Entry and Release of Poliovirus as Observed by Electron Microscopy of Cultured Cells

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Cells of differing culture types were inoculated with poliovirus at 37 C, sampled at intervals during the replicative cycle, and examined in thin sections by electron microscopy. The earliest samples, taken at 2 and 5 min postinoculation, showed virus particles adjacent to the exterior of the plasma membrane and others that had apparently penetrated it directly; later samples showed fewer such particles or none. Particles lying in the peripheral cytoplasm frequently appeared swollen and distorted in shape. No sign of virus entry by a pinocytotic process was found at any time. At 3 hr, and subsequently during the replication cycle, particles of progeny virus appeared in the cytoplasm. They were found free in the cytoplasmic matrix, aligned along the elements of filamentous complexes, and enclosed within vesicles. Some of the vesicles were found to be open to the extracellular space, indicating a likely mechanism of virus release.

Two events in the replication cycle of poliovirus that are amenable to study by electron microscopy are the entry of the infecting virus into the cell and the release of progeny virus from it. Mediation of the entry of poliovirus by pinocytosis was proposed by Mandel (18) as a corollary to studies on virus neutralization, a proposal in accord with the long-standing notion that pinocytosis is an important mechanism of virus entry (1, 9). Electron microscopic observations by Dales et al. (4) on the formation of poliovirus gave tentative support to the proposal by Mandel. The significance of this support is uncertain, however, inasmuch as the observations covered times only subsequent to the commencement of progeny virus formation, rather than times shortly after inoculation when the only particles visible would be the entering ones.

No electron microscopic findings have been reported on the mechanism of release of poliovirus, except for the common observation that, following prolonged periods of incubation, the structural integrity of the cells is severely damaged.

The present paper is an examination of the manner of entry of poliovirus particles into cells and the manner of release of progeny virus. The element of ambiguity inherent in the investigation by Dales et al. (4) was avoided by observing cell samples taken at several times during the replicative cycle, commencing immediately after inoculation. Since an "eclipse" interval, in which little infective virus is recoverable, is

known to intervene between the adsorption and entry period and the period in which progeny virus becomes fully assembled and released, it was anticipated that cytological examination would show the earlier period to be unambiguously distinguishable from the later one.

MATERIALS AND METHODS

Cells. Cells from human amniotic and chorionic membranes, made available by A. W. Makepeace and Sylvia McLeod (Kaiser Foundation Hospital, Oakland, California), were cultured by the method of Dunnebacke and Zitcer (8), in medium 199 containing 10% human serum and 10% calf serum. After cells began to grow, the human serum was omitted.

Cells were also used from mycoplasma-free cultures of HeLa, FL, and KB in Eagles MEM with 2% fetal bovine serum, and from HEP-2, BS-C-1, and Vero in L15 (Leibovitz) with 10% fetal bovine serum. Basic media were prepared from powdered mixtures (Grand Island Biological Co., Grand Island, New York).

Virus. Poliovirus type I, strain Brunhilde, was used to inoculate the cells. The stock virus, titered at 1×10^8 plaque-forming units (PFU)/ml, was prepared from frozen-thawed debris of 18- to 20-hr inoculated HeLa, HEP-2, or GMK cells, and clarified by centrifugation. Infectivity titers were determined by plaque assays on monolayer cultures of HEP-2 or GMK cells.

Ferritin. Ferritin, 1% diluted from 6X crystallized horse spleen ferritin (Pentex Chemical Div., Naugatuck, Conn.), was mixed with the virus inoculum in the experiments dealing with virus penetration. It was used to allow a distinction to be made between pinocytotic vesicles formed and closed prior to, and subsequent to, inoculation; the latter would be expected to contain ferritin.

Inoculation. Cells in monolayer culture were washed in two changes of buffer (Dulbecco) prior to application of the inoculum (37°C) which contained about 100 PFU/cell in a 1-ml volume. The inoculum was estimated to contain upwards of 3,500 virus particles per cell (27). For study of virus entry, a mixture of virus and ferritin was added to the amnion, Vero, and KB cells for 2, 5, 10, and 30 min and 1, 2, and 3 hr prior to fixation and subsequent electron microscopic examination. An additional sample was taken at 20 hr to assess the effectiveness of inoculation. For study of virus release, cells of all eight culture types were fixed at 3, 4, 6, 8, 12 and 20 hr following inoculation; other samples were taken for assay and cytological examination. Control cells were treated in parallel fashion, except for application of inoculum.

Electron microscopy. Cells were prepared for electron microscopy by removing the medium, and then by fixation in 1.6% glutaraldehyde for 15 min. They were then scraped from the culture dishes, pelleted, postfixed in 1% osmic tetroxide for 1 hr, dehydrated in ethyl alcohol and propylene oxide, and embedded in Epon. The sections, about 500 Å thick, were placed unsupported on 400 mesh copper grids, stained in 2% uranyl acetate and then by lead citrate for 5 min each, and observed in a Siemens Elmiskop I. