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Viruses of *Entamoeba histolytica*

I. Identification of Transmissible Virus-Like Agents

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This and a companion report deal with the identification and morphogenesis of viruses in axenized cultures of *Entamoeba histolytica*. There are probably two different types of virus each producing a different pathological picture in different amoebal strains, or, less likely, there is one type of agent having widely different morphological and morphogenetical pictures in different strains of *E. histolytica*. Both types of agent produce a lytic response in axenized amoebae and have been serially passaged to an extent assuring their replicating nature. One appears to replicate in the nucleus as multiple clusters of fine filaments which ultimately lyse the nucleus, causing cell death. The second type of agent appears to be a typical polyhedral virus, seen only in the cytoplasm and also resulting in lysis of the cell. A particle morphologically indistinguishable from this second agent is also found in late passages of the agent producing the nuclear pathology.

Prior investigations on protozoal viruses fall into two categories. First, there are isolated reports of morphological entities resembling virus particles in protozoa, and, second, there are more extensive studies of virus-like structures associated with the toxic R body of Kappa, a bacterial endosymbiont found in certain killer strains of *Paramecium aurelia*.

Perkins (10) found viruslike particles 46 to 53 nm in diameter and exhibiting five- and six-fold rotational symmetry, predominantly in nuclei of the oyster pathogen *Labyrinthomyxa marina*. Similarly, Terzakis (14) observed arrayed virus-like particles 35 to 55 nm in diameter both in retarded ookinetes and early oocysts of *Plasmodium gallinaceum* from the mid-gut epithelium of mosquitos previously fed on infective blood. Observations similar to those of Terzakis have been noted by Davies and Howells (4) in a preliminary report. Schuster (13) observed numerous virus-like particles (ca. 100 nm in diameter) in many nuclei of the amoeboflagellate, *Naegleria gruberi*. The particles appeared to arise in association with a precursor "generative" body and also occasionally exhibited what apparently is an unusual form of budding from the nuclear membrane. Cytopathology was not observed in these amoebae. The initial appearance of these viruslike particles was apparently triggered by transferring amoebae from axenic culture to a medium containing either *Aerobacter aerogenes* or *Escherichia coli*. Since similar particles appeared following the utilization of both bacteria as a food source, Schuster felt it unlikely that the particles were introduced via the bacteria. He also considered the possibility that the agent was introduced via chick embryo extract used as medium supplement. However, recent investigations by Dunnebacke and Schuster (7) apparently rule out this possibility. Furthermore, they demonstrated a cytopathic effect in chick embryo cells inoculated with cell-free extracts of the amoeboflagellate.

Miller and Swartzwelder (9) observed a small array of virus-like particles about 40 nm in diameter within the perinuclear cytoplasm in a single section of a trophozoite of the NRS simian strain of *Entamoeba histolytica* cultivated in the presence of bacteria.

Kappa is one of a number of bacterial endosymbionts associated with *P. aurelia* [reviewed by Beale et al. (3)]. In certain killer strains of *Paramecium*, the Kappa particles contain characteristic refractile R bodies which apparently are toxic for sensitive strains of *Paramecium*. Preer and Preer (12) described virus-like particles associated with R bodies. Subsequently Preer and Jurand (11) established a "close relationship between the virus-like particles, R bodies and toxic activity."

It is apparent that previous investigations have not uncovered a well established protozoan-virus system. The present and companion report will deal with the identification of agents from *E.*

histolytica which cause lysis of susceptible amoebae, can be continuously passaged, passes through a 0.2-μm filter, and possess morphological characteristics of known viruses.

MATERIALS AND METHODS

The techniques for axenic cultivation of *E. histolytica* used in this study have been described in detail (6). Salient features are presented here for background. Amoebae isolated from the host are cultivated in vitro with their naturally occurring bacterial associates. After establishment of these cultures, the amoebae are freed of the bacteria by microisolation procedures, or use of antibiotics, or both, and are induced to grow monoxenically with either one of two protozoan flagellates, *Trypanosoma cruzi* or *Crithidia* sp. (5). Finally, the amoebae with their flagellate associates are transferred to a specially devised medium for axenic growth. Here, the trypanosomatids die out within three subcultures, whereas the amoebae continue on in the axenic state. Antibiotics are never used after the amoebae have been freed initially of their bacterial associates.

Characteristically, newly initiated axenic cultures flourish for the first few transfers, decline sharply for several transfers, and then gradually and steadily improve and finally reach a point of stability permitting indefinite subculture. Stabilized cultures, however, may also undergo a temporary period of decline following major alterations in cultural conditions. In any event, during the period of diminished growth it is not unusual to observe lysed amoebae in the cultures. The possible relation of this lysis to viral activity will be discussed later.

Routinely, the axenic cultures were grown in 15 ml of TP-S-1-monophasic medium (6) contained in screw-capped culture tubes (16 by 125 mm), incubated at 35.5 C in stationary cultures and subcultured at alternate intervals of 76 and 92 hr. Subculturing was performed as follows. The old cultures were chilled for 5 min in ice-water, inverted several times to loosen amoebae attached to the walls of the tubes, and then centrifuged for 5 min at 850 $\times$ *g*. After this, the supernatant fluid was decanted; the sedimented amoebae were resuspended in a small amount of phosphate-buffered saline (*p*H 7.4) and then were transferred to tubes of fresh medium.

Viral infections were initiated as follows. The donor amoebal culture was chilled and centrifuged, and, after the supernatant fluid was decanted, the sedimented amoebae were frozen and thawed one or more times to lyse the amoebae. The lysed organisms were then resuspended in 1 to 2 ml of buffered saline and divided into two equal portions, and each portion was added to a tube of medium freshly inoculated with the recipient amoebae. To continue the virus line by passage, 2 ml of supernatant fluid from an infected culture was added to 13 ml of medium inoculated with the recipient amoebae. On occasion, the sedimented lysed amoebae from the infected culture rather than the supernatant fluid were used.

Six strains of *E. histolytica* were used. Strain 200:NIH (hereafter referred to as 200) was isolated in 1948 from proctoscopic material taken from a U.S. merchant seaman suffering from amoebic dysentery. The geographical origin of this strain is unknown. Strain F-22 was obtained from an amoebic brain abscess in 1947. In this case, the infection was known to have been contracted in the U.S.A.

Strain HK-9 was isolated from proctoscopic material from a Korean prisoner of war in 1951. Strain HB-301:NIH (hereafter called HB-301) was isolated in 1960 from the dysenteric stool of a patient who contracted the infection in Burma. Additional information on the above strains can be found in the publication of Diamond (6).

Strain ABRM was isolated in 1967 by B. Sepulveda and M. de la Torre (Hospital General-Centro Medical Nacional, Mexico City) from a rectal ulcer of a patient with amoebic dysentery. The amoebae were received in the form of a monoxenic amoeba-trypanosome culture and were axenized in this laboratory. Strain HU-21:AMC (hereafter referred to as HU-21) was isolated in 1970 by K. Juniper (Arkansas Medical Center, Little Rock, Ark.) from a patient with amoebic dysentery acquired in Arkansas. The amoebae were supplied to us in the form of an amoeba-bacteria culture. It was monoxenized with *Trypanosoma cruzi* and then axenized.

The following system of culture designation is employed. The donor-recipient relation of virus and amoeba is expressed thusly: the name of the donor amoebal strain (standing within parentheses) appears first, followed by an arrow and the name of the recipient or indicator strain. Attempt to detect a virus by employing the same donor-recipient combination more than once is indicated by a letter within parentheses preceding the name of the donor strain, e.g. (B), the second attempt. Absence of a letter indicates the first attempt. Viral passage and amoebal subculture designations immediately follow the name of the recipient or indicator amoebal strain. A virus passage is indicated by a capital P plus the appropriate number, e.g., P2, the second passage. Amoebal subcultures are indicated by the use of the capital letter S, followed by a numeral indicating the particular subculture in the series, e.g., S1, the first subculture. Ordinarily subcultures of infected amoebae were performed only on the first virus passage.

Thus (C) (ABRM) $\rightarrow$ HK-9,P6 indicates the third, C, attempt to isolate an agent from the donor amoebal strain (ABRM) employing HK-9 as the recipient or indicator amoebal strain. The HK-9 culture is infected with the sixth passage of this virus, P6, in HK-9 amoebae.

Details of procedures employed for the electron microscopic examination of sections of amoebae are detailed in the companion paper (8).

