Viruses of *Entamoeba histolytica*

II. Morphogenesis of the Polyhedral Particle (ABRM₂→HK-9)→HB-301 and the Filamentous Agent (ABRM)₂→HK-9

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The intracellular development of two morphologically different amoebal viruses has been studied by electron microscopy. One is a polyhedral agent which was observed as early as 24 hr after infection in the perinuclear cytoplasm. Subsequently, cell lysis occurred and particles were found in large number bound to membranes of disrupted amoebae. Other particles were found in phagocytic vacuoles suggesting a possible portal of entry into amoebae. The other virus is a filamentous particle which is first seen in small clusters in the nucleus after 24 hr of infection. The number of particles increases such that by 72 hr massive whorls of particles occupy a substantial part of the nucleus. After rupture of the nuclear membrane, clusters of filaments are widely dispersed throughout the cytoplasm. Still later, the cytoplasmic membrane disintegrates and clusters of filaments are found extracellularly, but free of cell membranes. The morphology of these agents is discussed in comparison with a variety of plant, animal, and bacterial viruses.

The identification of two morphological entities believed to represent different viruses naturally infecting *Entamoeba histolytica* is described in detail in a companion paper (3). One type consisted of a polyhedral virus about 70 to 75 nm in diameter; the other consisted of filamentous particles found initially in the nucleus. This report describes the morphogenesis of these agents.

The first to be described is the polyhedral particle as observed in strain HB-301 amoebae, fifth virus passage, and designated (ABRM₂ → HK-9) → HB-301, P5. As is explained in the above mentioned paper, (ABRM₂ → HK-9) indicates the virus source, ABRM being the viral donor and HK-9 being the original recipient amoebal strain. HB-301 is the amoebal strain in which the virus (ABRM₂ → HK-9) was passaged, and P5 is the fifth passage. Except for the first passage of this virus in HB-301 amoebae in which a few filamentous particles were encountered, we have observed only polyhedral particles in all passages examined.

The second agent to be described is the filamentous particle replicating in strain HK-9 amoebae. It is designated (ABRM)₂ → HK-9, P9, where (ABRM) indicates the virus donor strain of amoeba, HK-9 the recipient amoeba, and P9 the ninth passage of the virus in normal recipient amoeba (3). The development of this agent is complicated by the fact that this line also contains polyhedral particles of which a few were found in

the 3rd passage and many in passages beyond the 18th. An intermediate passage (ninth) of the filamentous virus in which no polyhedral particles have been encountered will be described in detail and compared with passages in which large numbers of the latter have been found.

MATERIALS AND METHODS

Tubes containing 15 ml of medium with infected or normal, axenized amoebae (3) were chilled for 10 min in an ice bath to detach organisms from the glass. Amoebae were pelleted by low-speed centrifugation (International Refrigerated Centrifuge; no. 269, 8 place, swinging bucket head) at 1,000 rev/min for 10 min at 4°C. The supernatant medium was decanted and the pellet was fixed by adding about 3 ml of 2.5% glutaraldehyde in pH 7.4 phosphate buffer. Samples were stored in fixative for at least 24 hr at 4°C, post-fixed in 1% osmium tetroxide in pH 7.4 phosphate buffer, and dehydrated in graded solutions of ethanol, terminating with propylene oxide. Dehydrated amoebae were embedded in Maraglas; and thin sections were cut with a diamond knife. Sections were stained with uranyl acetate followed by lead citrate and examined in an RCA EMU 3H electron microscope.

To examine crude homogenates for viruses by negative staining, frozen-thawed culture fluids were ultracentrifuged (Spinco, no. 30 head) for 1.5 hr at 30,000 rev/min, and the pellets were resuspended in distilled water. Equal volumes of suspension and 2% neutral phosphotungstate were mixed and placed on carbon-coated collodion grids for examination by electron microscopy.

RESULTS

Polyhedral particle (ABRM₂ → HK-9) → HB-301. Amoebae infected 12, 24, 32, 48, and 72 hr and uninfected amoebae of the same subculture have been examined in the electron microscope. Only details of 24- and 32-hr infected cultures will be presented since we have been unable to detect any evidence of virus replication as early as 12 hr, and 48- and 72-hr infected cultures differed only quantitatively from the 32-hr infected cultures.

