Cryptosporidium: Cellular Localization, Structural Analysis of Absorptive Cell-Parasite Membrane-Membrane Interactions in Guinea Pigs, and Suggestion of Protozoan Transport by M Cells

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In ilea of spontaneously infested guinea pigs, we examined the interface between the plasma membranes of cryptosporidia and absorptive cells using thin section and freeze fracture techniques. Initially, cryptosporidia invaginate microvilli, and the resulting redundant folds of membrane envelop the protozoan, thereby internalizing it in a membrane sac of host cell origin. Subsequently, a pentalaminar membrane fusion site develops at the base of the protozoan between the parasite's outer plasma membrane and the internalized host membrane. The membrane domains isolated by this fusion site are then modified: the host membrane disintegrates, and the isolated parasite membrane, which now directly contacts absorptive cell cytoplasm, becomes amplified. While cryptosporidia are restricted to the apex of absorptive cells, they may be found deep within the cytoplasm of M cells overlying Peyer's patches. Moreover, both intact and partially digested cryptosporidial organisms associate with macrophages subjacent to such M cells. These findings define the intracellular localization of cryptosporidia and suggest that cryptosporidial antigens may be sampled by intestinal lymphoid cells at sites underlying M cells.

Organisms of the genus Cryptosporidium (phylum Apicomplexa, class Sporozoea, subclass Coccidia) (1) produce a parasitic disease that involves the gastrointestinal, biliary, and/or respiratory epithelium of many animal species, including nonhuman mammals, reptiles, birds (2-4), and more recently, humans (5,6). Early reports of this disease in humans described an associated, protracted, watery diarrhea occurring in immunosuppressed patients, many of whom had acquired immunodeficiency syndrome (AIDS) (7,8). Recent epidemiologic studies utilizing techniques that identify Cryptosporidium oocysts in stool specimens have shown that cryptosporidiosis may also present as an acute, self-limited diarrheal disease in immunocompetent individuals and may account for 1%-10% of diarrheal disease among the population at large (9-11).

Tyzzer (12), who first described this protozoan, defined it as an extracellular parasite (13), a view that was not challenged until 1966 (14). Subsequently, several transmission and scanning electron microscopic studies have approached the issues of the anatomy of the host cell-parasite relationship and of the cellular localization of the protozoan (15-18). In general, such studies lacked detailed, high-resolution examination of parasite and host cell membranes, and thus, not surprisingly, they have come to divergent and discrepant conclusions (14,16) as to the intracellular or extracellular localization of the protozoan.

Using thin section and freeze fracture techniques, we performed detailed ultrastructural analysis of the small intestinal epithelium of guinea pigs spontaneously infected by Cryptosporidium. The anatomy of host and parasite membranes was carefully analyzed...
and, using the distinctive life cycle forms of the parasite, the sequential interactions between membranes of the two cell types were assessed.

Lastly, as the membranous epithelial (M) cells (19) overlying the gut-associated lymphoid tissue are capable of transporting both viral and bacterial organisms (20,21), we performed ultrastructural studies of Peyer's patch epithelium to see if we could identify transport of this parasite by the M cell.

Materials and Methods

Small intestinal mucosa from three adult male Hartley strain guinea pigs, Cavia porcellus (Elm Hill Farms, Chelmsford, Mass.) weighing 400-600 g, were studied. The spontaneous cryptosporidial infection in these guinea pigs was serendipitously discovered in the course of harvesting control tissues for other experiments. In our series of >300 guinea pigs used for experimental studies, these have been the only animals recognized as being infected by cryptosporidia. The guinea pigs had been fasted overnight but were allowed to drink water ad libitum. Under urethane anesthesia, segments of ileum, including Peyer's patches, were excised, opened longitudinally, and fixed by submerging them in a 4°C solution of 3% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4 for 2 h (22). Subsequently, tissues for light and electron microscopy were postfixed in 1% osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in Epon. Oriented 1-μm-thick sections were obtained and stained for light microscopy with toluidine blue in 0.2M phosphate buffer pH 7.0. Epon blocks were then trimmed and silver-interference-color thin sections were cut with a diamond knife. The sections were mounted on No. 200 hexagonal mesh copper grids and stained with uranyl acetate and lead citrate.

