

THE INTERACTION BETWEEN *TOXOPLASMA GONDII* AND
MAMMALIAN CELLS

II. THE ABSENCE OF LYSOSOMAL FUSION WITH PHAGOCYTTIC
VACUOLES CONTAINING LIVING PARASITES*

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In the past 15 yr there have been many advances in our knowledge of the intracellular events that follow phagocytosis of microorganisms (1, 2). Lysosomal constituents and metabolic products are delivered to the phagocytic vacuole, and in most instances there ensues rapid death and destruction of the microorganisms. However, certain bacteria or protozoans are clearly capable of surviving inside of cells, and in some instances, toxoplasma being an example, the parasites apparently are able to multiply only in an intracellular environment. Very few studies have been made on the mechanisms by which some parasites can evade or withstand the metabolic factors and hydrolases that usually kill microorganisms in the phagolysosomes of cells. Several different mechanisms might be responsible for parasite survival intracellularly. Some viruses may interact with the plasma membrane or with the vacuolar membrane so that the viral cores reach the host cell cytoplasm without encountering lysosomal and other factors that attack foreign materials in the vacuolar system (3). In some instances microorganisms may be able to prevent the transfer of lysosomal materials to phagocytic vacuoles. Recent experiments indicate that such a mechanism may operate in macrophages infected with tubercle bacilli (4) or with *Chlamydia* species in fibroblasts (5). Finally, certain parasites may be capable of withstanding the toxic substances and digestive enzymes delivered to the phagocytic vacuoles. *Mycobacterium lepraemurium* apparently is able to survive such exposure (6, 7).

Toxoplasma infections of macrophages in vitro provides an excellent model for further study of mechanisms operating to foster parasite survival at the level of the phagocytic vacuole and the lysosomal system. The macrophage is richly endowed with both primary and secondary lysosomes, and these can be observed by cytochemical procedures, or in the case of the secondary lysosomes by tagging with electron-opaque colloidal materials such as thorium dioxide or ferritin. As described in the preceding paper (8), about half of the toxoplasmas taken into macrophages thrive, whereas the other half die and are digested soon after phagocytosis. Toxoplasmas are large parasites with a complex ultrastructure, and it is possible to establish reliable morphologic criteria for distinguish-

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ing between living and dead parasites. This experimental model thus has allowed us to compare the delivery of lysosomal constituents to vacuoles containing living or dead parasites. In many instances this comparison could even be made in a single cell.

Materials and Methods

The parasites, cells, and media were prepared and used as described in the accompanying paper (8). The technique previously detailed for bringing toxoplasmas into contact with cells by centrifugation at 4°C was utilized for all these experiments (8). Zero time for each experiment was the time of warming of the toxoplasma-cell mixture to 37°C in a water bath. Specimens were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.4, and prepared for electron microscopy as described (8).

For thorotrast (thorium dioxide; Fellows Testagar, Detroit, Mich.) or ferritin (Pentex Biochemical, Kankakee, Ill.) labeling, the macrophages were first cultivated in 40% heat-inactivated newborn calf serum (HINCS)¹ (Grand Island Biological Co., Grand Island, N.Y.) and minimal essential media (MEM) (Microbiological Associates, Inc., Bethesda, Md.) for 28 hr. The medium was removed and the macrophages were cultured for an additional 18 hr in medium of the same composition containing thorotrast, diluted 1:40, or ferritin, 0.5 mg/ml. Finally, the cultures were washed twice with MEM and cultured for an additional 3 hr in fresh MEM-40% HINCS so that nearly all of the thorotrast or ferritin marker was located in cytoplasmic granules (4). The cover slips were then transferred to glass vials at 4°C and toxoplasmas were centrifuged onto the macrophages.

A preparation of toxoplasmas killed, but well-preserved morphologically, was made by exposure to glutaraldehyde as follows: A toxoplasma suspension in MEM was centrifuged for 10 min at 300 g, and the pellet was resuspended in 2.5% glutaraldehyde for 10 min at 4°C. The material was centrifuged and the pellet resuspended in 0.88 M sucrose for 10 min at 4°C. The toxoplasmas were then washed two times in a large volume of MEM and finally resuspended in MEM and 20% HIFCS to a concentration of 4×10^6 toxoplasmas/ml. The glutaraldehyde-killed toxoplasmas were taken up by macrophages (but not by fibroblasts or He³La cells) and were not toxic for the cells.

Specimens were prepared for acid phosphatase cytochemistry using standard Gomori techniques (9) as modified for electron microscopy (10). Specimens were fixed for 30 min in 2.5% glutaraldehyde at 4°C, washed once with 0.88 M sucrose and maintained in 0.88 M sucrose for 15 min at 4°C, overlaid with β -glycerophosphate for 30 min at 37°C, washed extensively with 0.05 M cacodylate buffer containing 5% sucrose, fixed in glutaraldehyde- and osmium-mixed fixative (1:2) for 75 min, postfixed in 0.25% uranyl acetate, and embedded in Epon.

Assessment of delivery to vacuoles containing the living or the degraded toxoplasmas of thorotrast or ferritin secondary lysosome label, or of acid phosphatase primary or secondary lysosome label, was made by counting 100 vacuoles, using sections from several levels of different blocks. Only those vacuoles that were situated within approximately 1 μ of three or more thorotrast- or ferritin-laden granules were counted.

RESULTS

Labeling of Macrophage Granules with Thorotrast.—Macrophages in culture when stimulated in the presence of 40% newborn calf serum formed large numbers of secondary lysosomal granules. These granules could be readily

¹ Abbreviations used in this paper: HINCS, heat-inactivated newborn calf serum; MEM, minimal essential medium.

labeled with thorotrast or ferritin by incubating the cells overnight with these materials. To insure that the label was primarily in secondary lysosomes rather than pinocytic vacuoles, a 3 hr chase period was used as recommended by

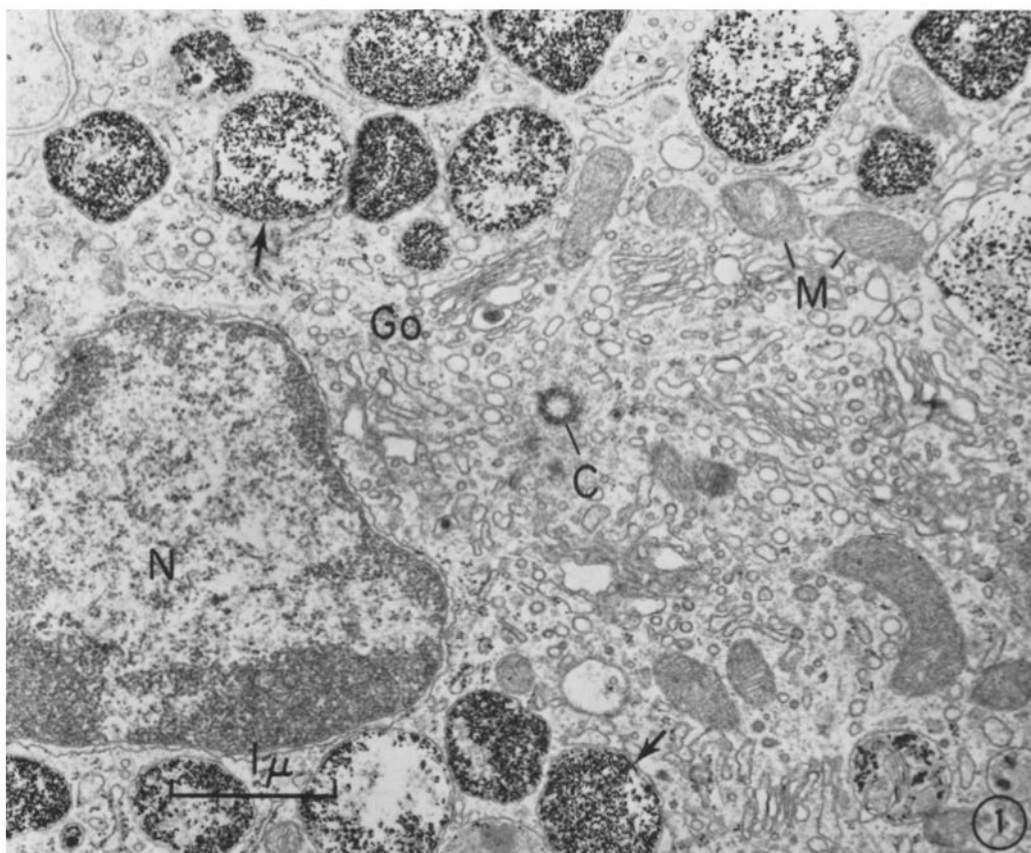


FIG. 1. Electron micrograph of the perinuclear region of a macrophage, illustrating the labeling of secondary lysosomal granules with thorotrast. The nucleus (*N*) is at lower left. In the centrosomal region are seen a centriole (*C*), and several stacks of flattened saccules and numerous small vesicles of the Golgi apparatus (*Go*). Microtubules and a few coated vesicles are also visible in the centrosomal area. At the periphery of the Golgi region are mitochondria (*M*) and numerous large, membrane-bounded granules (*arrows*) packed with the very electron-opaque thorium dioxide particles. $\times 22,000$.

