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Interactions Between *Toxoplasma gondii* and Its Host Cells: Function of the Penetration-Enhancing Factor of Toxoplasma

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A protein with a molecular weight of 70,000 to 150,000 which was extracted from merozoites of *Toxoplasma gondii* enhanced the host cell penetration of the merozoites. The optimal pH and temperature for penetration of merozoites coincided with those favoring the action of the penetration-enhancing protein. In addition, a dependence on Ca and Mg existed for penetration of merozoites, either in the presence or absence of this protein. No evidence was found that indicated that the enhancing effect on penetration elicited by the protein was due to increased phagocytic capacity of host cells (HeLa) or improved motility of the merozoites. Electron microscopy demonstrated that the protein, in high concentration, caused disruption of cytoplasmic membranes. In a 100-fold-lower concentration, which still caused a marked enhancement of penetration, no such effect was observed. However, the vacuoles surrounding the penetrated parasites seemed smaller than for merozoites penetrating in cultures to which no penetration-enhancing factor was given, and the membranes limiting the vacuoles demonstrated discontinuities more often. The penetration-enhancing effect of some known enzymes was studied. However, none of these enzymes seemed to correspond to the penetration-enhancing protein of toxoplasma. The mode of entry of toxoplasma merozoites into host cells is discussed. It is concluded that phagocytosis must play a less important role and that merozoites actively penetrate the cytoplasmic membranes of the host cells. The penetration is proposed to be a result of combined mechanical and chemical actions. It is suggested that an enzymatic function of the penetration-enhancing factor released by the merozoites is of importance. The membrane limiting the vacuole of a penetrated merozoite seems to be newly formed in the cell after penetration is completed.

The mode of entry of *Toxoplasma gondii*, a coccidian protozoan, into mammalian cells has been a matter of debate for many years. Pulvertaft et al. (29), Visher and Suter (39), and more recently Jones et al. (14) considered phagocytosis to be the process by which the merozoites reached an intracellular position. Wildfür (40), van der Zypen and Piekarski (37), and Bommer et al. (7) suggested that toxoplasma merozoites penetrated the cytoplasmic membrane mechanically and that structures found at the anterior end of the merozoites were used in the process of penetration. A third hypothesis proposed by Garnham et al. (11), Ludvik (16), and Lycke and Norrby (22) also suggested an active penetration but emphasized, in addition, the possibility of an enzymatic mode of merozoite penetration. From disintegrated toxoplasma merozoites Lycke and Norrby (22) extracted a protein which, if added with the merozoites to cell cultures, increased the number of intracellular parasites. Observations on synthesis and properties of this enzyme-like penetration-enhancing factor (PEF) suggested that it was synthesized and released by the merozoites (25) and was important for their ability to pass into the cells (27). The present paper reports studies on the function and characteristics of PEF and discusses the mechanism of entry into mammalian cells of toxoplasma.

MATERIALS AND METHODS

Toxoplasma parasites. The RH strain of *T. gondii* was used in all experiments. Parasites were collected from the peritoneal cavity of Swiss albino mice 3 days after intraperitoneal infection. Suspensions of parasites, free from exudate fluid and from most of the peritoneal cells, were prepared according to previously described techniques (19). These suspensions were used as test parasites in the cell culture assays of parasite penetration.

Test for assaying the penetration of Toxo-

plasma parasites. The cell culture technique employed was previously described (19). Materials to be tested or, as the control, Hanks balanced salt solution (BSS), were mixed with parasites and inoculated into HeLa cultures. After incubation at 37 C for 19 h, the cultures were read in a phase contrast microscope. The quotient between the number of toxoplasma parasites that had penetrated host cells and the number of exposed cells was determined, and this quotient times 100 was used to indicate the relative number of infecting parasites. The mean and the standard deviation of the mean of 127 standard tests were 50.9 and 1.3, respectively.