RESULTS

ABRM strain. Axenic cultures of ABRM strain were initiated with organisms from the amoeba-trypanosome culture mentioned earlier (Fig 1). On the 10th subculture, one of two tubes showed evidence of considerable spontaneous lysis. The cultures at this point were just beginning to show

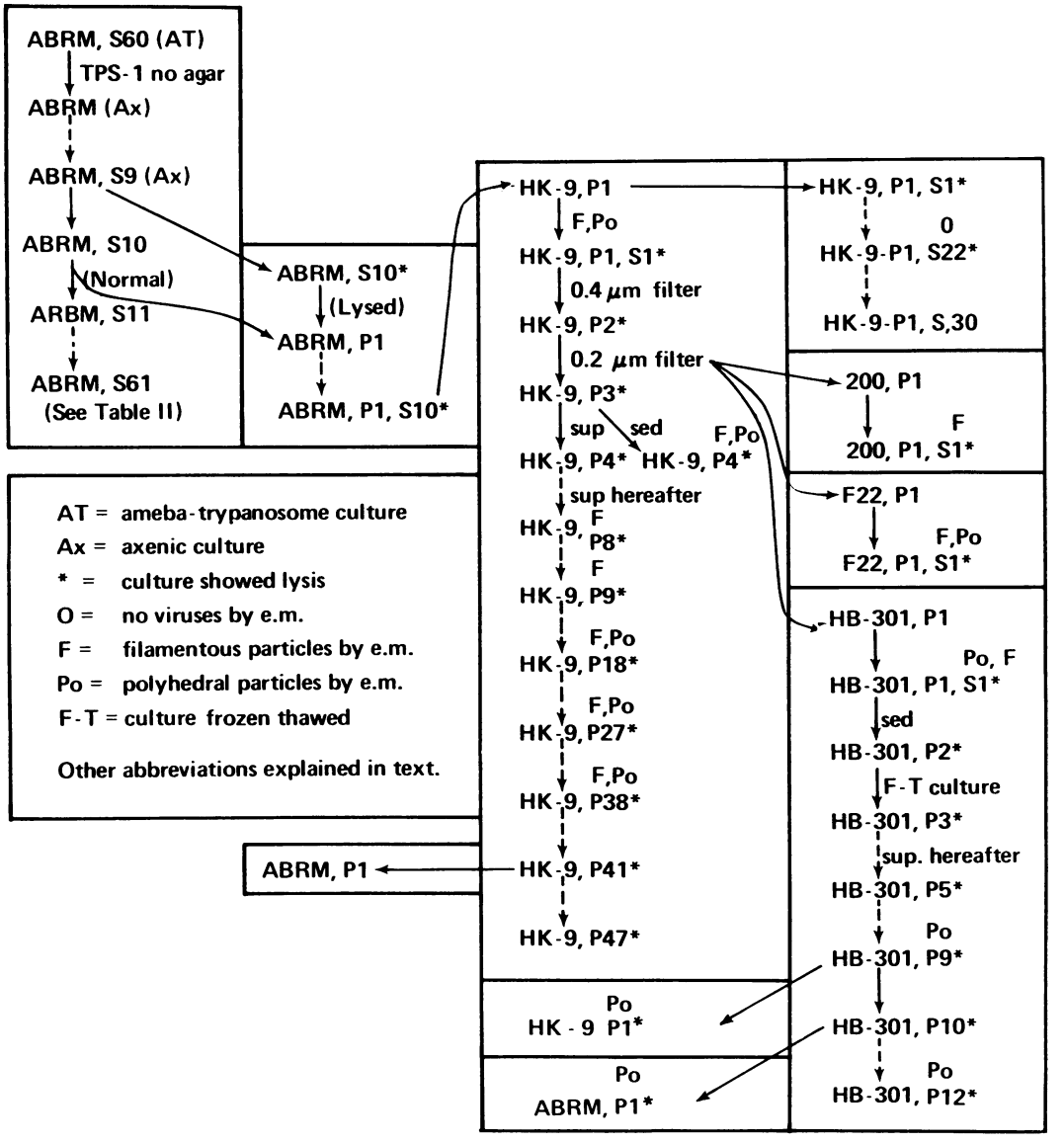

FIG. 1. *Passage history of original virus isolations involving ABRM as the donor amoebal strain.*

recovery from the characteristic period of decline and had not yet stabilized. Suspecting that the unusual amount of lysis might be due to viral activity, an attempt was made to induce lysis in the "normal" sister culture. To this end, the "normal" culture was split into two subcultures (11th subculture) and to one was added the pellet obtained by low-speed centrifugation of the lysed culture. This established the first passage line of ABRM agent in ABRM designated (ABRM) → ABRM, P1. Amoebae infected with this first passage line were subcultured twice weekly through the 10th subculture, (ABRM) → ABRM, P1, S10. The subcultures behaved erratically. Amoebal growth was always poor, and, to maintain the line, it was usually necessary to transfer the entire yield from an old tube to a new one. Lysis of amoebae, in varying amounts, was always observed.

The "normal" ABRM line has been subcultured 61 times. It too has had a history of erratic growth and has taken considerably longer to stabilize than other strains axenized in this laboratory. Cultures from the 24th and 42nd

subculture series which showed a moderate number of lysed cells were sectioned and examined by electron microscopy. No recognizable agents or specific pathology were detected, even though a substantial number of lysed cells were encountered during the examination and, as will be seen, a number of viral isolations have been obtained from late subcultures.

(ABRM) → ABRM, P1 to HK-9 strain. The passage of (ABRM) → ABRM, P1 into another "normal" strain (HK-9) can be followed schematically in Fig. 1. The 10th subculture of (ABRM) → ABRM, P1 was pelleted and the pellet was added to an apparently normal culture of HK-9 strain of *E. histolytica* which was in its 684th axenic subculture. This established the first passage of ABRM agent in HK-9, for the sake of brevity designated $(ABRM)_2 \rightarrow$ HK-9, P1. After 92 hr $(ABRM)_2 \rightarrow$ HK-9, P1 showed no obvious pathology by light microscopy and was subcultured. The subculture, $(ABRM)_2 \rightarrow$ HK-9, P1, S1, showed extensive lysis after 76 hr. On the 7th day of incubation, one tube was chilled in ice-water for 5 min and centrifuged at low speed. The supernatant fluid was passed through a 0.4-μm Millipore filter; tubes of normal HK-9 containing 10 ml of medium were inoculated each with 5 ml of filtrate, establishing $(ABRM)_2 \rightarrow$ HK-9, P2. Another tube, containing lysed amoebae infected with the first virus passage, was fixed in 2.5% glutaraldehyde, and the amoebae were prepared for electron microscopy. The light- and electron-microscopic appearance of these cells and those infected with subsequent passages of virus will be described at the end of this section.

The progeny of the few remaining amoebae of another tube of $(ABRM)_2 \rightarrow$ HK-9, P1 were subcultured twice a week through 30 subcultures. The early subcultures behaved unpredictably, showing, variably, slight to moderate lysis. Eventually they took on the appearance of normal axenic cultures, and, when examined in the electron microscope, the 22nd subculture, $(ABRM)_2 \rightarrow$ HK-9, P1, S22, appeared essentially normal.

$(ABRM)_2 \rightarrow$ HK-9, P2 showed extensive lysis in 76 hr and was subcultured $(ABRM)_2 \rightarrow$ HK-9, P2, S1. After 92 hr, these subcultures also showed extensive lysis. Pooled, clarified supernatant fluids from these cultures were then passed through a 0.2-μm filter (Fig 1), and were inoculated into three additional strains of axenized *E. histolytica*, as well as into normal HK-9. Within 76 to 92 hr of inoculation, each subsequent virus passage produced extensive lysis; consecutive passages of HK-9 have been continued twice a week through the 47th passage which represents approximately a $1/7.5^{47}$ dilution of the original ABRM material. Passages 2, 4, 8, 9, 18, 27, and 38 have been examined by electron microscopy.

For the first 24 hr of incubation, the gross appearance of the healthy and viral-infected culture is similar, except that there are fewer organisms in the latter. Viewed with the inverted microscope (100×) in situ, the majority of amoebae are seen moving slowly over the glass surfaces of the tube to which they are attached. The remainder of the amoebae are attached to each other in clumps. A few motile, multinucleate, giant amoebae are present.