Armstrong and Hart (4). Identical results were obtained in cells labeled with ferritin or with thorotrast. The thorotrast label seemed generally superior, however, since it was nontoxic, did not seem to alter the behavior of the lysosomes, and was easily seen even in stained sections. Only illustrations of

the thorotrast-labeled cells are presented here. Fig. 1 shows an electron photomicrograph of a macrophage after labeling with thorotrast. Numerous granules are seen to be labeled heavily with the marker. The label was occasionally seen also in vacuoles. Other organelles, such as Golgi vesicles and saccules, mitochondria, endoplasmic reticulum, and nucleus did not reveal the thorotrast label. The labeled cells showed no toxicity at the phase-contrast level and exhibited normal pinocytosis and phagocytosis.

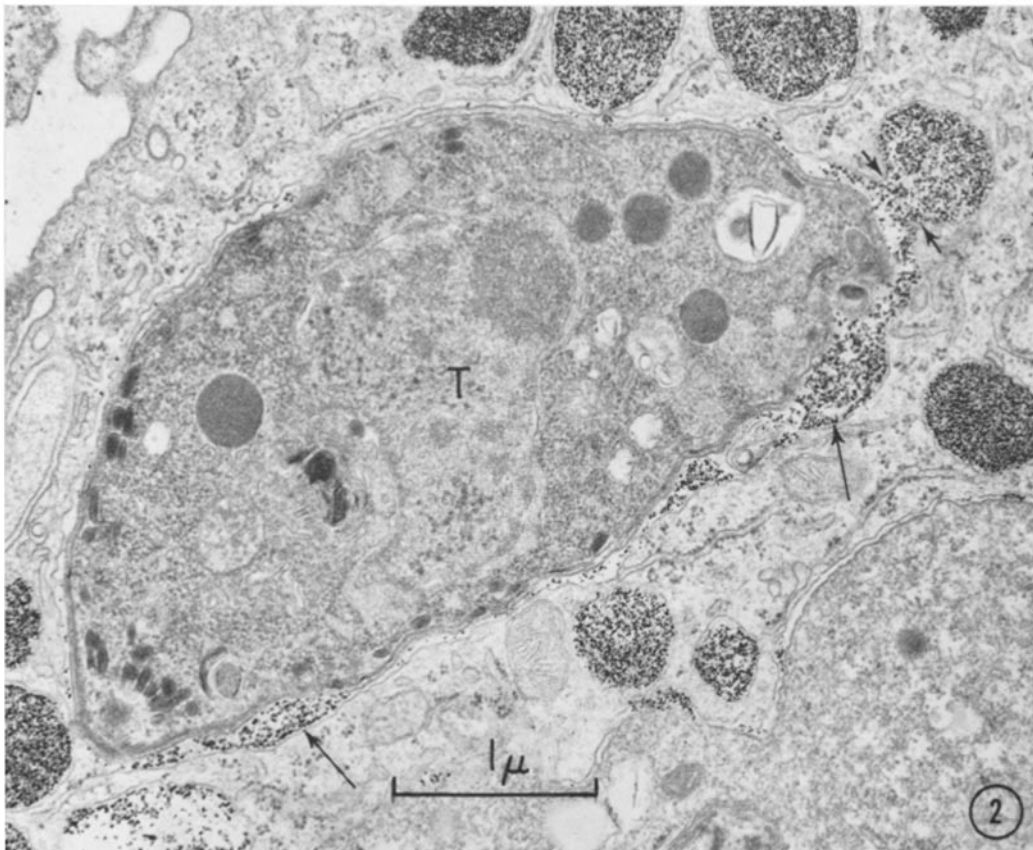


FIG. 2. A glutaraldehyde-killed toxoplasma 1 hr after phagocytosis by a thorotrast-labeled macrophage. The toxoplasma (*T*) remains reasonably normal morphologically, although there is some increase in overall electron opacity and the ultrastructure is not crisply defined (compare to Fig. 1 of the preceding paper, or to the cross section of a living toxoplasma in Fig. 7 below). The toxoplasma lies in a phagocytic vacuole, which contains abundant thorotrast marker (*long arrows*). This specimen was fixed during or shortly after the fusion between a granule and the vacuole, as illustrated at upper right (*two short arrows*). A portion of another toxoplasma in a vacuole is seen at the lower right. $\times 27,000$.

Delivery of Thorotrast from Macrophage Granules to Phagocytic Vacuoles Containing Dead Toxoplasmas.—To evaluate the reliability of the thorotrast label as a marker for fusion of secondary lysosomal granules with phagocytic vacuoles, we examined labeled macrophages that had engulfed killed toxoplasmas. Fig. 2 shows an example of delivery of thorotrast from secondary lysosomes to a vacuole containing a glutaraldehyde-killed toxoplasma. 90% of these vacuoles were positive for thorotrast (Table I).

As described in the accompanying paper, approximately half of the parasites which entered macrophages were digested in a few hours, whereas the other half remained viable, divided, and caused eventual rupture of the cell. The

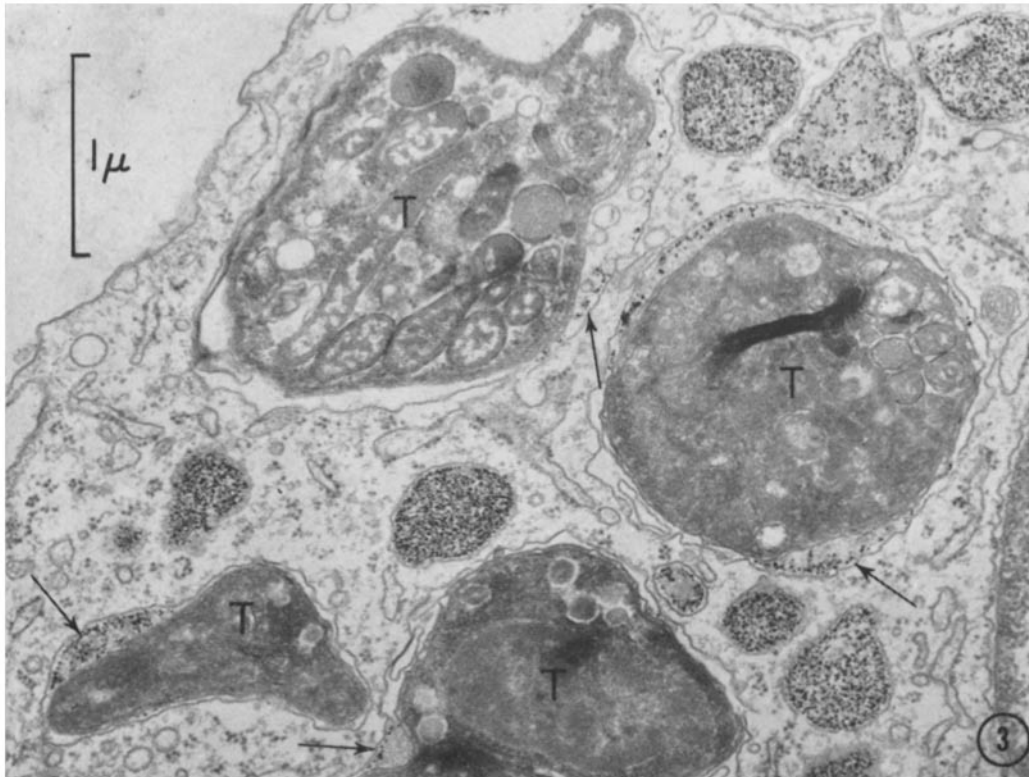


FIG. 3. A thorotrast-labeled macrophage fixed 1 hr after the ingestion of toxoplasmas. A rim of the nucleus is seen at the lower right, and a portion of the cell surface is at upper left. Four dead toxoplasmas (*T*) are present in this field. The organisms exhibit general increase in electron opacity and marked loss of ultrastructural definition, although some organelles are still recognizable (e.g., saccular or "paired" organelles in the parasite at upper left). Each dead toxoplasma lies in a well-defined vacuole, and each of these vacuoles contains the thorotrast marker (*arrows*) derived from macrophage granules. $\times 27,000$.