Extraction of PEF. The technique has been described in detail previously (22). Briefly, the merozoites were frozen and thawed three times, sonicated for 15 min, and centrifuged at 20,000 $\times$ *g* for 45 min. The supernatant obtained was incubated at 37 C for 1 h and centrifuged at 80,000 $\times$ *g* for 45 min to remove substances inhibiting PEF. After purification by precipitation with $(NH_4)_2SO_4$ and Sephadex G-200 chromatography, it was dialyzed against distilled water for 4 h. The dialyzed material was lyophilized and stored frozen at −90 C. Before use, lyophilized PEF was dissolved in BSS to a concentration of 2.2 mg of protein per ml. This was considered a standard solution of PEF (26). All preparatory work with PEF was performed at temperatures below 5 C.

NBT tests. Two kinds of nitroblue tetrazolium (NBT) tests were used. One was a modification of the method by Park et al. (28), using cover slip cultures with HeLa cells instead of blood smears. The percentage of cells with intracellular deposits of formazan, NBT-positive cells, was determined. The second method was a modified technique according to Baehner and Nathan (5), replacing the leukocytes with cultures of HeLa cells. The formazan produced by the cells was extracted with pyridine, and the amount of formazan recovered was determined spectrophotometrically.

Time lapse cinematography. A Zeiss microkinokamera was used. The magnification employed ranged from 120 to 200 times, and the speed was increased to 10 to 250 times the natural rate.

Electron microscopy. HeLa cell monolayer cultures in plastic 5-cm dishes were inoculated with toxoplasma merozoites. After varying periods of time, glutaraldehyde was added to the culture medium in a final concentration of 3 to 5%. After 2 min the medium was replaced by concentrated glutaraldehyde which, 30 min to 1 h later, was followed by an osmium tetroxide fixative for 60 min. Dehydration was performed stepwise using 70 to 95% methanol. The cell monolayers were separated from the dishes by a method described by Biberfeld (6). Briefly, epoxypropane was added to the culture dishes. After agitation for 5 to 10 s the monolayers separated and could be transferred to glass tubes, pelleted, washed in epoxypropane, and embedded. The method has the advantage of permitting selected areas of the monolayer to be removed before detaching the monolayer. The cells were embedded in Epon by centrifugation at 60 C for 30 min during the initial period of polymerization.

RESULTS

Characteristics of PEF. Biochemical characterization of PEF revealed that it was precipitable at 30% saturation with $(NH_4)_2SO_4$, moved electrophoretically as a slightly acid protein, and was sensitive to treatment with trypsin and phenol (5). It was therefore considered to be a protein. Using Sephadex G-200 chromatography, the molecular weight of PEF was estimated to be within the range of 70,000 to 150,000 (6).

For demonstration of the enhancing activity 1 ng of PEF protein was sufficient. The temperature and pH optima yielding the most pronounced enhancing effect were 37 C (26) and 7.6 (25), respectively.

The following experiments demonstrated that the presence of two divalent cations was important for the entry of toxoplasmas into the host cells. A constant number of toxoplasma merozoites were added to different salt solutions. The suspensions of merozoites were then inoculated into cell cultures, replacing the culture medium. The cultures were observed for intracellular toxoplasmas 8 h after inoculation of the cultures. Figure 1 illustrates the effect various concentrations of Mg. Similarly, the merozoite the effect of PEF. Almost identical results were obtained when Ca was replaced by increased concentrations of Mg. Similarly, the merozoites penetration in cell cultures to which no PEF was added was also favorably influenced by increasing the Ca and Mg concentrations. This dependence on Ca and Mg was not reflecting merely an effect of increased ionic strength. It seemed to be specific, as it was not observed when Ca or Mg ions were replaced by salts of Al, Co, or Mn. The anionic composition of the liquid medium, on the other hand, seemed less important.

Ability of HeLa cells to take up particulate material in the presence of PEF. Toxoplasma merozoites infect phagocytic cells such as macrophages, as well as cells not generally considered to be phagocytic. HeLa cells which do not exibit a particular phagocytic activity but are sensitive to toxoplasmas were observed in a series of experiments. NBT solution, ink particles, latex particles, or toxoplasma merozoites were added with or without PEF to the cell cultures. The influence of PEF on the passing from an extracellular to an intracellular position of the agents mentioned was then studied.