The appearance of the healthy culture remains essentially unchanged during the entire period of logarithmic growth (12 to 84 hr; Fig. 2). In striking contrast, by 36 hr the majority of amoebae in the infected culture have become detached from the tube walls and for the most part are rounded up and inactive. The cytoplasm of the amoebae in these cultures is noticeably denser than in organisms from healthy cultures. Lysed amoebae are present. Multinucleate giant cells are numerous. By 48 hr (Fig. 2B), lysis is marked and continues to increase as the culture ages until all or nearly all of the amoebae are involved.

The most prominent ultrastructural changes seen in early passages of infected HK-9 cultures have been in the nucleus. Large clusters of fine filamentous strands develop and accumulate within the nucleus (Fig. 3). Eventually the nuclear membrane ruptures (Fig. 4) after which many small clusters of strands are found widely distributed through the cytoplasm; finally, subsequent to rupture of the cell membrane, they are found extracellularly. The individual fine strands are about 10 nm in diameter; we have been unable to measure their length accurately. Similar structures have never been observed in any uninfected strain of *E. histolytica*. The morphogenesis of the filamentous particle in the ninth passage of $(ABRM)_2 \rightarrow$ HK-9 is described in a companion report (8).

Complicating this picture was the appearance of polyhedral, virus-like particles which, in certain passages, were seen associated with lysed cells. The latter were rarely found in passage 4 amoebae but were observed in large numbers in passages 18, 27, 38 (Fig. 5). On the other hand, passage 1 (4.5 days after infection), passage 8 (7 days after infection), and passage 9 amoebae (1, 2, 3, and 4 days after infection) revealed only filamentous particles, despite an intensive search for polyhedral forms. In late passages, both filamentous and polyhedral particles were found in the debris of lysed cells (Fig. 6). However, we have not seen morphological evidence suggesting that the two particles represent different stages in the development of a single agent.

A second successful attempt was made to isolate

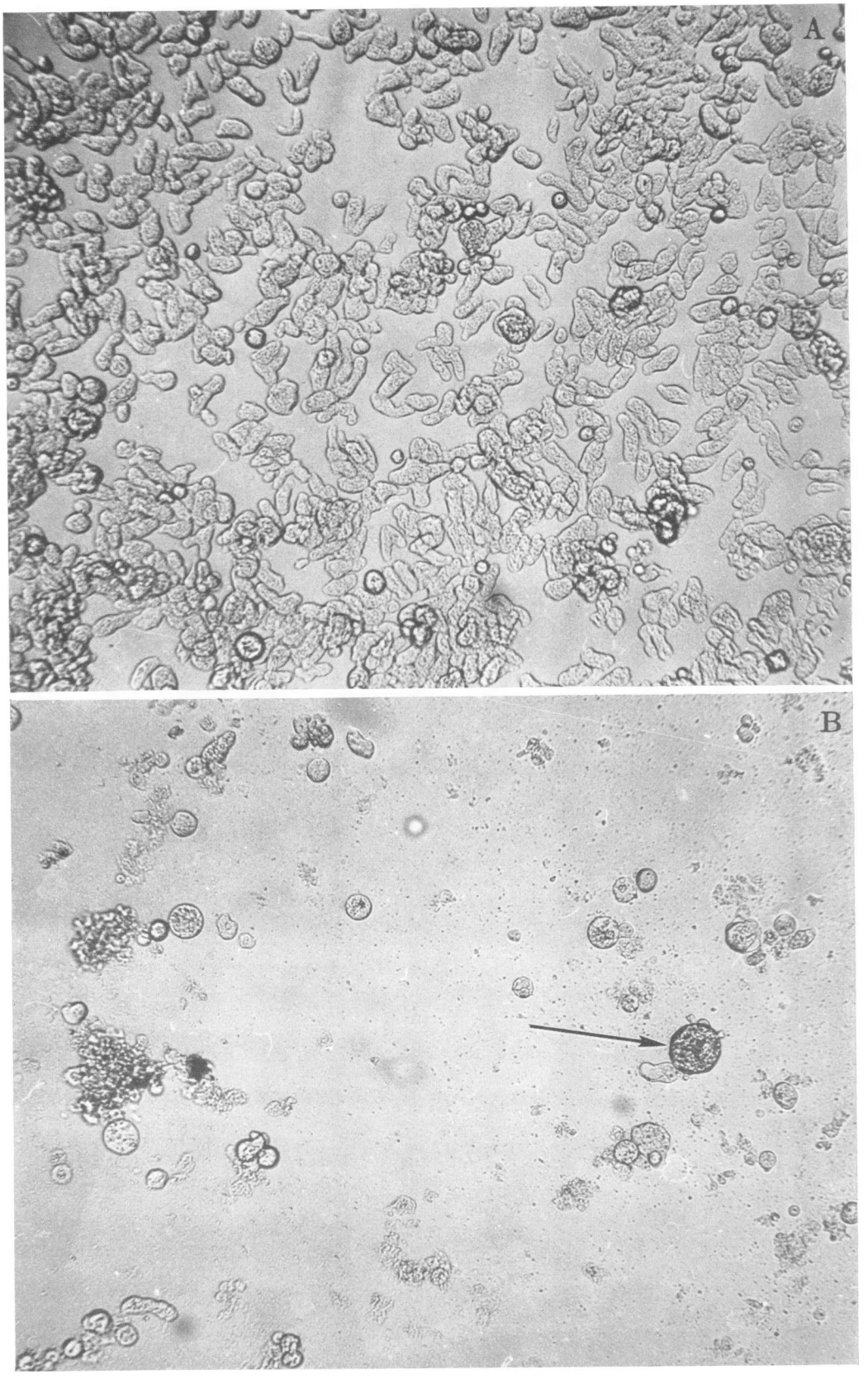

FIG. 2. (A) *Light micrograph of a culture of normal HK-9 amoebae showing a nearly confluent sheet of amoebae.* (*B*) *A culture infected for 48 hr with the* (*HB-301*) → *HK-9 virus strain. Extensive lysis is evident by absence of a sheet of amoebae, debris of lysed amoebae, and an occasional multinucleated, giant cell* (*arrow*).