TABLE I
Delivery of Thorotrast to Vacuoles Containing Various Types of Toxoplasmas

Content of vacuoles	Per cent of vacuoles positive for thorotrast
	%
"Normal" toxoplasmas	0
Degenerating toxoplasmas	88
Glutaraldehyde-fixed toxoplasmas	90

viable and nonviable intracellular toxoplasmas were readily distinguishable from one another by electron microscopy at 1 hr after infection. The nonviable toxoplasmas demonstrated at this time general increased electron opacity, loss of definition of cytoplasmic organelles, vacuolization, and separation or coalescence of membranes. Examination of specimens fixed earlier or later than 1 hr after uptake supported the conclusion that these changes were a stage in the degradation of the approximately 50% of parasites destroyed in macrophages; at 15–30 min only minimal morphologic changes were seen, whereas at 6 hr, there remained few identifiable features of the parasites that had been degraded. The numbers and the morphology of the surviving half of the toxoplasma population remained constant during the 6 hr after uptake, and all of these organisms then proceeded to multiply with a generation time of ~8 hr. In specimens fixed 1 hr after the phagocytosis of living toxoplasmas, it was possible to determine the frequency of delivery of thorotrast from secondary lysosomes to the vacuoles containing the dead parasites. Fig. 3 illustrates several of these degenerating toxoplasmas in a macrophage; each of the vacuoles contains thorotrast particles. As is shown in Table I, nearly 90% of the vacuoles containing these altered toxoplasmas were thorotrast positive.

Absence of Lysosomal Fusion with Vacuoles Containing Living Toxoplasmas.—In sharp contrast to the above findings establishing the fusion of macrophage granules with phagocytic vacuoles containing dead toxoplasmas, vacuoles containing living toxoplasmas, as indicated by the normal ultrastructure of the parasites or by their division and rosette formation at later time periods, remained thorotrast negative. This appeared to be an absolute correlation; in examinations of several different specimens and of hundreds of vacuoles containing the living toxoplasmas, we have never encountered a single instance of delivery of thorotrast marker from lysosomal granules to the phagocytic vacuole (Table I).

Morphologic study revealed frequently the presence of a thorotrast-positive vacuole containing a degenerating toxoplasma situated in the same cell nearby to a thorotrast-negative vacuole containing a living parasite (see below), thus establishing that the factors determining fusion were acting locally in the vicinity of the vacuole, rather than influencing physiology of the entire cell.

At later time periods after infection of thorotrast-labeled cells, electron microscope sections were obtained of dividing toxoplasmas in vacuoles. Fig. 4 shows a parasite dividing by endodyogeny, an unusual process by which two daughter cells are formed within the parent toxoplasma, as described in detail elsewhere (11). Later in the course of infection, many cells showed vacuoles containing rosettes of toxoplasmas. Fig. 5 shows such a cell fixed 20 hr after infection. At time periods beyond 6 hr after infection, all intracellular toxoplasmas appeared healthy morphologically and no thorotrast label was present in any of the vacuoles.

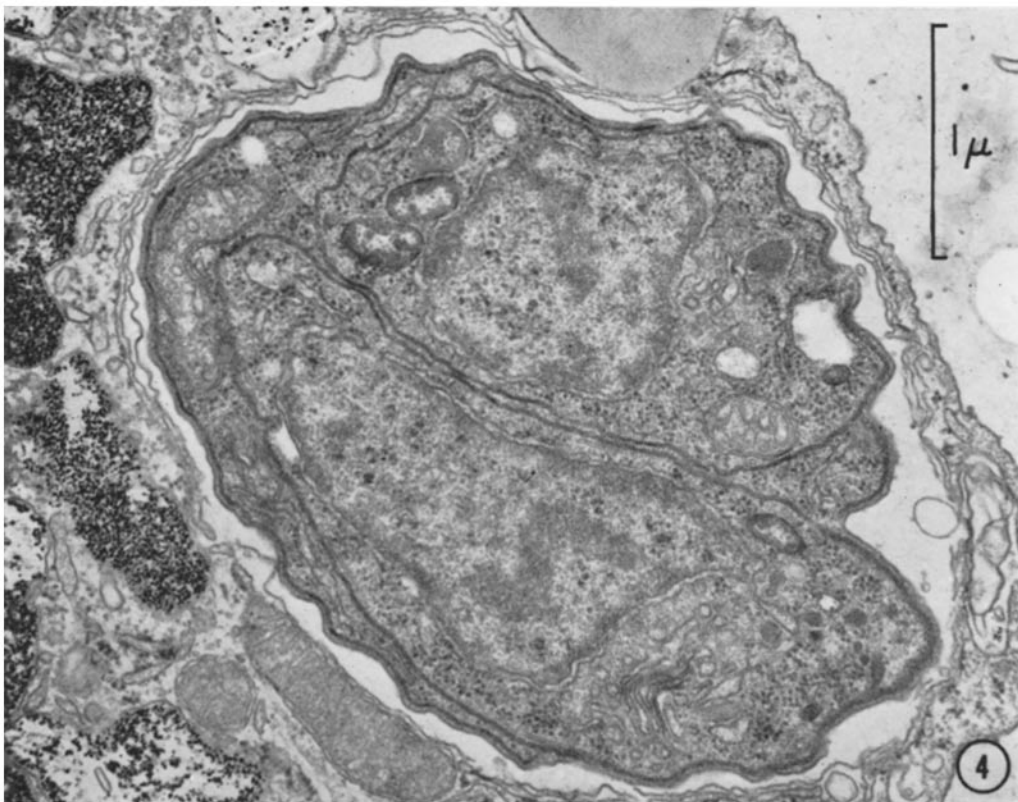


FIG. 4. A toxoplasma dividing by endodyogeny, in a phagocytic vacuole within a thorotrast-labeled macrophage. The vacuole is thorotrast negative, despite the presence of thorotrast-laden granules and vacuoles in the cytoplasm nearby. "Overcoating" of the vacuole by mitochondria and endoplasmic reticulum is evident. The toxoplasma has been separated into two daughter cells by a membrane that has an appearance identical with the inner membrane at the parasite surface. $\times 31,000$.