In addition, non-Peyer's patch mucosal samples were prepared for freeze fracture. The tissues were fixed as above, washed three times in sodium cacodylate buffer, embedded in a 3% agar solution, and chopped into 125-μm slices with a Smith-Farquhar tissue chopper.

Figure 1. A. Schematic diagram of a Cryptosporidium outlining the results of our structural findings for the early trophozoite phase. For descriptive purposes we have divided the organism into two general areas—that which indents the terminal web (B) and that which is above the terminal web (A). In the area above the terminal web five unit membranes are identified, labeled 1 through 5. These represent: 1, envelope's outer membrane; 2, envelope's inner membrane; 3, parasite's plasma membrane; 4 and 5, an inner pair of membranes intrinsic to the parasite. Together, membranes 3, 4, and 5 correspond to what others have termed the pellicle of the parasite. With the exception of the envelope's outer membrane (membrane 1), all membranes are also present below the terminal web at this early trophozoite phase. In addition, the area below the terminal web has a dense band subjacent to the parasite-absorptive cell interface. This dense band represents an absorptive cell cytoskeletal specialization (see Results). B. Electron micrograph of an early trophozoite showing the various membranes in both general areas of the parasite. This trophozoite is identified as an early form by the presence of rhoptries (R), the absence of endoplasmic reticulum, the nuclear size (N), and by its elliptical rather than oval shape. (This last feature cannot be fully appreciated in this micrograph because it contains only a segment of the parasite.) The electron-lucent appearance of the rhoptries suggests that they have released their contents. The envelope and parasite membranes have been labeled to correspond to those in the accompanying schematic diagram. Note that, focally, the parasite's plasma membrane is becoming closely apposed to membrane 2 (arrowhead). DB represents dense band. Uranyl acetate and lead citrate. Approximately ×96,000.
(Sorvall, Newton, Conn.). After equilibrating for 1 h in a solution of 25% glycerol in 0.1M sodium cacodylate buffer, each tissue slice was mounted between two gold disks, rapidly frozen in the liquid phase of partially solidified Freon 22, and stored in liquid nitrogen. Tissues were fractured at a stage temperature of $-110^\circ$C and a vacuum pressure of $<6 \times 10^{-7}$ mmHg in a Balzers 300 freeze-etch device (Balzers, Hudson, N.H.) equipped with a double replica attachment. The fractured tissue was replicated with platinum and carbon without etching, cleaned in commercial bleach, rinsed in distilled water, and mounted on Formvar-coated (Ernest F. Pullam, Inc., Schenectady, N.Y.) No. 200 hexagonal mesh copper grids. Both freeze fracture replicas and silver thin sections were examined and photographed in a Philips 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) at 60 kV. A total of 400 electron micrographs (206 of thin sections and 194 of freeze fracture replicas) were analyzed. Because in thin sections the various stages of the life cycle of Cryptosporidium, as well as the various maturational phases of trophozoites, are ultrastructurally distinctive (15), as briefly outlined below, we were able to draw...
conclusions from our material concerning the sequence of morphologic events associated with attachment of this parasite to absorptive cells. Early trophozoite forms are identified by their elliptical to ovoid shapes, absence of rough endoplasmic reticulum, and incomplete dedifferentiation of the apical complex (15). The apical complex, which ultrastructurally defines protozoans as members of the phylum Apicomplexa (23), consists of several specialized organelles that are believed to aid in parasite penetration into host cells. These include the rhoptries (electron-dense club-shaped organelles), the polar ring (an osmiophilic thickening formed by the inner membranous layer of the pellicle), and the micronemes (small, osmiophilic convoluted structures) (23). These organelles, which are identifiable in early trophozoites, disappear as the trophozoite matures (15). Ultrastructural evidence of trophozoite maturation also includes rounding of the parasite into a spherical body, enlargement of the nucleus and nucleoli, the development of the rough endoplasmic reticulum cysternal complex, and the development of a zone with interdigitated membranous folds at the host-parasite interface (15). The nucleus of the fully matured trophozoite finally undergoes a series of divisions followed by cytokinesis, and schizonts are formed.