The first period of the replication in which we have been able to detect ultrastructural changes in the amoebae was at 24 hr. As may be seen in the binucleate amoeba in Fig. 1, the general architecture of both nuclei and cytoplasm appears relatively normal (4, 8, 9). A number of prominent vesicles are seen and the cytoplasm characteristically contains a large number of densely staining glycogen granules. Also present within the cytoplasm are clusters of heavily stained rings (arrows) which are indistinguishable from structures normally found both in the periphery of nuclei and in the cytoplasm. In the latter location, they are often surrounded by electron-dense bars (9); their function in either location is unknown.

In the immediate perinuclear region, a number of elongated structures are seen interspersed with either empty polygonal particles of about 70 to 75 nm diameter or some of similar size and shape and containing electron-dense, internal material (Fig. 1-3). As may be seen in Fig. 2, these structures and polyhedral particles are dispersed throughout a large part of the perinuclear area. The elongated entities appear to be laminar sheets of variable spacing (12 to 15 nm; insert, Fig. 2). In Fig. 3 the polyhedral profile of these particles is readily apparent (arrows). Similar polyhedral particles have never been encountered either in normal, uninfected amoebae or in those infected for only 12 hr.

Lysed amoebae are encountered occasionally in normal and infected cultures. However, lysed amoebae with numerous polyhedral particles attached to, or otherwise associated with, membranous debris (Fig. 4) are seen only in infected cultures. They are rarely encountered as early as 24 hr but thereafter are found with increasing frequency. Concomitantly, otherwise normal appearing amoebae in the infected culture are frequently observed to have large cytoplasmic vacuoles containing a few to many polyhedral particles (Fig. 5). These are presumed to represent the ingestion of fragments of virus-lysed amoebae. In both such situations, the majority of virus particles appear to have an electron-dense core as distinguished from the empty appearance of most perinuclear particles (Fig. 3).

We have not observed polyhedral particles in any nucleus with intact membranes, although, as may be appreciated from Fig. 2 and 3, one cannot exclude the possibility of some nuclear participation in the production of components or in the process of assembly of this virus. In this regard, we have occasionally encountered crystalline intranuclear inclusions (Fig. 6) of unknown significance, which are indistinguishable from similar inclusions seen in nuclei of amoebae infected with the filamentous (ABRM)₂ → HK-9 agent (Fig. 12).

Only preliminary attempts have been made to examine this agent by negative staining. Crude, frozen-thawed cell culture fluids were ultracentrifuged and pellets were negatively stained. Polyhedral particles (Fig. 7) of about 80 nm diameter (edge to edge) have been observed with sufficient frequency to suggest that these represent the agent seen in sectional material. They appear to exist both in the "full" and "empty" form, but capsomeres have not been definitively resolved. Their slightly larger diameter (80 nm) in negatively stained preparations compared to sectioned viruses (70 to 75 nm) is probably due to a degree of particle flattening frequently encountered when the former technique has been employed with other viruses. Although some of the particles present images which are compatible with their having a short, ill-defined tail, we do not feel the presence of such an appendage to be firmly established at this time.

Filamentous particle (ABRM)₂ → HK-9. Infected cultures of the ninth passage of (ABRM)₂ → HK-9 (3) were examined after 24, 48, and 72 hr of incubation and compared with normal cultures of HK-9 amoebae. A section encompassing part of an amoeba from a 24-hr infected culture is seen in Fig. 8. Numerous vesicles and large glycogen granules constitute major constituents in the otherwise rather homogenous cytoplasmic matrix. No abnormal cytoplasmic elements can be detected. The nucleus, however, reveals distinct changes seen only in infected cultures. Bundles of fine filaments are seen in early stages of development in Fig. 8 and 9. The remaining nuclear inclusions (Fig. 9) are normally seen in *E. histolytica* (8). The perinuclear cytoplasm is normal.