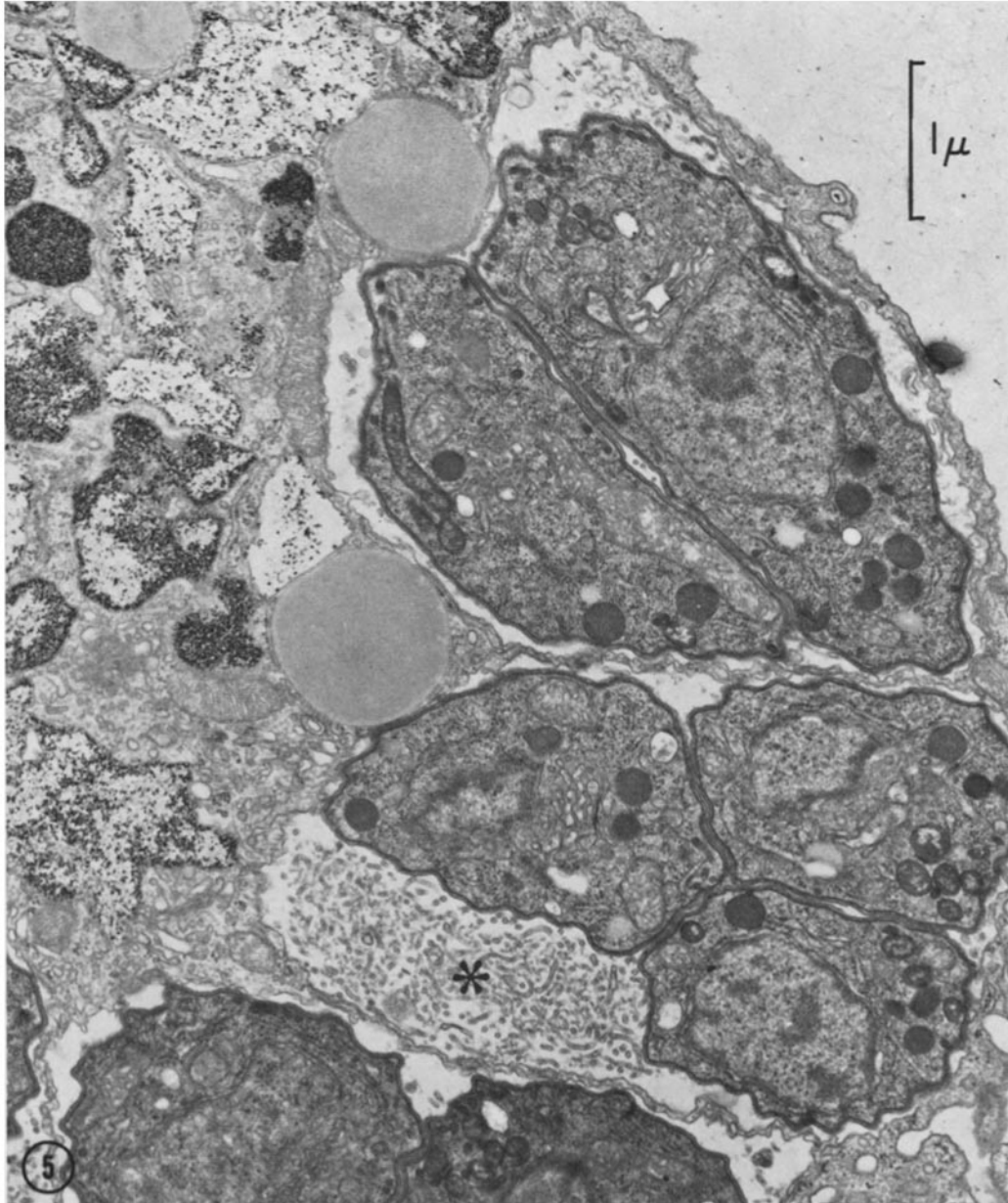
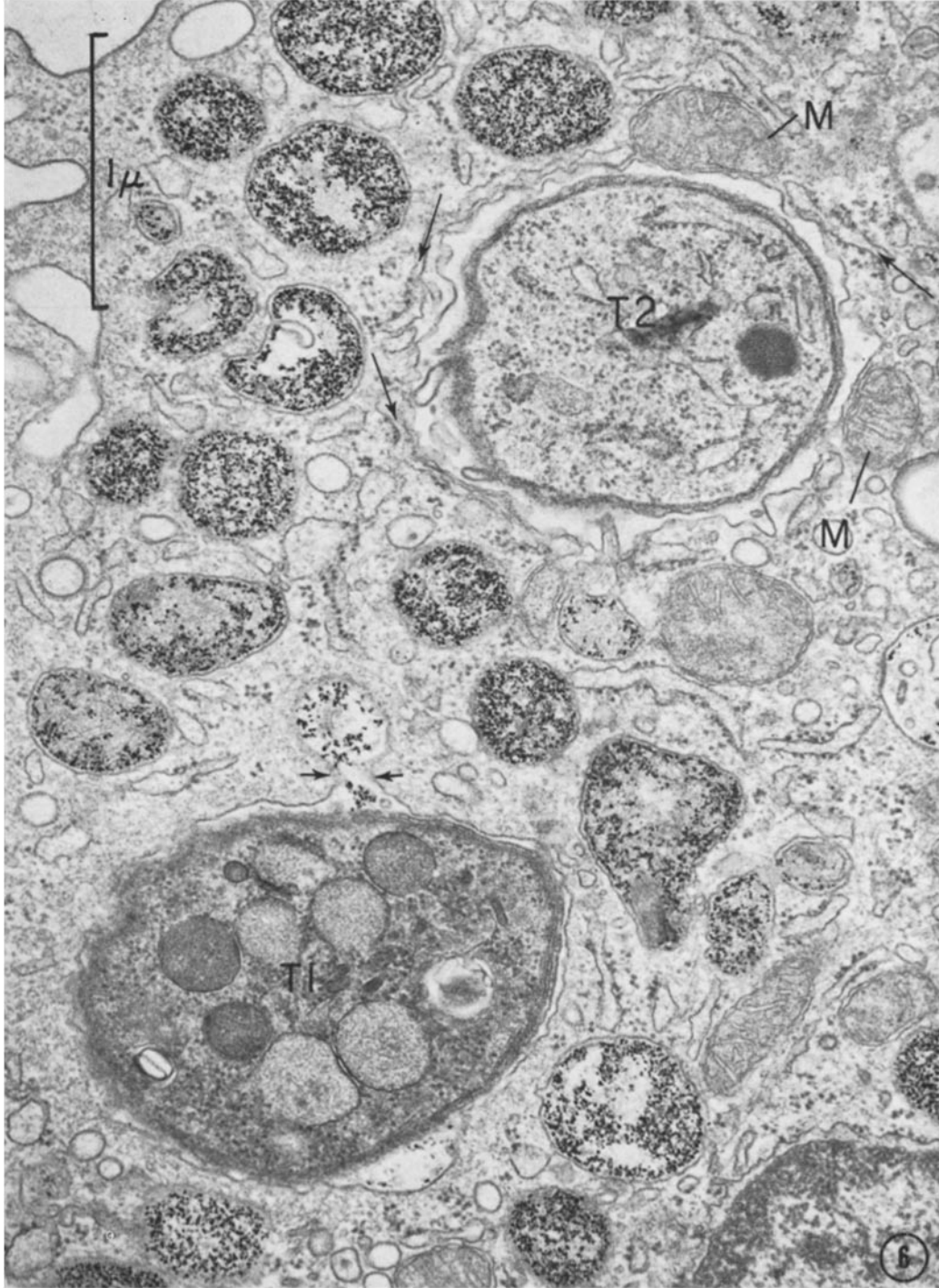


FIG. 5. A thorotrast-labeled macrophage fixed 20 hr after infection with toxoplasmas. The parasites have divided two or three times to form pairs or rosettes. The toxoplasmas exhibit normal ultrastructural appearance, and they lie in vacuoles that remain negative for thorotrast, despite the presence of numerous thorotrast-laden granules and vacuoles in the nearby cytoplasm, and despite the prolonged incubation period. Impressive arrays of microvillous structures (*) are seen in one vacuole. In some places mitochondria and endoplasmic reticulum are closely apposed to the vacuoles. $\times 22,000$.



For legend Fig. 6 see page 1182.

When thorotrast was added to the macrophage cultures after the cells had engulfed toxoplasmas, pinocytic vesicles, granules, and phagocytic vacuoles containing dead toxoplasmas all became labeled. 4 hr later the vacuoles containing living toxoplasmas remained devoid of thorotrast, indicating that they did not fuse with pinocytic vesicles or with other phagocytic vacuoles, in addition to their resistance to fusion with secondary lysosomes.

Envelopment of Vacuoles Containing Living Toxoplasmas with Mitochondria and Endoplasmic Reticulum.—In addition to the striking difference in acquisition of lysosomal label, the vacuoles containing living and dead toxoplasmas also differed in terms of their relationship to other organelles. As is illustrated in Figs. 6-8, the vacuoles containing morphologically normal toxoplasmas were surrounded by a layer of closely apposed mitochondria or endoplasmic reticulum, in contrast to the absence of such a relationship in vacuoles containing the degenerating parasites. The "overcoating" of vacuoles containing the living toxoplasmas was in some instances detectable within minutes of phagocytosis of the parasites, and the phenomenon was still apparent at 20 hr after infection (see Fig. 5). There was no detectable difference in the dimensions or in the trilaminar ultrastructure of the membranes of vacuoles containing living toxoplasmas as compared with the membranes of vacuoles containing degenerating parasites. No significant alteration was seen in the appearance of the thin membranes of endoplasmic reticulum and mitochondria adherent to the vacuoles containing living toxoplasmas.

One other morphologic feature notable in the vacuoles containing living toxoplasmas was the appearance of tiny invaginations of the vacuolar membrane. These often formed impressive arrays of microvilli in the phagocytic vacuole (see Fig. 5).

In order to determine whether the morphologic changes in the vicinity of vacuoles containing living toxoplasmas were seen *in vivo* as they were *in vitro*,

FIG. 6. A thorotrast-labeled macrophage, demonstrating differences between vacuoles containing living and dead toxoplasmas. A portion of the nucleus is seen at lower right, and the cell surface is at upper left. Numerous granules packed with thorotrast particles are distributed in the cytoplasm. The specimen was fixed 1 hr after entry of the toxoplasmas. The dead toxoplasma (*T1*) shows increased general electron opacity and some loss of ultrastructural definition. It lies in a vacuole that contains thorotrast; a granule has apparently just fused with the vacuole (*short arrows*). The cytoplasm surrounding the vacuole appears unaltered. The living toxoplasma (*T2*) is also located in a well-defined vacuole, but no thorotrast particles are seen within this vacuole. The vacuolar membrane has a highly irregular contour, and in several places mitochondria (*M*) or endoplasmic reticulum (*long arrows*) are closely apposed to the vacuole. $\times 40,000$.

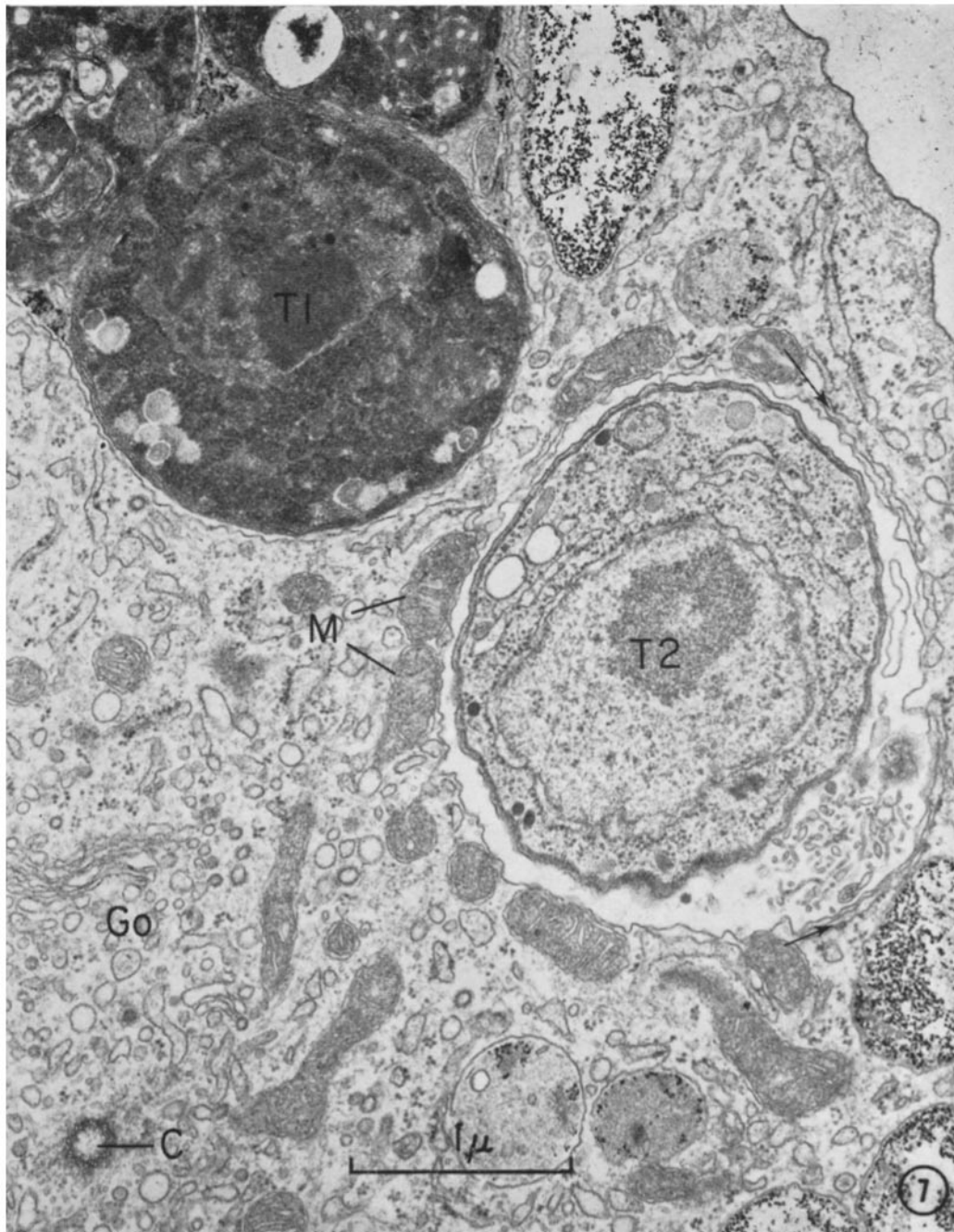


FIG. 7. A thorotrast-labeled macrophage 1 hr after uptake of toxoplasmas. Centriole (C) and Golgi region (Go) are at lower left; the cell surface is seen at upper right. The vacuole at upper left contains several dead toxoplasmas (e.g. T1), which show marked increase in electron opacity and loss of ultrastructural detail. The vacuole contains thorotrast, and the vacuolar membrane shows no notable association with cytoplasmic organelles. The living toxoplasma (T2) shows normal morphology; well defined in this cross section are nucleus and nucleolus, rough endoplasmic reticulum, dense granules, peripheral microtubules, and the inner and outer membranes. The vacuole contains no thorotrast particles. Microvilli are present in the vacuole at lower right. Host cell mitochondria (M) and endoplasmic reticulum (arrows) are in several places closely apposed to the vacuolar membrane. $\times 32,000$.

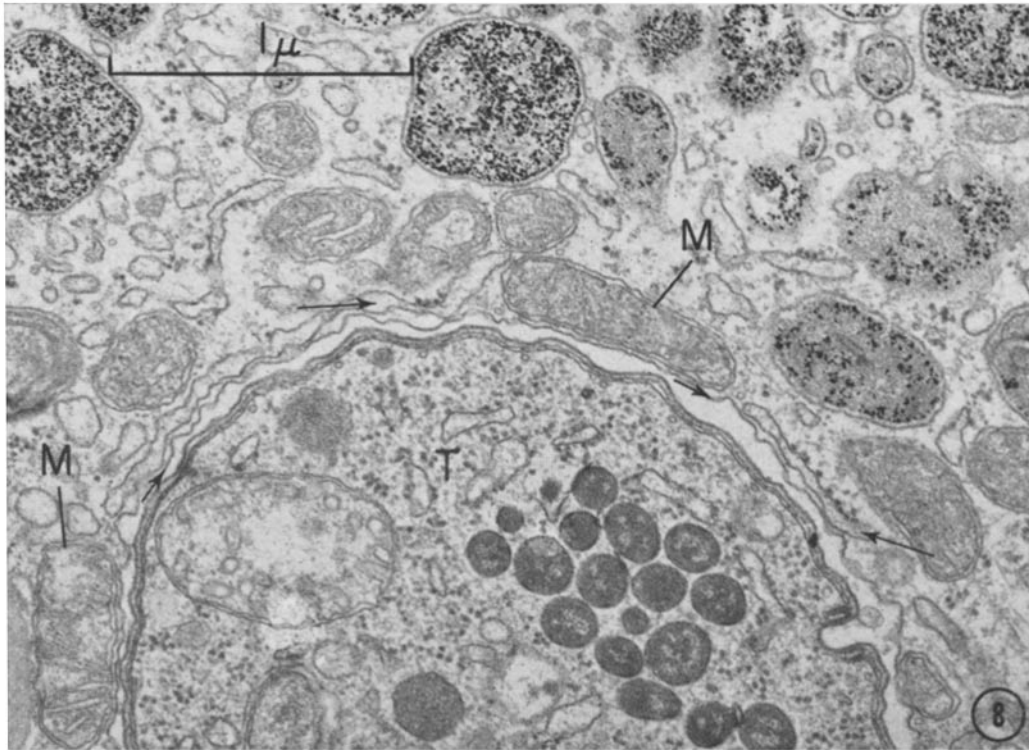


FIG. 8. A high magnification of a living toxoplasma in a phagocytic vacuole of a thorotrast-labeled macrophage. The toxoplasma (*T*) shows various cytoplasmic components, including a mitochondrion at left and the "paired" organelles in cross section at right. The protozoal membranes are well defined, and numerous microtubules (seen here in cross section as tiny circles) are located just beneath the inner membrane. The vacuole contains no thorotrast particles. The vacuolar membrane (*short arrows*) is not detectably altered or interrupted. Closely apposed to the vacuolar membrane are macrophage mitochondria (*M*), and stumps of endoplasmic reticulum (*long arrows*) with a few ribosomes attached to the outer leaflet. $\times 40,000$.

large numbers of toxoplasmas were injected intraperitoneally into mice; 2 hr later peritoneal cells were fixed *in situ* by injection of 2.5% glutaraldehyde, and were then harvested and processed for electron microscopy. The morphologic features recorded in the *in vitro* experiments reported above were the same as those seen in these specimens from the infected peritoneal cavity.

Vacuolar microvillus formation and vacuole envelopment by endoplasmic reticulum and mitochondria were also seen in the two other cell lines studied, L929 fibroblasts and HeLa cells. Figs. 9 and 10 show these changes in a fibroblast 3 hr after infection with toxoplasmas.

