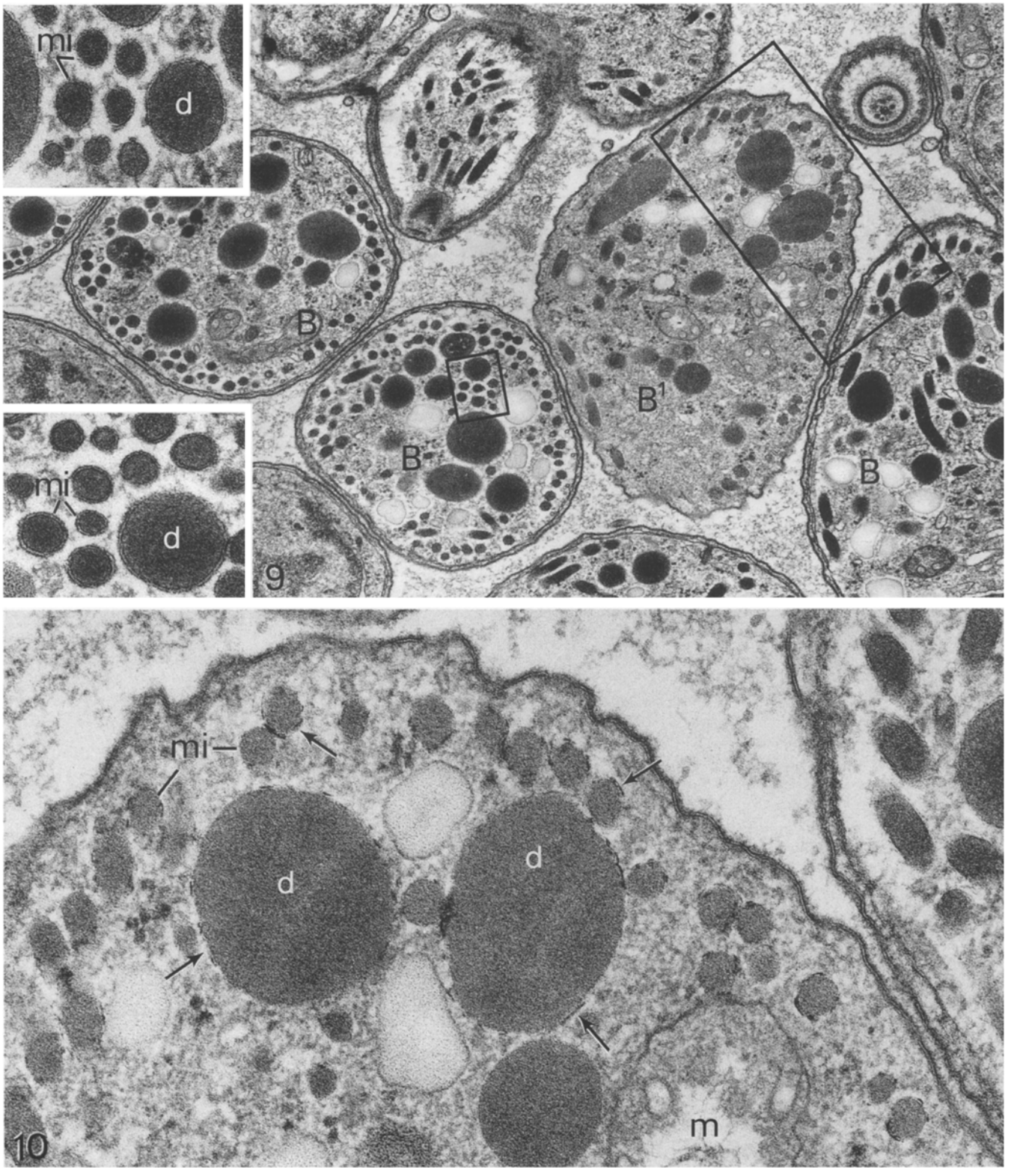


Fig. 9. Part of a cyst from a mouse that had been inoculated 6 months previously. On the right, a degenerating bradyzoite (B^1) displaying dense cytoplasm is adjacent to several intact parasites (B). ×33000. *Insets:* Higher magnifications of intact parasites in the same cyst, showing that the membranes around the micronemes (*mi*) and dense bodies (*d*) of some parasites are well preserved. The inset at *upper left* corresponds to the small box in Fig. 9. ×124000. The inset at *lower left* illustrates part of another parasite (area of cyst not shown). ×105000. **Fig. 10.** Higher magnification of the degenerating parasite (B^1) shown in Fig. 9 (*large box*) shows that the membranes around the micronemes (*mi*) and dense bodies (*d*) have fragmented (*arrows*). Note also the absence of the inner membrane complex and the ruptured mitochondrion (*m*). ×118000

The degeneration of many bradyzoites that we observed within cysts in vivo and the eventual death of some raise the question as to whether organisms escaping from older cysts initiate the formation of daughter cysts. Older cysts contain hundreds of organisms, and it is virtually impossible to examine every bradyzoite in an entire cyst, even in serial sections. If organisms capable of initiating new cyst formation are present, they are therefore difficult to identify. Presumably, the young and mature bradyzoites that we observed would be most capable of parasitizing new host cells and renewing cyst formation. Further studies of cysts produced in vivo may provide answers to these and other intriguing questions.

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