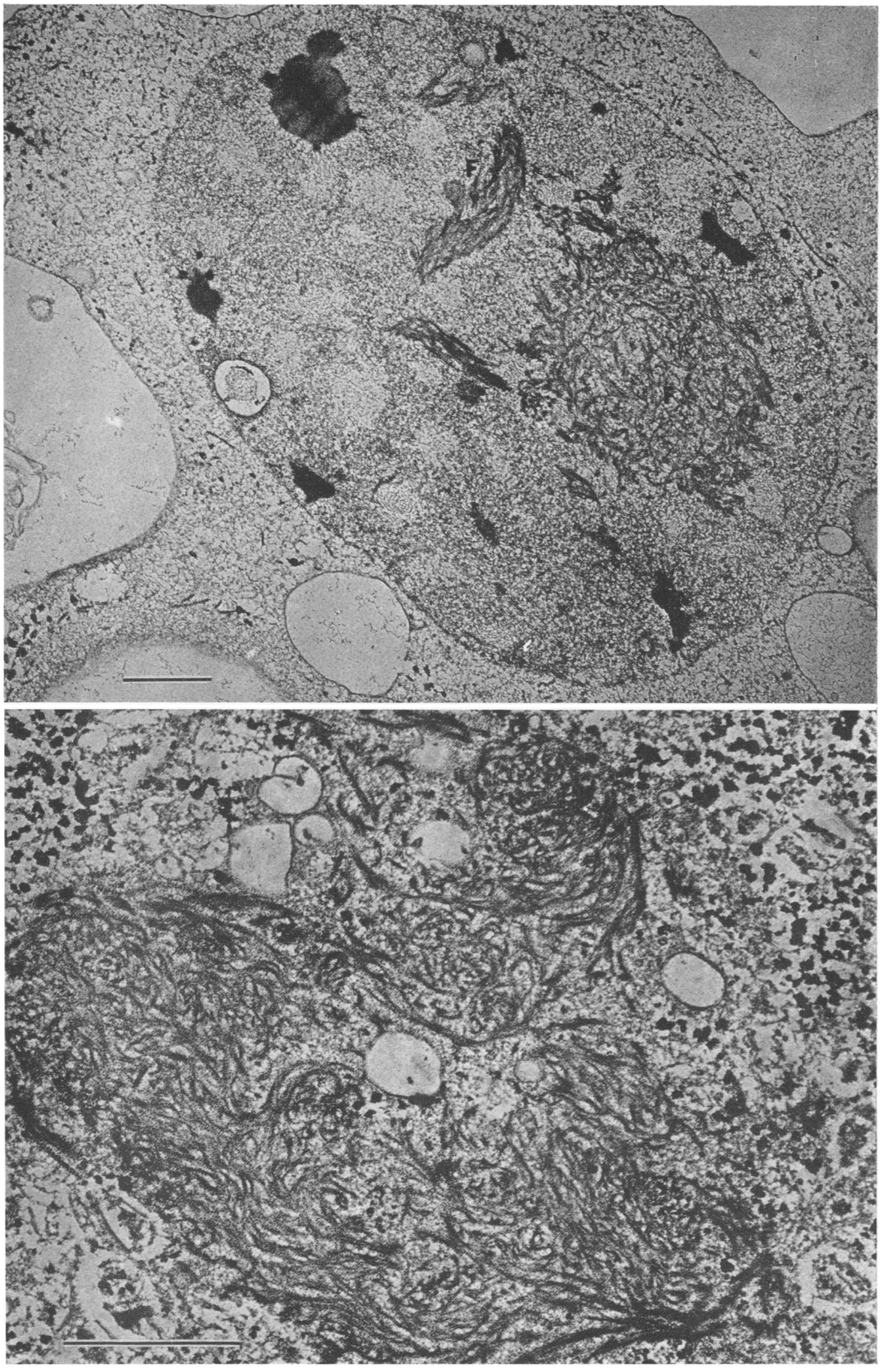

FIG. 3 and 4. *Cells from* $(ABRM)_2 \rightarrow$ *HK-9, P1, S1, cultures infected for 76 hr. (Top) Fig. 3, bundles of filaments (F) are restricted to the nucleus; (Bottom) Fig. 4, the bundles appear in a large cluster within the cytoplasm after lysis of the nuclear membrane.*

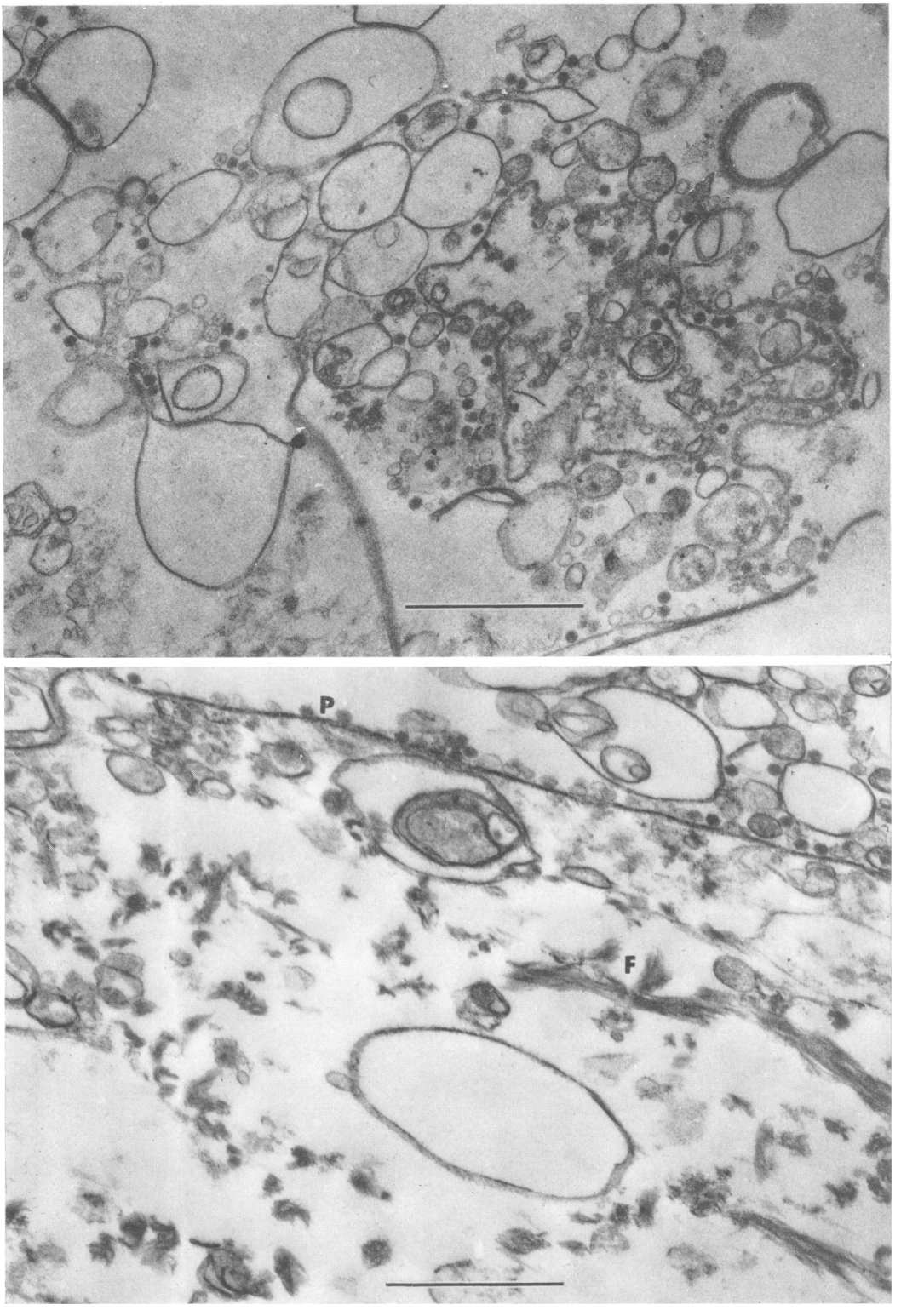

FIG. 5. *The 18th passage of* $(ABRM)_2 \rightarrow$ *HK-9 contained lysed cells with associated polyhedral particles.*
FIG. 6. *In the 27th passage of* $(ABRM)_2 \rightarrow$ *HK-9, occasionally filamentous* (*F*) *and polyhedral* (*P*) *particles were found nearby in the debris of lysed cells.*

an agent from ABRM in the HK-9 strain (Fig. 7a). Apparently normal ABRM in its 53rd axenic subculture was frozen-thawed and added to normal HK-9, establishing (ABRM) → HK-9, P1. This culture appeared normal in 76 hr and was subcultured. After 92 hr the subculture still appeared normal and was again inoculated with frozen-thawed ABRM (55th subculture), establishing (ABRM) → HK-9, P1, S2. After 92 hr it was thought to show a small amount of lysis, was passaged, and was found extensively lysed in another 76 hr. Although all passages showed lysis, only the second and sixth passages were examined by electron microscopy. In both passages, nuclear filaments and polyhedral particles were found in large number each associated with different lysed cells.

(ABRM)$_2$ → HK-9, P2 in other ameobal strains (200, F-22 and HB-301). Supernatant fluid of the second virus passage of (ABRM)$_2$ → HK-9 was passed, as previously noted, through a 0.2-μm filter and inoculated into apparently normal axenized cultures of strains HB-301, F-22, and 200 (Fig. 1). Each of these recipient strains failed to show any recognizable changes by light microscopy after 76 hr. Each was subcultured, and, after an additional 92 hr, all showed extensive lysis; the cultures were pelleted and fixed for electron microscopy.

(ABRM$_2$ → HK-9) → 200, P1, S1 revealed extensive development of nuclear filaments indistinguishable from those seen in (ABRM)$_2$ → HK-9 passages (cf. Fig. 8, 3, and 4). No other type of particle could be found. On the other hand, (ABRM$_2$ → HK-9) → F-22, P1, S1, that is, the same inoculum in the F-22 strain, revealed in some cells not only numerous, fine, intranuclear filaments (Fig. 9) (also seen later in the cytoplasm) but also a moderate number of polyhedral particles associated with other lysed cells (Fig. 10). The two types of particles were never encountered in the same cell, nor was there any other evidence suggesting that the filamentous form might be a precursor of the polyhedral particle. These two passages have not been carried further.