With the exception of toxoplasma merozoites, PEF did not affect the rate of cellular uptake of particulate material. Thus (Fig. 2) HeLa cells

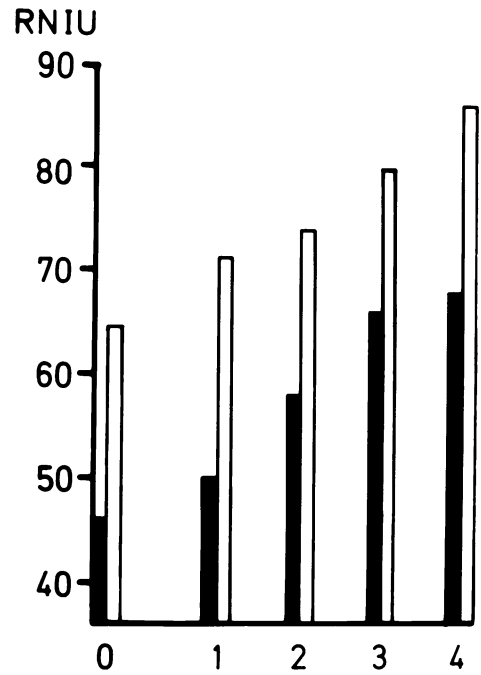

FIG. 1. *Penetration of toxoplasma merozoites into HeLa cells in the presence or absence of PEF. Relative number of infective units (RNIU) in an inoculum of 1.2 × 10⁶ merozoites was plotted against the concentration of Ca in the liquid medium. The Ca concentration is expressed as multiples of the Ca in Hanks salt solution (0.16 M). Presence of PEF (1:800 of a standard preparation), unfilled columns; PEF not added, filled columns.*

exposed to NBT solution reduced the dye to intracellular formazan at the same rate independent of the presence of PEF and/or toxoplasma merozoites in the culture medium.

Motility of toxoplasma merozoites after addition of PEF. The motility of toxoplasma merozoites in the presence or absence of PEF was studied using direct light microscopy observations and time lapse cinematography. Toxoplasmas incubated at 37 C in cell cultures or as cell-free suspensions of merozoites were observed. The motility of the merozoites was studied both when they were added to liquid medium containing PEF and when they were suspended in a PEF-free medium gradually replaced by PEF-containing fluid. Neither direct observations nor time lapse cinematography revealed significant differences in motility between merozoites present in the PEF-containing media and merozoites suspended in the liquid media without PEF. The merozoites moved with a speed ranging from 100 to 2,000 μm/s, depending upon when the merozoites were observed after harvest.

Morphological changes in HeLa cells treated with PEF. Cultures of HeLa cells were exposed to PEF and toxoplasma merozoites and observed by electron microscopy. Two concentrations of PEF were used, purified standard PEF diluted 1:8 and 1:800.

PEF caused destruction of cells in its high concentration. This was initiated by breakages of the cytoplasma membranes (Fig. 3 and 4) and ended in a disorganization of the cells. The percentage of cells demonstrating defects in the plasma membranes after exposure to PEF is illustrated in Fig. 5. It is seen that after 4 h all cells of cultures to which PEF had been added in high concentrations revealed defects in membranes, whereas the controls demonstrated damage of membranes in 17%. Sometimes loose membranes, as if stripped off from the cells, were seen in preparations of cells exposed to PEF for 4 h. The changes observed seemed specifically due to effects elicited by PEF, as altering the pH from 7.6 to 6.6 and/or the temperature from 37 to 20 C inhibited cellular destruction. Using the low concentration of PEF, no morphological changes of treated cells were detectable, although in this concentration PEF had the capacity to enhance host cell penetration of the toxoplasma merozoites by 28 ± 3% (seven tests).

Toxoplasmas in the state of entering or

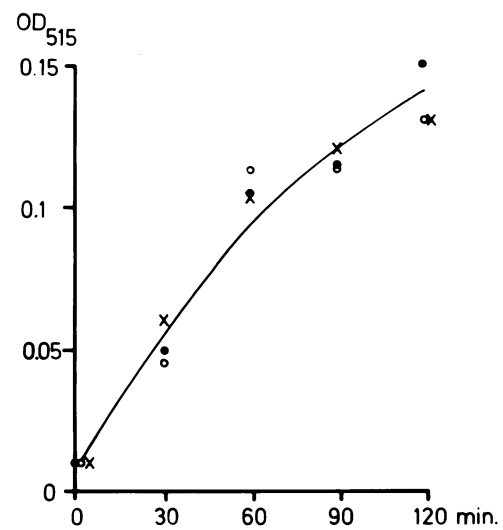

FIG. 2. *Concentration of formazan extracted from HeLa cells exposed to NBT (×), NBT and PEF (○), or NBT and PEF and toxoplasma merozoites (●). Optical density was plotted against time of exposure.*