Cultures infected for 48 hr typically show a greater number of amoebae with nuclear filaments. The clusters of filaments are usually larger than those seen at 24 hr and present a whorled appearance (Fig. 10). By 72 hr many nuclei show massive whorls of filamentous material (Fig. 11 and 12). In addition, another type of nuclear inclusion is occasionally encountered in infected cells. An example may be seen in Fig. 12 which presents a section of the nucleus of a 72-hr in-

FIG. 1. A binucleate (N) amoeba from culture infected for 24 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9 \rightarrow HB-301$). Cytoplasmic vesicles (V) and glycogen granules (G) as well as clusters of rings (arrows) are among normal structures. At regions X and Y, seen in higher magnifications in inserts, a few 70-nm particles (P) and laminated structures (L) represent early morphological changes associated with infection. Bar = 1 μ m.

FIG. 2. An amoeba from a culture infected for 24 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9 \rightarrow HB-301$), showing an extensive perinuclear accumulation of empty and full viral particles (P) and laminar bodies (L). Insert shows details of the laminar structures. Bar = 1 μm .

FIG. 3. A large number of "empty" and a few "full" particles in the perinuclear region. A hexagonal contour is easily seen in many particles (arrows). An infection for 24 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9$) $\rightarrow$ HB-301. Bar = 1 μ m.

FIG. 4. A small portion of the cytoplasm of a lysed cell from a culture infected for 24 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9$) $\rightarrow$ HB-301 showing characteristic accumulation of 70-nm particles, "full" and "empty", in close proximity to cytoplasmic membranes. An attachment between particles and membranes is not apparent. Many particles present a hexagonal contour (arrows). Bar = 1 μ m.

FIG. 7. Several polyhedral particles (70 nm diameter) from crude pellets of infected cultures herein described ($ABRM_2 \rightarrow HK-9 \rightarrow HB-301$, and from the same virus line passed in another amoeba ($ABRM_2 \rightarrow HK-9 \rightarrow 200$ (3)).

infected amoeba. In addition to the large filamentous clusters, there appear several poorly stained inclusions which have a periodic (perhaps laminated) structure (inserts) and which closely resemble nuclear inclusions rarely seen with amoeba infected with the polyhedral agent (Fig. 6). The significance of these inclusions in either virus-amoeba-system is not known.

Throughout these stages of infection, the cytoplasm continues to appear essentially normal. Rarely, however, by the 48th hr and frequently at 72 or more hr of infection, the membranes of nuclei containing clusters of filaments are ruptured or absent (see Fig. 3, 6, 7 in reference 3). In such nuclear remnants, filaments often appear somewhat separated and their individual nature can be recognized (Fig. 13). Individual filaments are about 10 nm in diameter; their length cannot be determined in sectioned material. Images of some

particles suggest the presence of a longitudinal periodicity.

At a still later stage of infection, small bundles of filaments are found diffusely scattered throughout the cytoplasm (Fig. 14). These bundles are often associated with densely staining beaded structures which are about 30 nm in diameter and which clearly differ in morphology from individual filaments as seen in the nucleus. Their role, if any, in the infectious process is not known.

As a final stage of cell destruction, the cell membrane lyses as evidenced by bundles of filaments and other cytoplasmic elements being found extracellularly (Fig. 15).

We have never encountered similar filamentous structures either in normal amoebae or in amoebae infected with polyhedral viruses derived from amoebae other than the $ABRM$ strain (3). Likewise investigations in other laboratories (4, 8, 9)

FIG. 5. A typical cytoplasmic vacuole in a cell of an otherwise normal appearing amoeba from a culture infected for 32 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9 \rightarrow HB-301$). The vacuole contains a few polyhedral particles. Bar = 1 μ m.

FIG. 6. Intracellular inclusion (I) in an amoeba from a culture infected for 74 hr with the polyhedral virus ($ABRM_2 \rightarrow HK-9 \rightarrow HB-301$). Perinuclear virus particles are indicated by arrow. Bar = 1 μ m.