Absence of Fusion of Primary Lysosomes with Vacuoles Containing Living Toxoplasmas.—The above studies indicated that the secondary lysosomal granules of macrophages did not fuse with vacuoles containing living toxoplasmas. Macrophages also have primary lysosomes, which morphologically appear as Golgi-associated vesicles. The primary lysosomes by definition have not fused with pinocytic or phagocytic vacuoles, and thus could not be labeled

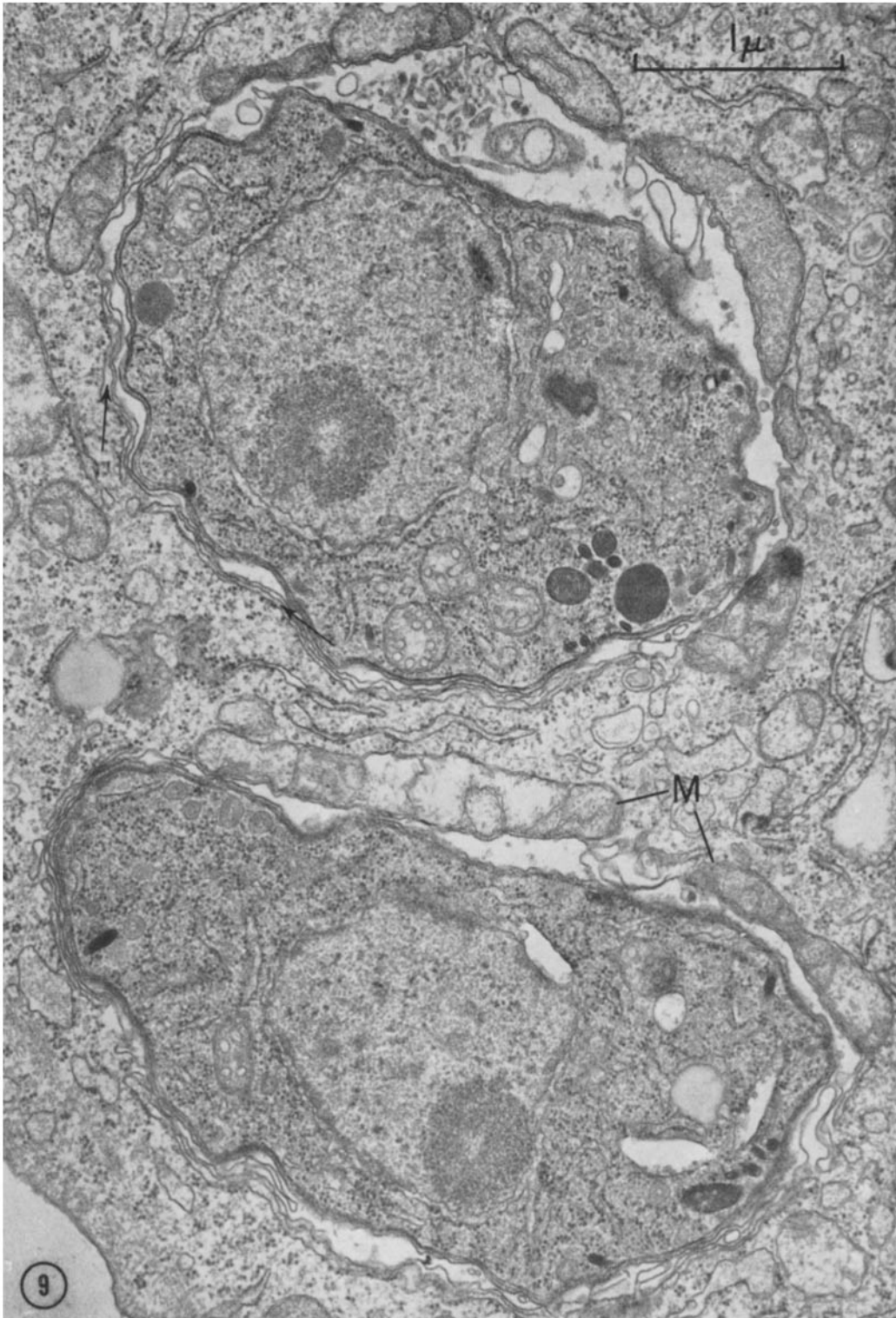


FIG. 9. Two toxoplasmas in a mouse fibroblast fixed 3 hr after parasite entry. The toxoplasmas show normal ultrastructural detail. They are located in vacuoles. Closely apposed to the vacuolar membrane are poorly fixed mitochondria (*M*) and strips of endoplasmic reticulum (*arrows*). $\times 31,500$.

with thorotrast or other particulate markers taken in by endocytosis. The acid phosphatase cytochemical reaction detects enzyme in both primary and secondary lysosomes, and studies were therefore done to determine whether or not vacuoles containing living toxoplasmas acquired this lysosomal hydrolase.

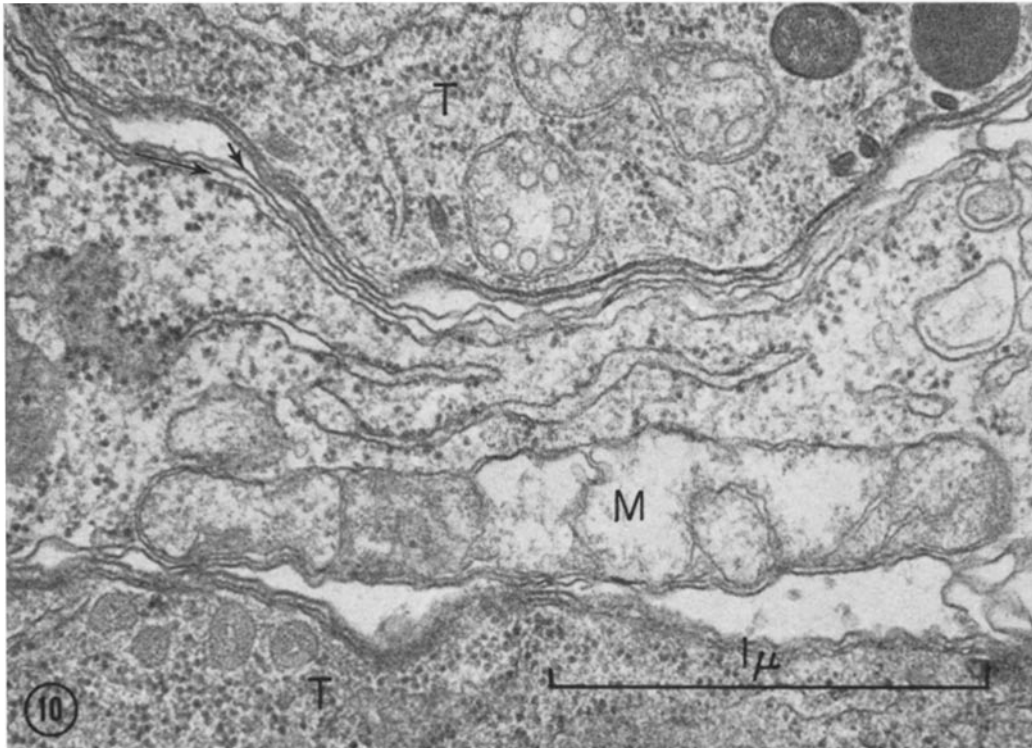


FIG. 10. A higher magnification illustrating the "overcoating" of vacuoles containing toxoplasmas in fibroblasts. The toxoplasmas (*T*) appear normal. The vacuolar membrane (*short arrow*) is well defined, and appears to be closely associated with or bound to the fibroblast endoplasmic reticulum (*long arrow*) or to mitochondrial (*M*) outer membrane. $\times 57,500$.