Figure 3. Electron micrograph of a maturing trophozoite (P) that has lost its rhoptries and is beginning to develop endoplasmic reticulum. This trophozoite, as are all trophozoites shown, is attached to an absorptive cell (AC). Note that the envelope cytoplasm contains parallel arrays of microfilaments seen best when tangentially sectioned (arrowheads). The inset shows an envelope at high magnification that consists of an electron-dense cytoplasmic core sandwiched between two membranes (1 and 2). Underlying the trophozoite-absorptive cell interface is a dense band (DB), but no microvillus rootlets are seen at this site. Uranyl acetate and lead citrate. Approximately ×48,000. Inset: Approximately ×204,000.
Figure 4. Electron micrograph of the interface between an absorptive cell (AC) and a maturing trophozoite (P). Toward the base of the parasite, as shown by the arrowheads, the parasite plasma membrane (membrane 3 in Figure 1 schematic) and the internalized microvillus membrane (membrane 2 in Figure 1 schematic) form a discrete pentelaminar membrane fusion site. Two membrane domains are isolated by this fusion site, a disk of membrane 3 (parasite origin) and a disk of membrane 2 (host origin). Dense band can be seen below membrane 2. Uranyl acetate and lead citrate. Approximately ×167,000.

Results

Ileal Villus Epithelium

Light microscopy. The architecture of the small intestinal mucosa of infested animals was altered, showing a 2:1 villus height to crypt depth ratio. (Normally, a 3:1 to 4:1 ratio is present.) A sparse infiltrate of polymorphonuclear leukocytes was present in the lamina propria and the epithelium. These findings were interpreted as evidence of a mild enteritis.

Organisms, measuring from 2 to 4 μm, were associated with the villus absorptive cell brush border.

Transmission electron microscopy. With the exception of oocysts and free zites (sporozoite or merozoite), multiple examples of the various Cryptosporidium life cycle forms (15) were identified by thin section. We predominantly observed various maturational forms of trophozoites, however, and have concentrated on detailing sequential anatomic alterations accompanying this phase of the life cycle. In freeze fracture replicas, identification of the specific life cycle stages of individual parasites using the above, previously described, thin section criteria was not always possible. As outlined below, however, thin section examination did show structural rearrangement of specific membrane domains during trophozoite maturation. Thus, by using these characteristic alterations in membrane surfaces to identify various maturational forms, we were usually able to distinguish these forms in replicas as well as in thin sections. Because of the number of membranes dealt with, we will refer to the schematic diagram in Figure 1 in the following descriptions of our electron microscopic images. For descriptive purposes, we divide the protozoan into two general areas—that which indents the terminal web, and that above the terminal web where the parasite lies within the microvillus border (Figure 1).

We will first describe the appearance of membranes surrounding early or immature trophozoites. In the area above the terminal web (Figure 1), five unit membranes were associated with these early trophozoite forms. The outermost membrane (labeled 1 in Figure 1) was continuous over the superior pole of the parasite and occasionally could be seen to be covered by glycocalyx. Furthermore, this outer membrane was in continuity with the absorptive cell apical membrane (Figure 1B).
Figure 5. High power electron micrograph of the interface of an absorptive cell (AC) and a mature trophozoite (P) identified as such by the structural characteristics of the cytoplasm (absent rhoptries, large size, presence of endoplasmic reticulum). Although the pentalaminar fusion site is still present (arrow), the isolated disk of host membrane (membrane 2 in Figure 1 schematic), previously located at the site marked by the arrowheads, has been lost. The disk of parasite plasma membrane isolated by the fusion site has undergone extensive infolding and amplification and now has direct access to the host cell cytoplasm, although host cell cytoskeletal elements do not penetrate into the interdigitations produced by these folds. Lastly, the two inner membranes of the pellicle (membranes 4 and 5 in Figure 2 schematic) have been lost. E, envelope; MV, microvilli. Uranyl acetate and lead citrate. Approximately ×105,000.

ity of this outer membrane, both over the parasite (Figure 2A) and with the apical membrane of adjacent microvilli (Figure 2B), was confirmed in freeze fracture replicas. Also, as seen in the inset of Figure 2A, the P fracture face of this outer membrane had a high intramembrane particle density similar to that seen on the P faces of absorptive cell microvilli. The cytoplasm lying directly under this outer membrane was of the same electron density as the microvillus core cytoplasm and contained parallel arrays of microfilaments (Figure 3).