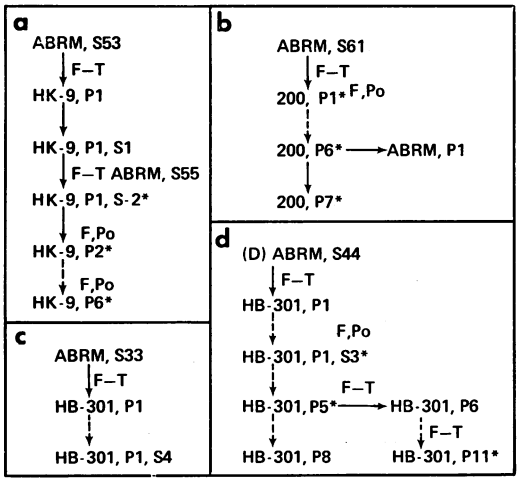

FIG. 7. *Apparently normal, axenically cultivated ABRM amoebae, at indicated subculture levels, were frozen-thawed (F-T) and employed as virus donors to infect HB-301, HK-9, and 200 amoebae strains. The first attempt (c) to infect HB-301 amoebae failed; all others (a, b, d) succeeded and both filamentous (F) and polyhedral (Po) particles were observed in lysed (*) cultures.*

When (ABRM$_2$ → HK-9) was used to "infect" HB-301 strain amoebae, an entirely different picture was observed by electron microscopy. On the first subculture of passage one (ABRM$_2$ → HK-9) → HB-301, P1, S1, the dominant particle, was a polyhedral agent which was found in large numbers associated with either the membranes of lysed cells (Fig. 11) or with phagocytic vacuoles, presumably representing ingested fragments of lysed amoebae (Fig. 12). The detailed morphogenesis of the fifth passage of this agent is presented in a companion paper (8). A few fine nuclear filaments were encountered in the first passage, but were never observed subsequently in the 5th, 9th, or 12th passages.

An infectivity assay on the eighth passage of this agent was performed. Serial 10-fold dilutions of the supernatant fluid were made and applied in the usual manner to normal HB-301 amoebae, employing six tubes per dilution. Tubes inoculated at 10^0 to $10^{-2.0}$ dilutions were noticeably lysed in 92 hr. Tubes receiving a higher dilution of inoculum were subcultured after 92 hr, and, within an additional 76 hr, all tubes at $10^{-3.0}$ dilution and two of six tubes at $10^{-4.0}$ dilution showed extensive lysis. The apparent titer was thus $10^{-3.7}$ Assay tubes constituted the ninth passage.

Since the (ABRM$_2$ → HK-9) → HB-301 line of polyhedral agent could have originated in either ABRM or HK-9 amoebae, we hypothesized the existence of a relative resistance of ABRM or HK-9 amoebae to this agent passaged in the recipient strain of HB-301. The ninth passage of (ABRM$_2$ → HK-9) → HB-301 was inoculated (2 ml to 13 ml of medium plus amoebae) into normal HK-9, and the 10th passage pool was inoculated into ABRM (Fig. 1). In both instances cultures were lysed in 76 hr, and relatively large numbers of polyhedral particles were found associated with lysed amoebae. However, when the 41st passage of the (ABRM)$_2$ → HK-9 strain was added to ABRM cultures, no lysis was detected. As will be subsequently described, the HB-301

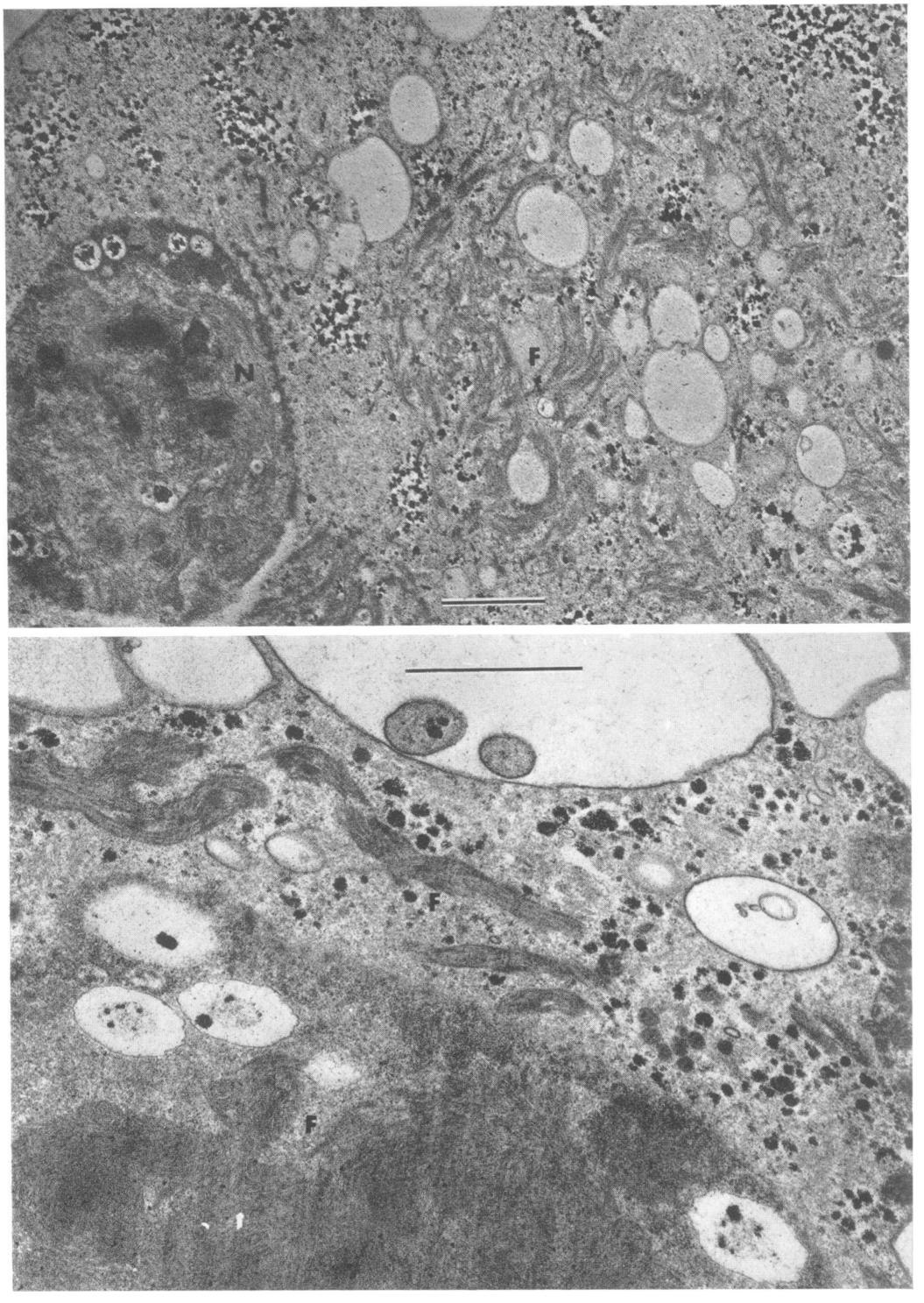

FIG. 8. *(Top) Filamentous particles in (ABRM$_2$ → HK-9) → 200, P1, S1 are observed both within the nucleus (N) and in the cytoplasm in an amoeba from a culture infected for 76 hr.*

FIG. 9. *(Bottom) Cells from cultures infected for 76 hr with (ABRM$_2$ → HK-9) → F22, P1, S1, replicating the filamentous agent.*