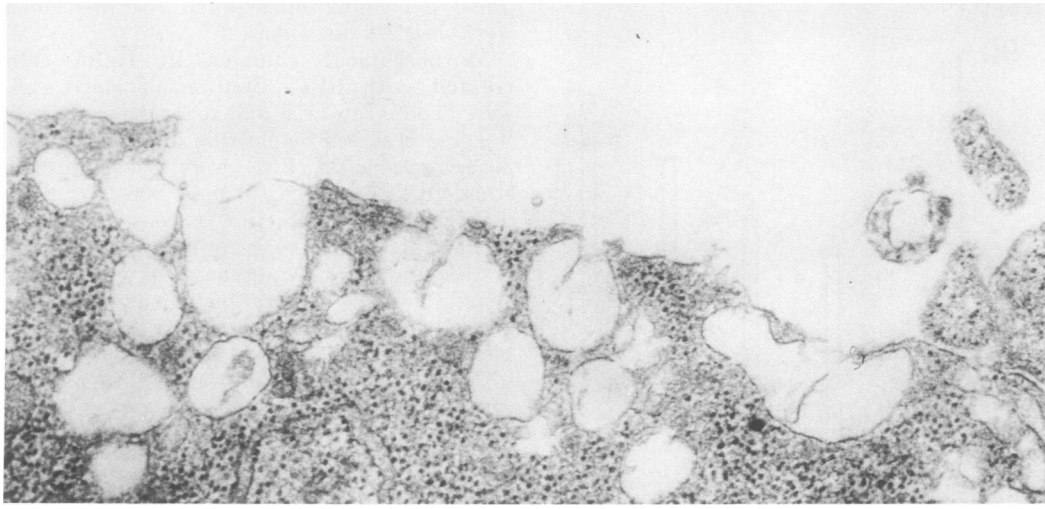

FIG. 3. *Electron microscopy of the cytoplasmic membrane of a HeLa cell exposed to PEF of toxoplasma. A 1:8 solution of a standard preparation of PEF was added for 90 min. Note breakages of cell membrane and beginning vacuolization. Magnification, 49,000×.*

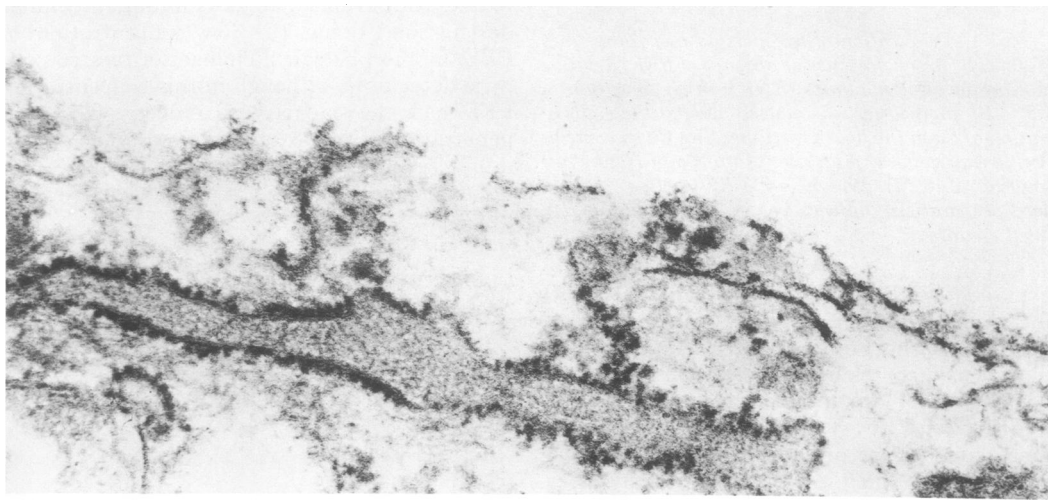

FIG. 4. *Electron microscopy of cytoplasmic membrane of a HeLa cell exposed to PEF of toxoplasma. A 1:8 solution of a standard penetration of PEF was added for 150 min. Note disruption and disorganization of cell membrane. Magnification, 61,600×.*

shortly after they had gained an intracellular position were studied in the following experiments. A number of cell cultures were inoculated with a large number of merozoites and after varying periods of time, the inoculated cells were washed in Hanks balanced salt solution, fixed, and studied in the electron microscope.