Moreover, in fractured parasites, the host cell cytoplasm was shown to be continuous with the cytoplasm underlying this outermost membrane surrounding the parasite (Figure 2B). The second membrane (labeled 2 in Figures 1 and 3) separated this host cell cytoplasmic extension, which enveloped the parasite, from a space that surrounded the parasite. Thus, the two outermost membranes, which we will refer to as the parasite’s envelope membranes, are of host cell origin and contain between them a narrow rim of host cell cytoplasm. The inner envelope membrane represents internalized microvillus membrane that has been invaginated by the protozoan during the process of attachment. Within the zone of the brush border, the fracture planes of these envelope membranes revealed an undulating pattern that, on cross-fractured planes, seemed to corre-
The inner three unit membranes (Figure 1), which intimately associated with the parasite and were separated from the envelope membranes by the aforementioned space, had no continuity with host cell membranes and thus represented membranes intrinsic to the parasites. These three membranes were also identified in individual merozoites within schizonts and correspond to the three-membrane pellicle that characterizes zoites belonging to the class Sporozoa, subclass Coccidia (23,24). The pellicle or covering of the parasitic surface consists of an outer membrane or plasma membrane and an inner pair of membranes (Figure 1B) that are closely opposed to one another. By freeze fracture, the protozoan's plasma membrane (labeled 3 in Figure 1) revealed "P" faces with low intramembrane particle areal densities, as did the most exterior of the inner duet of membranes, labeled 4 in Figure 1, whereas the remaining membrane, labeled 5 in Figure 1, had an intermediate number of large intramembrane particles (Figure 2A).

In areas where the early trophozoites indented the terminal web, four, instead of five, unit membranes were identified: the inner envelope membrane, and the three membranes of the so-called parasite pellicle (Figure 1). A narrow space separated the inner envelope membrane from the parasite’s plasma membrane. Thus, the inner envelope membrane separates, at this stage of the life cycle, the parasite from direct contact with host cell cytoplasm (Figure 2B).

Within the terminal web, at sites near the parasite’s interface with the absorptive cell, there was an electron-dense structure, which has previously been referred to as the “dense band” (15) (Figures 3 and 4). An ultrastructural periodicity was sometimes seen, but no unit membrane structure was ever demonstrated on thin section and no membrane fracture plane was visualized in this area in freeze fracture replicas (Figure 2B). Thus, the dense band represents a host cell cytoplasmic specialization and not the true interface between parasite and host cell. Microvillus rootlets were absent in the terminal web region under the parasite (Figure 3).

Analysis of trophozoites at different phases of maturation, as identified by changes in organelle composition, revealed that consistent, maturation-dependent modifications in membrane structure occurred. First, the inner pair of membranes of the parasite pellicle regressed, leaving the outer or plasma membrane as the only membrane over most of the lateral and lower surfaces of the protozoan (Figure 5). Second, early in maturation, the inner envelope membrane of host origin and the parasite’s plasma membrane approximated one another at a level just below that where the parasite indented into the terminal web (Figure 1B). Subsequently, these two membranes joined to form a pentalaminar membrane fusion site (Figure 4). These fusion sites isolate two distinct membrane domains, one of parasite plasma membrane and the other of internalized...
Figure 7. After maturation, a trophozoite undergoes divisions to produce multiple merozoites (MZ), as seen in this electron micrograph of a first-generation schizont (P). Note the three-membrane pellicle around each individual merozoite. These merozoites will eventually be released and will attach to other absorptive cells. At this stage cytoplasmic structures such as micronemes (MN), which also characterize immature trophozoites, reappear in merozoites. The membrane organization at the absorptive cell (AC)-parasite interface still consists of an amplified parasite plasma membrane that has direct contact with host cell cytoplasm and rests above the dense band (DB). Uranyl acetate and lead citrate. Approximately $\times 37,000$.