FIG. 8. An amoeba from a culture of *HK-9* amoebae infected for 24 hr with the $(ABRM)_2 \rightarrow HK-9$ agent. Small clusters of filaments (F) in the nucleus represent the only abnormal structure detectable in this organism; glycogen granules (G) and numerous vesicles (V) are among the normal cytoplasmic constituents. Bar = 1 μ m.

FIG. 9. At higher magnification, filaments (F) are seen in the nucleus of another amoeba from a culture infected for 24 hr with the $(ABRM)_2 \rightarrow HK-9$ agent. The densely staining ring structure (R) and endosome (E) are observed in uninfected amoebae. Bar = 1 μ m.

FIG. 10. Large filament clusters (F) generally have a whorled appearance in amoebae infected for 48 hr with the $(ABRM)_2 \rightarrow HK-9$ agent. Bar = 1 μ m.

FIG. 11. Large whorled masses of filaments characterize amoebae of cultures infected for 72 hr with the $(ABRM)_2 \rightarrow HK-9$ agent. Bar = 1 μ m.

FIG. 12. In addition to whorls or tightly packed filaments (F), large pale inclusions (I) are infrequently observed. In the inserts, two of them are seen to be highly ordered structures. Bar = 1 μ m.

have not revealed such particles in normal amoebae. Two of these studies employed normal, axenized HK-9 amoebae originating in this laboratory. [The D-9 strain (4) is actually the HK-9 strain.]

As was briefly alluded to previously, an early passage of this agent in HK-9 amoebae produced predominantly intranuclear filamentous particles although an occasional lysed cell revealed polyhedral particles, but no filaments (3). The ninth passage herein described has not, on the other hand, shown any evidence of polyhedral viruses. However, from the 18th passage on, polyhedral particles have been frequently encountered in large number. In the latter, some cells contain filaments and others polyhedral particles (Fig. 15). Occasionally both types of particle are found in association with debris of lysed cells, but this could result from the intermixing of debris of different cells during low-speed centrifugation of the amoebal cultures prior to fixation. Despite an intensive search, we have never encountered evidence suggesting that both types of particle are actively replicating within an amoeba. However, as may be seen in Fig. 16, we have observed cells with nuclei containing filamentous particles and with membrane-bound cytoplasmic inclusions containing polyhedral particles. The latter are presumed to represent phagocytosed debris from cells lysed by the polyhedral agent.

DISCUSSION

No previous ultrastructural investigations have revealed 70-nm polyhedral particles or filamentous structures in *E. histolytica*. Miller and Swartzwelder (14) did, however, present a single section of an apparently normal trophozoite of *E. histolytica* showing several 40-nm viruslike particles. The nature of their particles remains obscure.

Observations herein presented on the morphogenesis of the polyhedral virus ((ABRM₂ → HK-9) → HB-301) suggest that the assembly into complete particles takes place in the perinuclear cytoplasm. Among the animal viruses the group of large pox viruses, typified by vaccinia virus, is the only known class of deoxyribonucleic acid (DNA) viruses which is clearly assembled exclusively in the cytoplasm (5). The other DNA viruses are typically "nuclear" in assembly, although, as in the case of adenoviruses (6) and

possibly others (5), some of their protein components may be synthesized in the cytoplasm and accumulate in the nucleus where complete virus particles are first noted. A wide variety of ribonucleic acid (RNA) viruses (except for the nucleocapsid portion of myxoviruses and probably leukoviruses) are "cytoplasmic" in assembly or mature by budding from cytoplasmic or plasma membranes (5). Thus the criterion of site or assembly would favor this virus as being an RNA virus. However its size, morphology, and maturation in such intimate association with the nuclear membrane render any such conclusion extremely doubtful. We are currently attempting to definitively identify its nucleic acid type, both by purification of the agent and by means of metabolic inhibitors.

The virus is clearly polyhedral in form, but it cannot be concluded at this time that it is icosahedral. However, in view of the occasional appearance of a five-sided profile, we deem it likely that it will be proven by subsequent investigations to be an icosahedral virus.

Likewise we are unable to conclusively state whether or not this virus has a tail such as has been demonstrated on numerous bacteriophages (1) and the blue-green algal virus (10, 18). Otherwise, there is considerable morphological similarity between this polyhedral amoebal virus and both the DNA-containing icosahedral viruses of algae (11), which are about 60 nm in diameter, and a variety of polyhedral bacteriophages (1, 11), also containing DNA and ranging in diameter from about 50 to 100 nm.