All merozoites which were found in close contact with the cells were observed to have their anterior end placed against the plasma membrane. Damage of the membrane, as noticed with high concentrations of PEF, was never observed, and those areas of the membranes which were in close contact with the anterior end of the merozoites seemed intact as a rule (Fig. 6). The findings were the same whether merozoites were studied with cells inoculated in the presence of PEF or if the toxoplasmas were observed without PEF. Intracellular merozoites observed 1 to 2 h after the inoculation of the cultures were regularly found

in vacuoles. However, it was a general impression that for merozoites which had penetrated in the presence of PEF the space between the merozoite and the surrounding cytoplasm of the cells was less than for intracellular merozoites observed in cultures inoculated without PEF. For both types of cultures the vacuoles surrounding the merozoites demonstrated a limiting membrane-like structure only partly. The intracellular merozoites often seemed to be localized within the cytoplasmic matrix.

PEF of some different enzymes. It was previously (20) reported that lysozyme and hyaluronidase increased the number of toxoplasma merozoites if the enzymes were added to cell cultures together with the merozoites. These studies were extended, testing also acid phosphatases, β-glucuronidase, and β-galactosidase. In the tests the enzyme was added to the merozoites and the mixtures were inoculated into cell cultures. The number of penetrating parasites was determined for different concentrations of the enzymes, and the results were compared with that of controls with or without PEF but inoculated with the same number of toxoplasmas. The differences in penetrating parasites expressed as percentage of the controls were calculated. In Table 1 are listed the minimal enzyme units necessary to increase the number of penetrating parasites by 10 and 30%, respectively.

All five enzymes tested increased the number of intracellular toxoplasmas if added together with the merozoites to the cell cultures. However, considerable amounts of the enzymes were necessary to achieve this effect. The concentrations, relative to the concentration of PEF required for enhancement of penetration, were 56 times larger when hyaluronidase, the most potent of the enzymes, was tested.

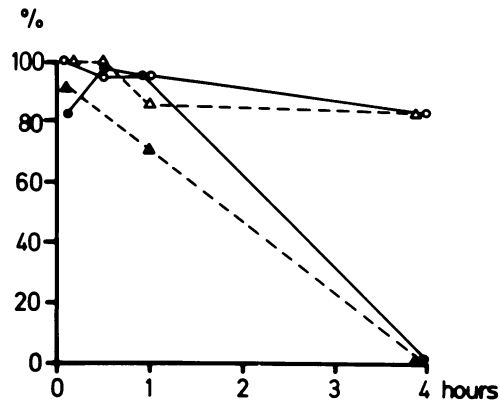

FIG. 5. *Percentage of HeLa cells demonstrating damage of cytoplasmic membrane after exposure to PEF of toxoplasma. PEF was added in a 1:8 concentration of a standard preparation to HeLa cells and incubated up to 4 h. Results with cells to which PEF were added are indicated by filled symbols, whereas those of controls without PEF are represented by unfilled symbols. The combined effect of toxoplasma merozoites and PEF is demonstrated by triangles; results with PEF alone are demonstrated by circles.*

DISCUSSION

T. gondii is infective for a great variety of mammalian cells from different species. In cell cultures intracellularly located, merozoites can be seen shortly after inoculation of the cultures. Some authors (4, 15, 29, 39) have suggested that the merozoites are phagocytized and that this is how they enter the host cells. Jones et al. (14) and Klainer et al. (15), who most recently have emphasized the importance of phagocytosis, observed by electron microscopy that micropseudopods were surrounding the parasites. Jones et al. saw no penetration of merozoites through the plasma membrane or lodgement of toxoplasma in the cytoplasmic matrix. Klainer et al. noted "some suggestion that the organism does actively penetrate L-cells," but the finding of folding of the cell membrane over the parasite made these authors emphasize the process of phagocytosis for the entry of toxoplasma merozoites into host cells. However, there are several findings, by the same and other authors, which in our opinion invalidate the hypothesis of phagocytosis. Toxoplasma merozoites are equally infective for phagocytic cells such as macrophages as for nonphagocytic cells such as fibroblasts and epithelial cells. HeLa cells and macrophages infected under similar conditions of cell density and parasite-to-cell ratio revealed during the first hours of infection a higher mean number of merozoites in the macrophages, but by 6 h the number of parasites was similar in the two types of cultures (14). Lysosomal constituents were not delivered to phagocytic vacuoles of macrophages containing living merozoites, but killed toxoplasmas were engulfed and digested in phagocytic vacuoles (13). Antibody-treated toxoplasmas were phagocytosed and killed by normal human macrophages (4). On the other hand, toxoplasma merozoites treated by antibody, heating, or glutaraldehyde fixation were not taken up by HeLa cells or fibroblasts (14, 21). Neuraminidase, which enhances the phagocytic activity of monocytes, did not affect the infection with toxoplasma merozoites (20). It seems unlikely, therefore, that the merozoites merely are passively taken up by the host cells. Evidently a more active participation of the