Microvillus membrane (inner envelope membrane) (Figure 4). We were not able to obtain good quality replicas of this fusion zone at this particular stage of development and thus are unable to comment on its freeze fracture appearance. The internalized microvillus membrane domain isolated by the pentalaminar membrane fusion site underwent progressive dissolution (Figure 5). In one fortunate fracture plane where this membrane was visualized while undergoing dissolution, it had, at its base, structural characteristics similar to those observed in replicas of detergent-extracted membranes (Figure 6). This process of host membrane dissolution led to the establishment of a direct contact between the domain of parasite plasma membrane isolated by the fusion sites and the host cell cytoplasm. Subsequently, again as suggested by analysis of different phases of maturation, this latter parasite membrane domain was amplified to form a complex system of membrane folds—a structure which has previously been referred to as the “feeder organelle” (2,16) (Figure 5). This structure was seen in replicas as multiple membrane facets.

The structural organization of the membranes at the parasite-host cell interface during the schizont or gamete stage of the life cycle was comparable to that described above for the mature trophozoite (Figure 7). However, even though most of the mature trophozoite pellicle was composed of a single unit membrane, the individual merozoites within a schizont had the characteristic three-membrane pellicle as mentioned earlier (Figure 7).

Mucosa Overlying Peyer's Patches

Light microscopy. The follicle-associated dome epithelium was not structurally disturbed. Organisms were rarely identified in the brush border
of epithelial cells that overlie the domes of Peyer's patches. Presumptive M cells were identified by their characteristic structural appearance, which includes attenuated brush borders, clearer cytoplasm relative to absorptive cells, and an association with aggregates of intrusive interepithelial lymphoid cells.

Transmission electron microscopy. Cryptosporidia were identified associated with follicular dome absorptive cells and with both mature (25) and immature M cells (25). Ultrastructural criteria used for the identification of M cells were those outlined in detail in the literature, which include microvilli that are shorter, wider, and more irregular than those of absorptive cells; terminal webs that are less well defined than in absorptive cells; increased cytoplasmic vesicles; and the association with intrusive phagocytic lymphoid cells (19,25,26). The association of cryptosporidial organisms with dome absorptive cells was identical to that described above for villus absorptive cells. In striking contrast, these parasites were identified deep within the cytoplasm of both immature and mature (Figure 8) M cells.

All the organisms seen within M cells were either in the merozoite or trophozoite life cycle stage. The structural modifications of membranes that were described as occurring at the parasite-absorptive cell interface were not seen in M cells, although the sample we were able to examine in our material was limited. Additionally, in no instance were dense bands noted adjacent to internalized parasites. Surface attachment of parasites to M cells was also not observed in our sample. Both intact and partially digested cryptosporidial organisms were seen in association with macrophages underlying such M cells (Figure 8B).