TABLE II

Acid Phosphatase Reactivity of Vacuoles Containing Various Types of Toxoplasmas

Content of vacuoles	Per cent of vacuoles positive for acid phosphatase
	%
"Normal" toxoplasmas	4
Degenerating toxoplasmas	83
Glutaraldehyde-fixed toxoplasmas	68

A modification of a cytochemical technique previously described (10) for acid phosphatase activity gave the best results. In macrophages fixed 1 hr after phagocytosis of toxoplasmas, 83% of the vacuoles containing degenerating parasites exhibited acid phosphatase reaction product, whereas only 4% of vacuoles containing apparently viable parasites were positive for acid phos-

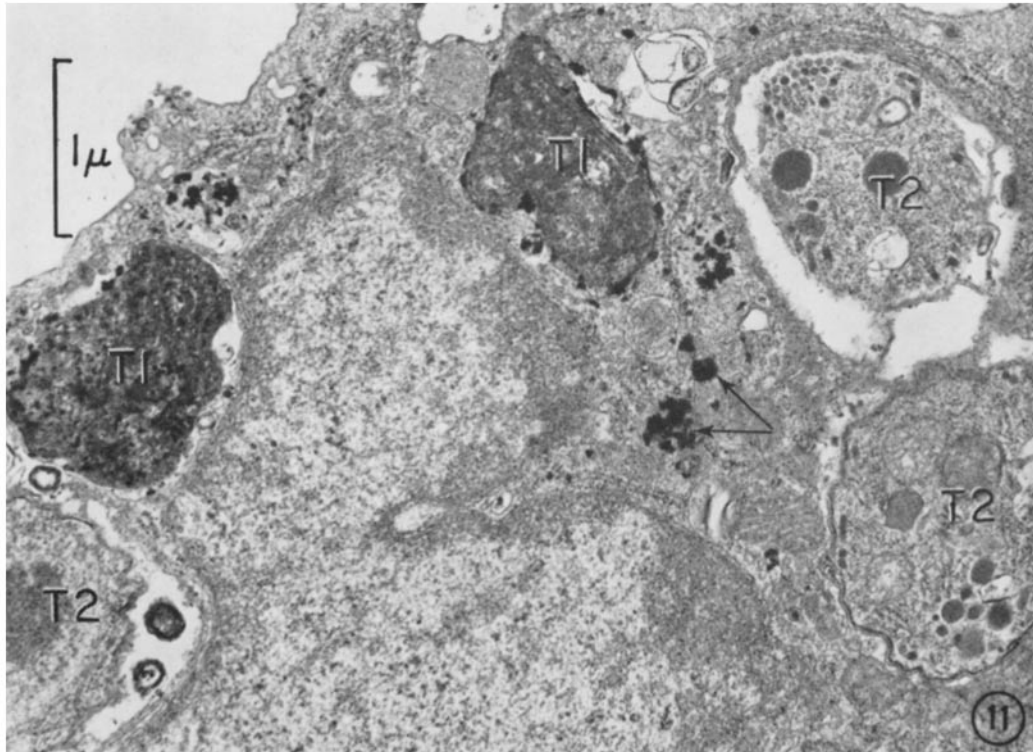


FIG. 11. Acid phosphatase reactivity of vacuoles containing apparently living or dead toxoplasmas in a macrophage fixed 1 hr after phagocytosis of the parasites. The electron-opaque lead phosphate reaction product is seen in the Golgi region (*arrows*), and in vacuoles containing degenerating toxoplasmas (*T1*). In contrast, the vacuoles containing apparently living toxoplasmas (*T2*) are negative for acid phosphatase. $\times 23,500$.

phatase (Table II). 68% of vacuoles containing glutaraldehyde-killed parasites were positive for acid phosphatase. Fig. 11 illustrates the difference in acid phosphatase reactivity of vacuoles containing living and degenerating toxoplasmas.

At later stages of macrophage infection by toxoplasmas, vacuoles containing rosettes of parasites remained negative for acid phosphatase, as illustrated in Fig. 12. These observations were thus consistent with the view that primary

lysosomes as well as secondary lysosomes did not fuse with vacuoles containing living toxoplasmas.

Attempts were also made to study acid phosphatase delivery to vacuoles containing toxoplasmas in mouse fibroblasts. These vacuoles were negative, but the general level of acid phosphatase reactivity in fibroblasts was so low that it was difficult to place significance on this result.

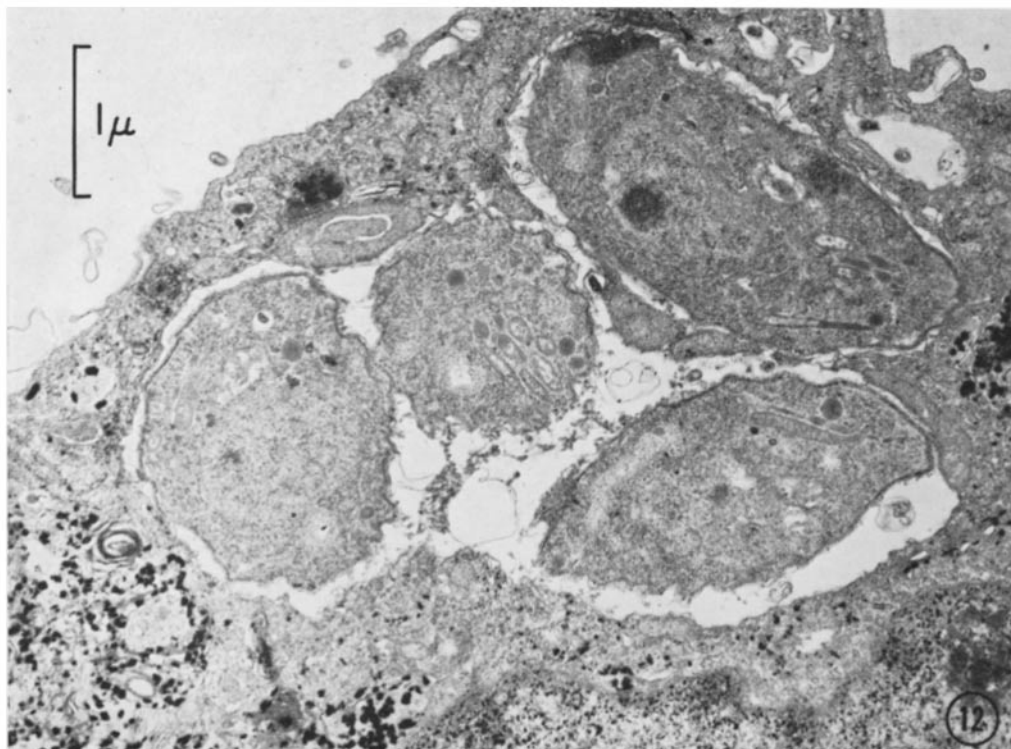


FIG. 12. Acid phosphatase reaction on a macrophage fixed 18 hr after infection with toxoplasmas. The organisms have multiplied to form pairs or rosettes. The vacuoles containing toxoplasmas remain negative for acid phosphatase. A positive reaction is seen in debris-filled vacuoles nearby and in perinuclear cytoplasmic vesicles. Nonspecific reaction product is present over the nucleus. $\times 20,000$.

DISCUSSION

The results of this study establish that lysosomal constituents are not delivered to phagocytic vacuoles harboring living toxoplasmas. This was almost certainly due to effects exerted by the parasite locally, probably as a result of secretion of some substance that altered the vacuolar membrane. There was no evidence of effects on general physiology of the cell, such as movement of cytoplasmic organelles, or over-all fusibility of lysosomes, since infected cells

exhibited normal shape and motility when observed by phase-contrast cinematography, and frequently we saw in a single cell adjacent vacuoles containing living and dead parasites, with normal delivery of lysosomal factors to the vacuole containing the dead toxoplasma. The lack of lysosomal fusion with vacuoles containing living toxoplasmas may reflect a failure to activate the fusion mechanism (i.e. toxoplasmas, unlike most ingested particles, may not alter the vacuolar membrane in such a way as to make it fusible with lysosomes) or it may reflect blocking by some unusual membrane alteration of the normal fusion-promoting mechanism. Whether the living toxoplasmas act by blocking the fusion mechanism, or whether they simply fail to activate it, might be determined if some procedure could be devised for introducing living and dead toxoplasmas into a single vacuole.

Envelopment of the vacuoles containing viable toxoplasmas with endoplasmic reticulum and mitochondria occurred in the early minutes after ingestion, and remained prominent throughout the course of parasite multiplication in the cell. The mechanism accounting for this overcoating is unknown; all we can say is that the vacuolar membrane is altered in such a way that it becomes attractive for these cytoplasmic organelles. The functional significance of the close approximation of endoplasmic reticulum and mitochondria to the vacuoles containing viable toxoplasmas is also unclear, but several possibilities come to mind: (*a*) the phenomenon may reflect a response of the host cell to the abnormal vacuole, attempting to segregate it by a process somewhat analogous to some types of autophagy; (*b*) the overcoating may account, at least in part, for the absence of lysosomal fusion, by preventing contact between lysosomes and the phagocytic vacuole; or (*c*) the close apposition of endoplasmic reticulum and mitochondria may well have significance in relation to provision to the vacuole of factors necessary for parasite multiplication.

Envelopment of organelles or of cytoplasmic foci with cytomembranes is an early step in formation of autophagic vacuoles, but there are several differences between the various types of autophagy (12, 13) and the process reported here. The enveloping membranes in autophagy are commonly smooth endoplasmic reticulum or Golgi elements, not the mitochondria or rough endoplasmic reticulum seen around the toxoplasma vacuoles. Furthermore in autophagy lysosomal hydrolases are usually delivered to the vacuole and digestion of the contents occurs, whereas in the toxoplasma vacuoles lysosomal delivery does not occur.