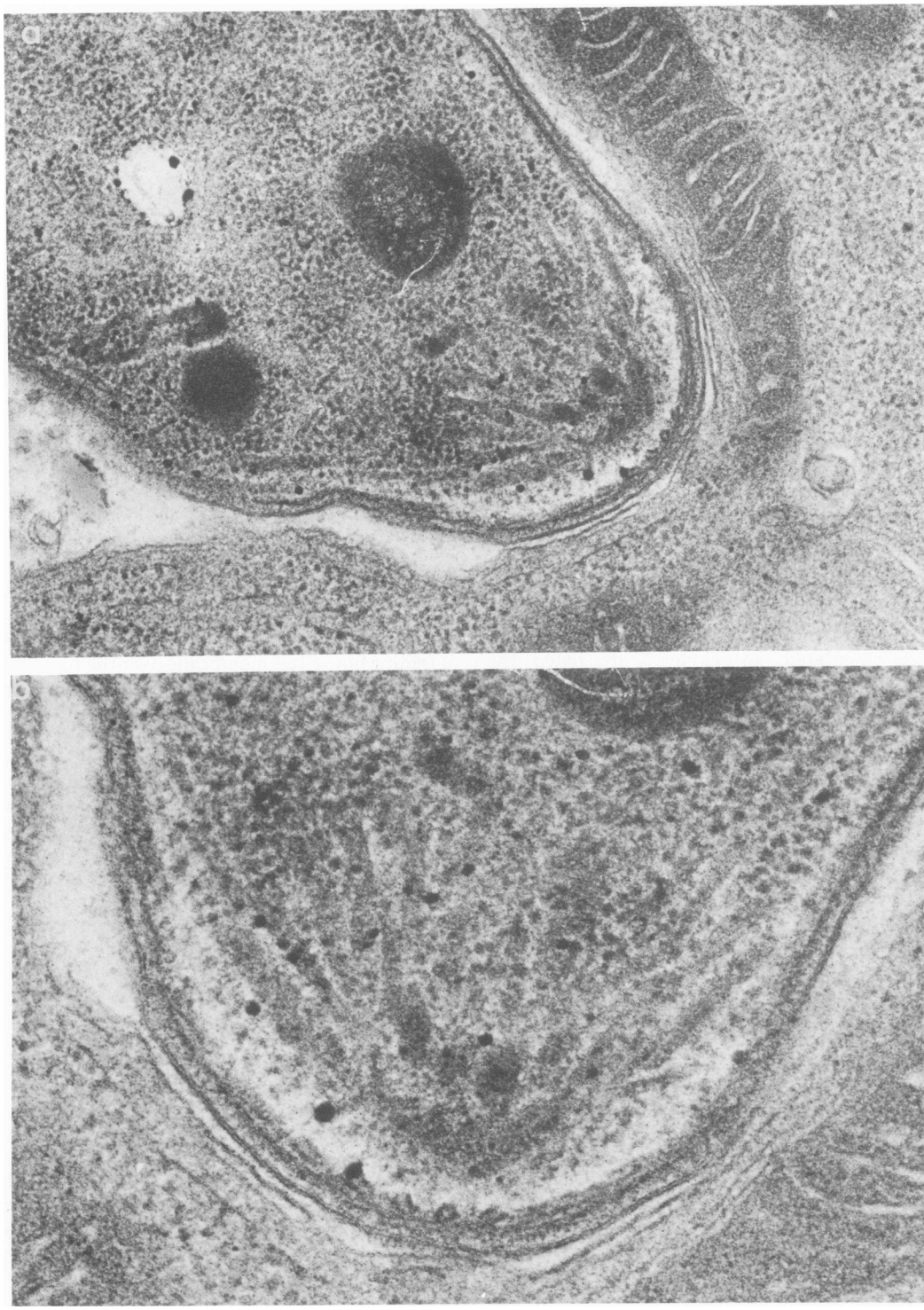

FIG. 6. *Penetrating toxoplasma merozoite 360 min after inoculation of a HeLa cell culture (top: magnification, 88,000×; bottom: magnification, 176,000×). Note preserved cytoplasmic membrane close to anterior end of the merozoite.*

TABLE 1. *Enzymes increasing the number of penetrating toxoplasma merozoites*

Enzyme	Concn required for increasing the no. of penetrating merozoites with[a]	
	10%	30%
Acid phosphatases	0.5 (0.1)	5
β-Glucuronidase	4.5 (0.5)	30
β-Galactosidase	3,500 (0.5)	12,000
Lysozyme	100 (0.1)	2,000
Hyaluronidase	1 (0.01)	14

[a] The minimal enzyme concentrations required are expressed in units and, within brackets, in milligrams per milliliter. The results are based on eight to twelve experiments per enzyme.

merozoites has to be considered, i.e., a process of penetration.

In the study of Jones et al. (14) the merozoites and the host cells were cooled, brought into close contact with each other by centrifugation, and subsequently warmed to stimulate entry of merozoites into cells. This procedure might have created too artificial conditions. Thus, the merozoites seemed to be positioned in a random manner without any evidence that the anterior end of the merozoites first contacted the cell plasma membrane. These observations are not compatible with the generally accepted view that it is with the anterior end of the merozoites that the contact between toxoplasma and cell is established (7, 12, 16, 18, 35, 37, 40, 41). Similarly, *Lankesteria culiculis* (34), *Besnoitia jellisoni* (9), *Eimeria alabamensis* (33), *Eimeria larimerensis* (31), *Eimeria tenella* (24), and *Eimeria callospermophili* (30) all have been found to attach to the cells with the anterior end first.

Specific organelles, i.e., the conoid, the polar ring, the paired organelle, and the rhoptries, are found in the anterior end of the merozoite. It has been assumed that these organelles are, in some way, involved in the process of cell penetration. Bommer et al. (7) described, on the basis of time lapse cinematography, that the toxoplasma merozoites perforated the cell membrane using their extended anterior pole. Similarly, the style-shaped tip of *Besnoitia jellisoni* suggested to Fayer et al. (9) that piercing of the plasma membrane occurred. They noted that the parasites evidently made an opening in the cell membrane and that the body of the parasite, as for toxoplasma, was constricted when it passed through the cell membrane. Interruption of the host cell membrane has also been observed with *Eimeria larimerensis* (31) either at the initial site of entry or after the membrane became invaginated to some extent.

A secretory function of paired organelle, toxonemes, and rhoptries has been assumed by several authors (11, 16, 22, 35, 38). In particular, the paired organelle has been believed to be associated with secretion of an enzyme involved in the host cell penetration of coccidian merozoites. In agreement with this, the treatment of host cells with enzymes was found to increase the number of penetrating toxoplasma parasites (20). It was therefore of interest when a PEF could be extracted from disintegrated toxoplasma merozoites (22). The nature of this factor is consistent with that of a protein with an enzyme-like function. Two observations emphaxize the importance of PEF for the penetration of toxoplasma. Firstly, the amount of PEF extractable from the merozoites was reduced if the merozoites were allowed to penetrate host cells shortly before the extraction (5); secondly, inhibitors of PEF also reduced the penetrating capacity of toxoplasma merozoites in the absence of PEF (7). The assumption that PEF is important for penetration of toxoplasma may be considered further substantiated by the findings reported in the present study, namely, that the same characteristic dependence on temperature, pH, and concentrations of Ca and Mg of the liquid medium is rate determining for the penetration-enhancing action of PEF as for the penetration of merozoites in the absence of PEF.