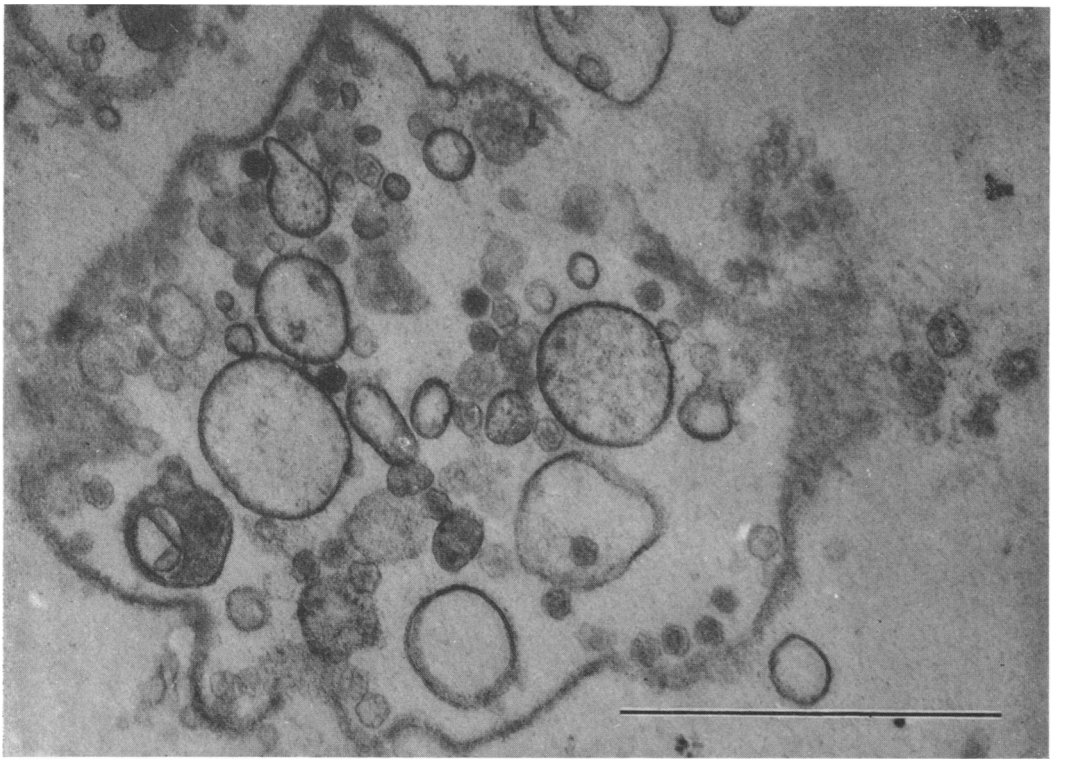

FIG. 10. *Cells from cultures infected for 76 hr with (ABRM$_2$ → HK-9) → F22, P1, S1, replicating a polyhedral virus.*

strain carries a particularly virulent agent which alone is capable of rapidly lysing HK-9 and other amoebae.

Other virus isolations from ABRM amoebae. Several additional attempts have been made to unravel these somewhat confusing observations. In particular we wished to know if the ABRM strain would induce other amoebal strains to produce both filamentous and polyhedral particles which would suggest that the ABRM strain contains two viruses. Normal ABRM amoebae were frozen-thawed and inoculated onto normal HB-301 cultures with no evidence of lysis after four subcultures (Fig. 7c). A second attempt in which one subculture was involved also failed. The third and fourth attempts were successful. Lysis was observed on the first subculture of the third attempt. The fourth trial (Fig. 7d) involved three successive applications of frozen-thawed ABRM amoebae. Lysis was first apparent after the second application and increased in amount with the third application of frozen-thawed material. Examination of (D) (ABRM) → HB-301, P1, S3 revealed a moderate number of polyhedral particles and a relatively small number of cells with nuclear filaments. On continuous passage, lysis diminished, and, by passage seven, the culture appeared normal. This line was discarded after the eighth passage. A second line was initiated with a lysed fifth passage culture which had been stored in the frozen state. Lysis of HB-301 amoebae occurred after six additional passages in which frozen and thawed amoebae from the preceding passages were used as the inoculum each time.

ABRM (61st subculture) was frozen-thawed and inoculated into normal cultures of the 200 strain (Fig. 7b). Both nuclear filaments and polyhedral particles were found in substantial number, but the two did not appear in the same cells. When the sixth passage of the (ABRM) → 200 agent was simultaneously added to normal cultures of ABRM and 200 amoebae, the former appeared completely resistant to a lytic effect, whereas the latter (200) were again lysed establishing the seventh passage (Fig. 7b).

HK-9 as a virus donor strain. Normal axenized HK-9 amoebae (in 706th subculture) were pelleted by centrifugation and then frozen-thawed to kill the amoebae. The thawed pellet was added to a normal culture of the HB-301 strain establishing the line (HK-9) → HB-301, P1 (Fig. 13a). This culture appeared normal after 76

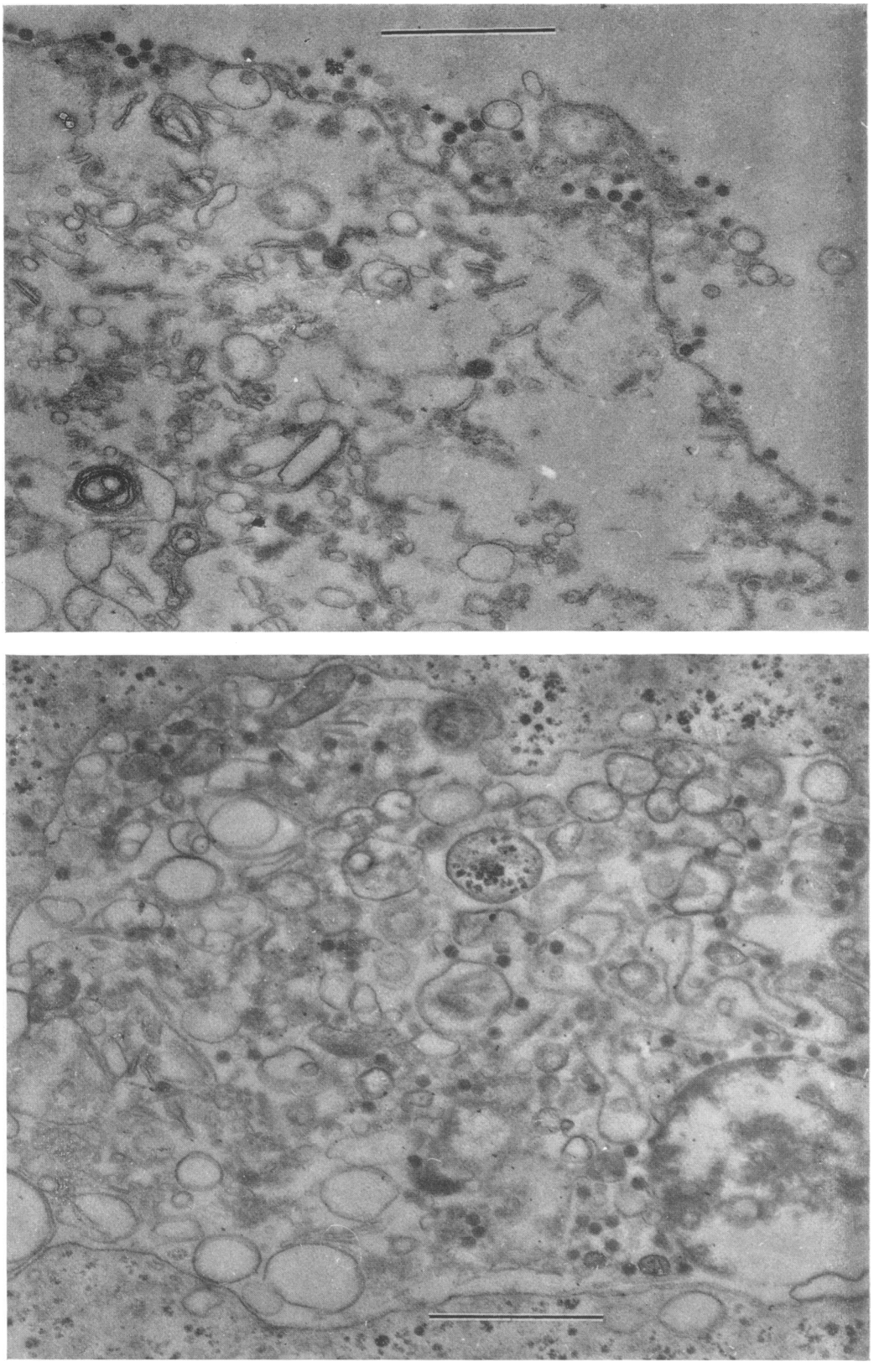

FIG. 11 and 12. *(ABRM$_2$ → HK-9) → HB-301, P1, S1 cultures infected for 76 hr. Lysed cells are associated with a polyhedral virus (Fig. 11, top) which is also often found within phagocytic vacuoles within the cytoplasm of otherwise normal HB-301 amoebae (Fig. 12, bottom).*