To our knowledge there have been no previous reports of this type of association between vacuolar membranes and mitochondria or endoplasmic reticulum. It represents an impressive example of membrane recognition within cells, resulting in membrane-to-membrane attraction without membrane fusion. No alterations were evident in the thickness or the trilaminar ultrastructure of the membrane of the vacuole containing living toxoplasmas, or in the adjacent mitochondrial or endoplasmic reticulum membranes.

Another morphologic feature, previously described by Sheffield and Melton

(11), indicating alteration of the membrane of the vacuole by living toxoplasmas was the presence of microvillus protrusions of the membrane into the vacuole, forming in many cases arrays of small vermiform structures (see Fig. 5, for example). Protrusions of vacuolar membrane have also been seen in cells infected with another protozoan, *Eimeria bovis* (14). These invaginations clearly increase markedly the surface area of the vacuolar membrane, and they may be of importance in transferring to the vacuole some cytoplasmic factors required for parasite survival or growth.

One interesting observation not yet fully explained is the heterogeneous behavior of toxoplasmas taken into macrophages: approximately half the parasites survived and these were situated in vacuoles that did not acquire lysosomal constituents, whereas the other half of the toxoplasmas were situated in phagolysosomes and were rapidly killed and degraded. Does this variation in outcome reflect a heterogeneity in the parasite population, or a heterogeneity in the phagocytic cells? The toxoplasmas used for infecting the macrophages were freshly harvested from the peritoneal cavity of infected mice. They had a uniform appearance and were over 95% viable, at least in terms of trypan blue dye exclusion. One interpretation is that half of the infecting toxoplasmas, although viable, did not produce or deliver the substance responsible for altering the vacuolar membrane so as to prevent lysosomal fusion. This hypothesis seems reasonable and it is in keeping with the fact that alteration in parasite to macrophage infectivity ratio did not alter the proportion of parasites that survived and died. As mentioned earlier (8), these "defective" parasites may be the same ones that were incapable of inducing phagocytosis in HeLa cells.

It is also possible that the division of the intracellular toxoplasmas into living and dead populations is a reflection of cellular factors, not variability in the viability or virulence of the parasites. Although there may be some variations from cell to cell in the capacity of macrophages to destroy toxoplasmas, such variation could not account entirely for the differences in fates of ingested parasites, since, as mentioned above, living and dead toxoplasmas were frequently seen in vacuoles lying side by side in the same cell. The fate of toxoplasmas in macrophages might be determined in the first moments after their uptake by the rate of conversion of the vacuole to the resistant (i.e. no longer fusible with lysosomes) state in comparison with the rate of contact and fusion with lysosomes. For instance, one might speculate that it takes 1 min for the parasite to alter the vacuolar membrane, and that there is a 50% chance of a vacuole encountering and fusing with a lysosome during that 1st min. If this were the case, one might expect that highly activated macrophages, containing large numbers of granules as a result of culture in high serum concentrations, would kill a higher proportion of ingested toxoplasmas than would unstimulated monocytes or macrophages with few cytoplasmic granules. However, when these cells were compared, it was found that toxoplasmas in them behaved

the same, i.e., approximately half of the parasites died and half survived and multiplied.

The thorotrast marker for lysosomes was readily visualized and was not cytotoxic, whereas the ferritin used by us and by Armstrong and Hart (4) was difficult to detect in stained sections, and many lots of ferritin were cytotoxic for macrophages. With either of these markers for secondary lysosomes it was clear that vacuoles containing living toxoplasmas did not fuse with macrophage granules, nor did they fuse with pinocytotic vesicles or with other phagocytic vacuoles.

The study of acid phosphatase cytochemistry at the ultrastructural level established with our material that this enzyme does not appear in vacuoles containing living toxoplasmas, thus suggesting that neither primary nor secondary lysosomes fused with these vacuoles. This conclusion requires a note of caution, however, since it is possible that the living toxoplasmas altered the vacuolar conditions so as to block acid phosphatase reactivity at this site, even if the enzyme had been delivered from primary lysosomes. This possibility seems unlikely, but we cannot rule it out. Primary lysosome markers not dependent on enzyme activity would be required to exclude this possibility; such markers are not available.

The results reported in this study were obtained using the highly mouse-virulent RH strain of *Toxoplasma gondii*. Preliminary studies using less virulent strains of toxoplasmas, which form cysts in mice, have shown similar ultrastructural changes in the infected cells. Further investigations on these attenuated strains may allow us to identify some of the properties of toxoplasmas that contribute to their virulence. At present, only the generation time in tissue cultures has been correlated with degree of virulence (15).

Acquired immunity to toxoplasmosis is apparently very effective in protecting against reinfection (16). Antibodies and complement will kill the parasites *in vitro* (17), so that humoral immunity no doubt plays some role, but most recent workers have emphasized the importance of acquired cellular immunity (18). The role of "activated" macrophages in toxoplasma infections (19) has been shown to be analogous to the cellular immunity to listeria infection, studied by Mackaness (20). In preliminary observations on interaction between the RH strain of toxoplasma and activated macrophages obtained from mice infected with *Listeria monocytogenes*, we have not been able to detect any differences from the results reported here. Further studies need to be made on this point, however, especially with less virulent strains of toxoplasmas and with macrophages obtained from animals immunized with other agents, including toxoplasmas.

The striking correlation between failure of delivery of lysosomes to the vacuole and survival and growth of toxoplasmas, as contrasted with death and digestion of toxoplasmas in vacuoles converted to phagolysosomes, suggests

that lysosomal factors exert antimicrobial, or more properly antiprotozoal action in macrophages. The nature of antimicrobial agents inside of macrophages has never been clearly established (1). No information is available on whether or not toxoplasmas also inhibit production or delivery to the vacuole of nonlysosomal metabolic products, such as lactic acid and hydrogen peroxide.

Granulocytes have in their cytoplasm large numbers of azurophil primary lysosomal granules, and of specific granules that do not contain acid hydrolases (10). Both of these granule types fuse with phagocytic vacuoles containing various microorganisms or particles (21). It was therefore of interest to observe the fate of toxoplasmas in granulocytes, and to determine if this fate was correlated with degranulation as was the case in macrophages. We have attempted to make these observations, under various conditions, but thus far have been unable to stimulate uptake of viable toxoplasmas by neutrophils. In thin slide-cover slip preparations polymorphonuclear leukocytes turn aside when they encounter toxoplasmas; apparently the organisms have a surface that does not attach to granulocytes. Dead toxoplasmas, or toxoplasmas coated with antibody, are engulfed by polymorphonuclear leukocytes, and degranulation follows phagocytosis of these organisms.

SUMMARY

Electron microscope methods have been used to study delivery of macrophage primary or secondary lysosomal contents to phagocytic vacuoles containing living or dead toxoplasmas. Secondary lysosomes were labeled by culturing the cells in colloidal thorium dioxide (thorotrast) or in ferritin. Acid phosphatase cytochemistry was employed for detection of primary as well as secondary lysosomal constituents. These various lysosomal labels were present in nearly all vacuoles containing toxoplasmas killed with glutaraldehyde, or in vacuoles containing those parasites undergoing degeneration 1 hr after the uptake of living toxoplasmas. In contrast, at times ranging from 1 to 20 hr after infection, no vacuoles containing morphologically normal, apparently viable toxoplasmas were thorotrast or ferritin positive, and only rarely did these vacuoles react for acid phosphatase. In many instances vacuoles containing viable toxoplasmas and no lysosomal markers were situated in the same cell nearby to vacuoles containing degenerating toxoplasmas and lysosomal constituents, thus indicating that the determinants of lysosomal fusion were operating locally in the immediate vicinity of the phagocytic vacuole, and not operating to influence general cell function. Thus, some toxoplasmas are able to prevent the delivery of lysosomal contents, and apparently the phagocytic vacuole provides for these parasites a sheltered microenvironment ideal for their growth.

Morphologic evidence indicated that living toxoplasmas altered the phagocytic vacuolar membrane in macrophages, fibroblasts, and HeLa cells. Within minutes after phagocytosis, the vacuole became surrounded by closely apposed strips of endoplasmic reticulum and mitochondria; somewhat later, microvillous

protrusions of the membrane into the vacuole were seen. These morphologic features of phagocytic vacuoles containing living toxoplasmas may be of importance in relation to the absence of lysosomal fusion, or they may serve some function in protecting the host cell or in nourishing the parasite.

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