Jones et al. (14) proposed that enzymes or other factors released by toxoplasma merozoites would induce or stimulate phagocytosis in cells not usually phagocytic. However, the experiments we reported with NBT, ink, or latex particles did not suggest that PEF or toxoplasma merozoites could stimulate phagocytic activity of HeLa cells. Neither was there any evidence that the motility, i.e., the capacity of mechanic penetration, would be increased by the presence of PEF. On the other hand, there was an obvious damage of membranes of HeLa cells to which PEF was added in high concentration. Breakages of cytoplasmic membranes with subsequent disorganization of cellular morphology occurred in all cells exposed for 4 h to a high concentration of PEF but was detectable already after 1 to 2 h. In a low concentration no morphological changes were seen in the plasma membranes, but the modifying of the cell membrane was reflected in the enhanced penetration observed. Rudzinska and Trager (32) have presented observations on the penetration of *Babesia microti* into erythrocytes. They observed that partial lysis of the erythro-

cyte membrane occurred at the place of entry of the parasites.

Some of our findings suggested that, shortly after the merozoites had penetrated the cells, the membrane limiting vacuoles surrounding the parasites was partly discontinous. The question arises whether the membrane is a newly formed one or represents part of a partly invaginated plasma membrane. As previously mentioned, penetration of partly invaginated host cell membrane has been described with *Eimeria larimerenis* (31). Zaman and Colley (41) have reported that the membrane, also of macrophages, was interrupted when the toxoplasma merozoites were penetrating. They found that host cell reaction to toxoplasma resembled that of *Eimeria callospermophili* and *Eimeria tenella*. Within the cytoplasm the parasites became surrounded with newly formed membrane after penetration. However, it must be taken into account that, for example, fixation for electron microscopy involves numerous potential artifacts influencing the interpretation of the results.

Akao has demonstrated the presence of aspartate aminotransferase (2) and adenosine triphosphatase activity (1) of the toxoplasma pellicle and peroxidase activity in the mitochondria (3). Histochemical studies and respirometry have revealed that the mitochondrial enzymes are fully represented in *Toxoplasma gondii* (8, 10). Of the proteolytic enzymes, acid phosphatases and leucyl aminopeptidase have been observed in merozoites (17). It should be observed that we have found, as reported in the present study, that several proteolytic enzymes were able to enhance the penetration of toxoplasma. Therefore, the possibility exists that the action of PEF represents more generally induced enzyme effects on the cell membrane. However, this does not seem to be particularly plausible. The differences in concentrations of PEF, on the one hand, and those of the enzymes studied, on the other hand, indicated that of the enzymes, alone or in combination, the amounts required for demonstration of enhancement of penetration were considerably larger than those of PEF. The differences in optimal pH and temperature do not suggest identity between PEF and any of the enzymes studied. Absence of hyaluronidase and diffusion factor in preparations of toxoplasma has excluded the possible influence of this particular enzyme (36).

At present we consider the mode of entry of toxoplasma merozoites into host cells best described as an active penetration through the cytoplasmic membrane of the host cell. (Phagocytosis may be of importance for the infection of macrophages and for other true phagocytic cells, although it is probably not the only way for establishing infection even in these cells.) We propose that the cytoplasmic membrane of the host cell is affected mechanically and chemically by the merozoites, and that the membrane limiting the vacuole which surrounds penetrated parasites is newly formed after the penetration, as suggested also by Zaman and Colley (41). A penetration-enhancing protein produced by the merozoites seems responsible for chemical modifications of the cell membrane. Studies on the blocking of this factor may be important for development of therapy, as administration of the factor together with virulent or avirulent toxoplasma strains increased their pathogenecity for experimental animals (23).

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