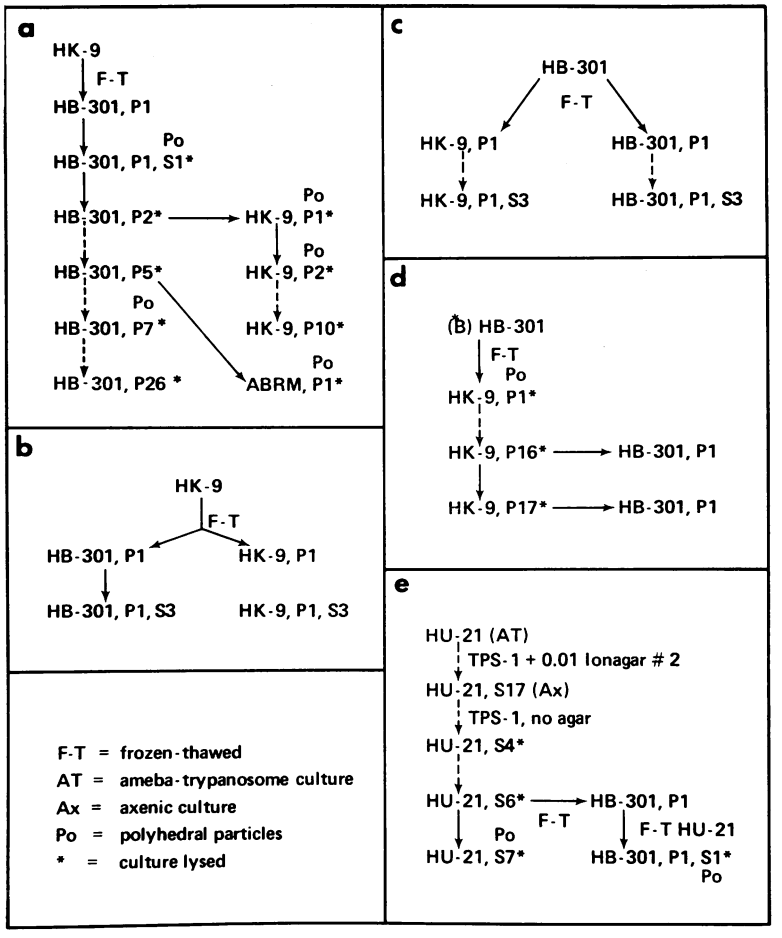

FIG. 13. *Attempts to isolate viruses from HK-9, HB-301, and HU-21 amoebal strains. Schemes labeled a, d, and e were successful; b and c were unsuccessful.*

hr and was subcultured. In 92 hr the subculture showed extensive lysis, and the lytic factor was passaged 26 times through normal HB-301 amoebae (HK-9) → HB-301, P26, all cultures showing extensive lysis within 76 to 92 hr. The first subculture of passage one and the seventh passage have been examined by electron microscopy. All showed substantial numbers of polyhedral particles, indistinguishable from those previously described, associated with lysed cells. The supernatant fluid from passage two was inoculated back into normal HK-9 amoebae and caused extensive lysis in the first passage, without subculture (Fig. 13a). This line (HK-9 → HB-301) → HK-9 has been examined by electron microscopy in first and second passage; both showed large numbers of polyhedral particles. The fifth passage (HK-9) → HB-301 was inoculated into normal ABRM cultures and again produced extensive lysis in 76 hr and many polyhedral viruses (Fig. 13a).

On the other hand, in a second experiment attempting to establish (B) (HK-9) → HB-301, P1, material from a frozen-thawed HK-9 pellet was simultaneously applied to normal HK-9 and HB-301 cultures (Fig. 13b). No lysis was produced in either strain even after three subcultures. This failure to infect HB-301 amoebae with frozen-thawed HK-9 amoebae suggests that the latter may carry a relatively small number of infectious particles.

HB-301 as a virus donor strain. The reciprocal experiment was performed; normal HB-301 amoebae were similarily frozen-thawed, and added to normal HK-9 and HB-301 amoebae (Fig. 13c). In neither case was lysis produced even after three subcultures. A partial repeat of this experiment, employing a later subculture of HB-301 amoebae, and establishing (B) (HB-301) → HK-9, P1, resulted in rapid and extensive lysis of the recipient HK-9 amoebae in 76 hr (Fig. 13b).

Polyhedral particles were abundant in electron microscopic sections. The 16th passage of (B) (HB-301) → HK-9 was simultaneously inoculated into normal cultures of HK-9 and HB-301. The HK-9 cultures were extensively lysed after 92 hr, whereas the HB-301 amoebae appeared unaffected. A second attempt to induce lysis with (B) (HB-301) → HK-9 in HB-301 also failed (Fig. 13b). Thus, in both experiments, the HB-301 strain appeared to be resistant to the agent it caused HK-9 amoebae to produce.

Virus in HU-21 amoebae in early axenization. One of the more important questions regarding these agents is whether they play a role in the spontaneous decline of cultures noted during early axenic passages. We have been able to examine this with amoebal strain HU-21. This strain had been axenized from a monoxenic amoeba-trypanosome culture in TP-S-1 medium containing 0.01% Ionagar no. 2. On the 17th axenic subculture, it was transferred to agar-free medium. As expected with this change in culture conditions, the cultures soon entered a period of decline. Many giant cells were observed, and a moderate number of lysed cells were evident. Examination with the electron microscope of amoebae from the seventh subculture in agar-free medium revealed a few polyhedral particles associated with cellular debris of the lysed cells (Fig. 13e). Frozen-thawed amoebae from the sixth subculture series were passed to normal HB-301 establishing (HU-21) → HB-301, P1. No lysis was apparent in 92 hr; subcultures were made and frozen-thawed HU-21 amoebae from the seventh subculture were added. Extensive lysis was present in 76 hr. Moderate numbers of polyhedral particles were observed in sections (Fig. 14).

Absence of nonviral agents. Attempts were made to detect the presence of agents other than viruses. All of the amoebal lines are regularly monitored for the presence of bacterial contaminants through use of the common sterility test media. Cultures showing gross contamination were excluded from these studies. None of the experimental cultures have shown light- or electron-microscopic evidence of the presence of bacteria, yeasts, or molds. Possible contamination with pleuro-pneumonialike organisms (PPLO) has been investigated. Over a period of years, all of the "normal" axenic cultures have been repeatedly examined by cultivation on a variety of media known to support recognized PPLO (1, 2). Sam-

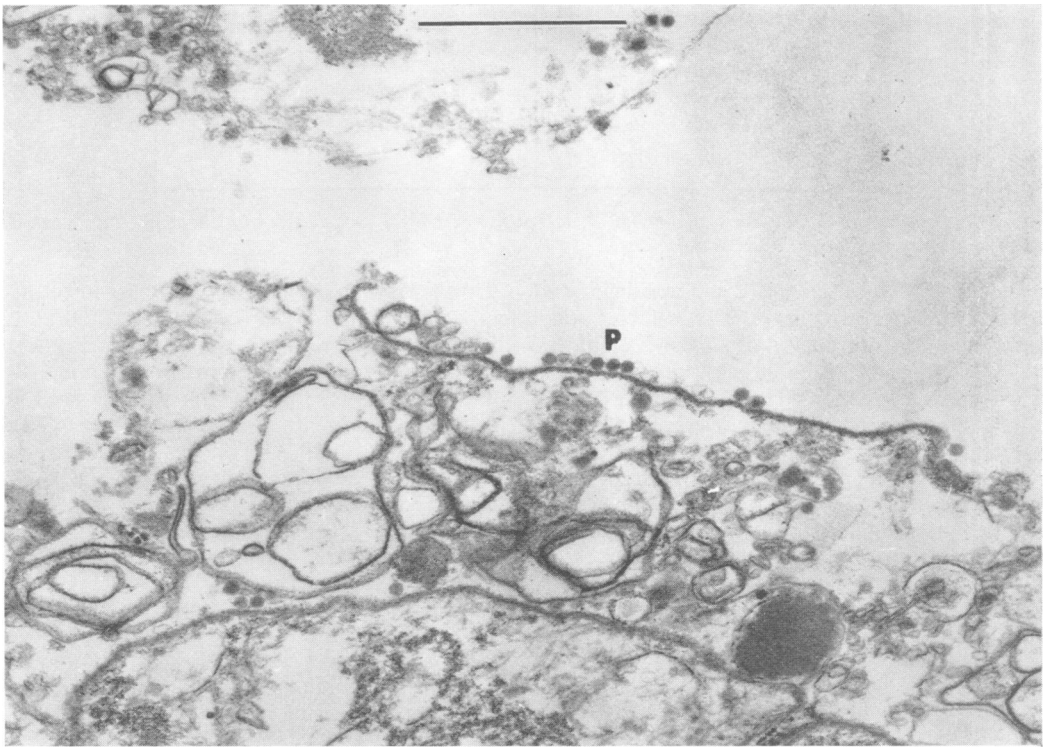

FIG. 14. *Polyhedral particles (P) produced by HB-301 amoebae infected with frozen-thawed HU-21 amoebae which were in the process of being axenized.*

ples of the spontaneously lysed ABRM have been included in this study. All such attempts have failed to show any evidence of the presence of PPLO.