Discussion

Using electron microscopy of thin sections and freeze fracture replicas we demonstrate defini-
Figure 9. Schematic diagrams depicting the progression of cryptosporidial trophozoite-absorptive cell interactions based on our observations of various phases of trophozoites. As cryptosporidia invaginate absorptive cell microvilli (A), the resulting redundant folds of apical membrane envelop the organism (B). This results in the intracellular localization of the organism within a sac of internalized microvillus membrane. The plasma membrane of the Cryptosporidium subsequently fuses toward its base with this invaginated host membrane (B). The two membrane domains isolated by this process subsequently undergo drastic alteration (C and D). The host membrane dissolves (C), and the isolated parasite plasma membrane, which now directly contacts host cell cytoplasm, becomes amplified (D). During this progression, the inner two membranes of the parasite pellicle (labeled 4 and 5) disappear, particularly toward the base of the parasite (B and C).
tively the intracellular localization of cryptosporidial trophozoites. The specific sequence suggested by our structural findings is outlined in Figure 9. These organisms were shown to be enveloped by a thin host cell-derived envelope. The host cell-derived envelope most likely results from the parasite invaginating the microvillus membranes during early attachment. Such membrane invagination is the method of host cell entry for other coccidia and perhaps for all sporozoans (27–30). Our structural observations suggest that the resulting redundant folds of apical membrane subsequently surround the protozoan and eventually fuse over the superior pole of the organism. The inner membrane of the envelope would thus represent an internalized sac of microvillus membrane that initially separates the parasite plasma membrane from direct contact with the host cell cytoplasm. Our data provide evidence that, at a specific anatomic domain, the surface of the parasite plasma membrane subsequently achieves intimate contact with the host cell cytoplasm. This association appears to be achieved by a series of remarkable structural events requiring modifications at highly specific membrane domains on both host cell and parasite.

After envelopment by the host cell, the plasma membrane of the parasite and the internalized microvillus membrane focally form a discrete pentalaminar membrane fusion site, which may represent a variation of the “moving junction” seen as Plasmodium morozoites indent host cells (30,31). This fusion site isolates both a disklike host cell and a disklike parasite membrane domain that subsequently undergo drastic alteration: the host membrane dissolves, and the isolated parasite membrane, which now directly contacts host cell cytoplasm, eventually becomes amplified. This latter membrane amplification has been referred to as the “feeder organelle” (2,16)—a label that suggests, logically considering our findings, that this is the site at which nutrient uptake from the host cell cytoplasm occurs. We also show that the dense bands underlying the parasite attachment site are not unit membranes but appear to be areas of modified host cell cytoskeleton. The function of this band is unclear, but it might serve to anchor the parasite to the host cell or, alternatively, resist further invasion into the absorptive cell cytoplasm. We have previously described similar cytoskeletal specializations at the sites where filamentous bacteria invaginate into the cytoplasm of rodent ileal absorptive cells (32).

We found Cryptosporidium to have pellicles composed of three membranes: a plasma membrane and an underlying pair of membranes. These latter two membranes were closely apposed to each other and, in some developmental stages, were not discernible, particularly at the base of the organism. Although this three-membrane pellicle is characteristic of the protozoans belonging to the subphylum Apicomplexa (23), especially in the motile stages (sporozoites and merozoites), it had not been previously described for Cryptosporidium. The inner membrane complex of the pellicle is thought to originate from the flattening of vesicular organelles, such as endoplasmic reticulum, during schizogony. As mentioned above, there is partial disappearance of this inner membrane complex with trophozoite matura-tion—a process that also affects the specialized organelles of the apical complex, and has been described as occurring in other coccidia and plasmodia (24,33).

Cryptosporidial organisms are the first example of a parasite to be identified within the cytoplasm of M cells. Although Giardia muris has been identified within macrophages in Peyer’s patch lymphoid follicles, it was not clear if these organisms gained access to these macrophages by way of transport through M cells or by invasion between dome epithelial cells (34,35). Membranous epithelial cells previously have been shown to endocytose and transport other microorganisms (including viruses and bacteria) from the lumen to underlying phagocytic lymphoid cells (20,21,32,34). This path of entry presumably permits antigenic sampling by the intestinal immune system and may represent an essential component of mucosal immunity. Competence of the intestinal immune system and its ability to respond to the stimulus presented via M-cell endocytotic transport may explain why cryptosporidiosis is a self-limited disease in immunocompetent hosts.

The intracellular location of Cryptosporidium might potentially explain the difficulties encountered in eradicating this organism in immunocompromised patients (4). We speculate that, as is the case with other intracellular organisms (36), therapeutic agents that will effectively eradicate cryptosporidiosis have to be able to penetrate absorptive cell plasma membranes. This suggestion should sensitize investigators to the possibility that while the in vitro culture system for Cryptosporidium allows progress in the study of areas such as the protozoan’s life cycle (37,38), such a system may have limitations as a screening method for agents that will eradicate the organism under in vivo conditions.

References