When we first encountered both polyhedral and filamentous particles in infected amoebal cultures, we hypothesized that these represented different morphogenetic stages of a single agent and made an intensive effort to find intermediate or transitional forms. This effort was notably unsuccessful.

Furthermore, as described in a companion paper (3), we have only encountered the filamentous particle when the ABRM strain of amoeba, at one stage or another, was involved as the virus donor. Isolations of at least four other agents from other strains of amoebae have revealed only polyhedral particles.

These observations lead us to conclude that there are two morphologically distinct viruses, one filamentous, the other polyhedral. Both types of agent are probably present in ABRM amoebae.

FIG. 13. Filaments in the remains of a nucleus of an amoeba from a culture infected for 72 hr with the (ABRM)₂ → HK-9 agent. Individual filaments of about 10 nm diameter are resolved. Bar = 1 μm.

FIG. 14. At late stages of infection with the (ABRM)₂ → HK-9 agent, filament clusters (F) are found scattered throughout the cytoplasm among vesicles (V), glycogen granules (G), and reticular elements (R). Densely staining beaded structures (arrows) of unknown significance are often seen in proximity to filaments. Bar = 1 μm.

FIG. 15. Cellular remnants of lysed amoebae from a 92-hr infected culture of the 27th passage of $(ABRM)_2 \rightarrow$ HK-9. In some regions, the debris contains polyhedral particles (P), whereas in others filamentous particles (F) are evident. Bar = 1 μ m.

FIG. 16. An amoeba from the 38th passage of $(ABRM)_2 \rightarrow HK-9$ at 36 hr after infection. Filamentous particles (F) are being replicated in the nucleus; a number of polyhedral particles are present in a cytoplasmic vacuole (arrow). The remaining perinuclear cytoplasm shows no evidence of replication of a polyhedral virus. Bar = 1 μm .

The filamentous particle was preferentially replicated in early passages in HK-9 amoebae; the polyhedral particle may have become adapted to this amoebal strain upon repeated passage. On the other hand, a polyhedral virus, probably derived from ABRM amoebae, preferentially replicated in HB-301 amoebae infected with an early passage of (ABRM)₂ → HK-9 (3).

Except for the intranuclear development of helical nucleocapsids of certain RNA viruses, the morphology and morphogenesis of the filamentous particle is unusual as far as animal viruses go. There are, however, numerous plant and bacterial viruses which, when mature, appear as fine flexible filaments. Rice leaves infected with hoja blanca virus revealed fine flexuous, threadlike particles in both cytoplasm and nucleus (17). Individual particles were 8 to 10 nm in diameter and were observed in a variety of cell types. Similar filaments were also observed in the intestinal epithelial cells of infective insect vectors. Clover yellow mosaic virus was seen to produce spiraled clusters of filaments of 8 to 10 nm diameter in the cytoplasm of infected pea leaves (16). Similarly citrus tristeza virus in the leaves of infected lime plants formed aggregates of flexuous filaments, which, upon isolation, were found to be about 11 nm in diameter (15). Potato virus X also develops within the cytoplasm of the host cell as masses of filamentous particles (7). A large number of additional filamentous plant viruses 10 to 13 nm in diameter have been reviewed (2, 13, 19). Of those investigated, all have been found to have a helical structure (19) and all are presumed to contain RNA.

On the other hand, a number of filamentous DNA-containing bacterial viruses have been identified and reviewed in detail by Marvin and Hohn (12). These also are morphologically similar to the filamentous amoeba virus.

Thus, whereas the intranuclear development of this amoeba agent might favor its being a DNA virus, its morphology is of little aid in predicting its nucleic acid type. As in the case of the polyhedral agent (3), we are attempting to isolate the agent to answer this and many other questions which arise upon identifying a new virus type.

Further investigations are needed to determine the taxonomic positions of these new viruses, the first to be isolated in the phylum *Protozoa*.

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