DISCUSSION

The data presented here and in the companion paper demonstrate the presence of at least one type of viral agent indigenous to *E. histolytica* in axenic culture. Strains ABRM, HK-9, HB-301, and HU-21 have all induced a lytic response coupled with the production of virus-like particles in one or more heterologous amoebal strains. One isolate has been serially passaged 47 times at a 1:7.5 dilution, others 34 and 14 times, clearly demonstrating their capacity to replicate. The agents have passed 0.4- and 0.2-μm filters in consecutive passages and they resemble known viruses with respect to size and morphological characteristics. The polyhedral particle, about 70 nm in diameter, closely resembles polyhedral or icosahedral viruses well known in bacterial, algal, insect, and vertebrate systems. The filamentous particles are similar to a number of known filamentous viruses of plants and bacteria as is discussed in the companion paper (8).

Originally we were not entirely certain as to whether these were different morphological manifestations of one type of virus or whether there was a filamentous virus and one or more possibly related polyhedral viruses, although now we favor the latter view. A summary of the most significant features of our experience to date in identifying these agents is presented in Table 1.

As may be seen by comparing lines 1 to 7 with 8 to 23, the only identification of filamentous particles has occurred where ABRM has been involved as a donor strain, and, in each such instance where an agent has been found, filaments have been observed in greater or lesser quantity. [Lines 21 and 22 do not represent a contradiction to this statement. This triple donor virus line originated from the ($ABRM_2 \rightarrow$ HK-9) $\rightarrow$ HB-301 isolate which is shown on line 5 to have contained filaments in its first passage and to be free of filaments in the latter passages employed in lines 21 and 22.]. Also, in all but one such instance (line 6) polyhedral particles have also been found in variable quantity. In that one case we have only examined the first passage of the ($ABRM_2 \rightarrow$ HK-9) $\rightarrow$ 200 isolate. As noted previously, several relatively early passages of the $(ABRM)_2 \rightarrow$ HK-9 isolate failed to reveal polyhedral particles, although other early passages contained a few, and later passages many

TABLE 1. *Summary of attempts to isolate and passage viruses*

	Donor	Recipient	Type of particle	
			Filaments	Polyhedra
1	$(ABRM)_2$	HK-9	++++	Early passage + Late passage ++++
2	B(ABRM)	HK-9	++++	++++
3	(ABRM)	HB-301	+	++
4	(ABRM)	200	++	++
5	($ABRM_2 \rightarrow$HK-9)	HB-301	Early passage + Late passage —	++++
6	($ABRM_2 \rightarrow$HK-9)	200	++++	—
7	($ABRM_2 \rightarrow$HK-9)	F-22	+++	++
8	(HK-9)	HB-301	—	++++
9	B(HK-9)	HB-301	—	—
10	(HK-9)	HK-9	—	—
11	(HB-301)	HK-9	—	—
12	B(HB-301)	HK-9	—	++++
13	(HB-301)	HB-301	—	—
14	(HU-21)	HB-301	—	++++
15	(HK-9$\rightarrow$HB-301)	HK-9	—	++++
16	(HK-9$\rightarrow$HB-301)	ABRM	—	++++
17	($ABRM_2 \rightarrow$HK-9)	ABRM	—	—
18	B(ABRM$\rightarrow$HK-9)	ABRM	—	—
19	(HB-301$\rightarrow$HK-9)	HB-301	—	—
20	(ABRM$\rightarrow$200)	ABRM	—	—
21	($ABRM_2 \rightarrow$HK-9$\rightarrow$HB-301)	ABRM	—	++
22	($ABRM_2 \rightarrow$HK-9$\rightarrow$HB-301)	HK-9	—	++
23	HU-21 early axenization		—	+

polyhedral viruses. Since HK-9 amoebae have been shown to induce the production of polyhedral particles, it is possible that the polyhedral agent found in the $(ABRM)_2 \rightarrow$ HK-9 isolate may have originated in the HK-9 strain and been "activated" by the filamentous particle presumably originating in ABRM. An alternative possibility is that the uncloned ABRM amoeba cultures contain both agents which is supported by the observation that ABRM, on two occasions, has proven resistant to both types of particle contained in late passages of the $(ABRM)_2 \rightarrow$ HK-9 isolate (lines 17, 18) as well the (ABRM) $\rightarrow$ 200 isolate (line 20).

On the other hand, the filamentous particle might represent a precursor of the polyhedral particle. In this case, however, one would not expect to find isolates such as those involving HK-9, HB-301, and HU-21 (Table 1, lines 8, 11, 12, 14) producing only polyhedral agents without any evidence of filamentous forms. The occasional occurrence in late passages of $(ABRM)_2 \rightarrow$ HK-9 (lines 1 and 2), of both types of particles in cells in advanced states of degeneration, is amenable to interpretations other than the above precursor hypothesis.

We interpret these data as indicating that each of the donor strains has its own indigenous virus, ABRM probably having two. Whether these represent simple carrier cultures or a lysogenic relationship cannot be answered at this time. The process of axenizing monoxenically cultivated amoebae is accompanied by a lytic response, and, in the case of HU-21, this lysis was associated with a small number of polyhedral viruses which proved virulent for HB-301 amoebae. During axenization, the surviving amoebae develop a relatively stable population of cells over successive subcultures. Stable cultures, nonetheless, have a low level of associated virus particles. The associated virus is detectable by the inoculation of susceptible strains of amoebae. It is apparent that axenized cultures have some degree of resistance to the virus which they induce susceptible strains to produce (Table 1, lines 17 to 20). An apparent exception can be seen on line 15 where the (HK-9) $\rightarrow$ HB-301 virus isolate produces extensive lysis within one-half week when put back into HK-9. However, in three of four attempts, we have observed that "normal" HB-301 amoebae, when frozen-thawed, induce a similar lysis in HK-9 within the same brief period of time. Thus, it seems probable that the virus indigenous to HB-301 is extremely pathogenic for HK-9, and, this virus, rather than the (HK-9) $\rightarrow$ HB-301 isolate, may have caused the lysis in the HK-9 amoeba noted in line 15 of Table 1.

As to the origin of these agents in *E. histolytica*, we can only speculate at this time. The experience with HU-21 (line 23) suggests that amoebae are associated with virus prior to axenization.

We have questioned the possibility of the viruses arising from the bacterial associates present in the gut of the human host or in the cultures in which the amoebae were isolated. Preliminary attempts to cultivate these viruses in *Shigella dysenterii* and three strains of *E. coli* have been unsuccessful.

The trypanosomatids employed in the monoxenic cultivation of the amoebae have also been considered as possible sources of virus. However, this involves two organisms, *Crithidia* sp. and two strains of *Trypanosoma cruzi*, and the presence of viruses indigenous to amoebae is no less probable than some indigenous to two flagellates and also capable of lysing amoebae.

The possibility exists that rabbit blood and horse and bovine serum, employed as supplements in the various media used for monoxenic and axenic cultivation of the amoebae, could serve as viral sources. However, we have been unable to produce cytopathology with these agents in vertebrate cells including chick and mouse embryo cells and human fibroblasts, nor any pathology in suckling or adult mice inoculated by intracerebral, intraperitoneal, or subcutaneous routes. It seems improbable to us that a filamentous agent and two or more polyhedral agents (based on studies of host range specificity) which would appear in amoebae as a result of exposure to the blood or sera would not affect at least one of the vertebrate systems studied. The remaining ingredients of the culture media, except for a vitamin supplement, were heat-sterilized.

At this time we are faced with two major problems which have as yet not been solved. First, it appears likely that most, if not all, of our amoebal strains have a virus, therefore we do not have a recipient or indicator strain which we know to be free from its own agent. This situation must be solved before serological identification and classification of these agents can be attempted. Second, we have only a relatively crude tube assay system further complicated by the fact that amoebal cultures must be subcultured twice a week. Solutions to these problems are being actively pursued to be able to perform quantitative studies on better defined virus-amoeba systems.

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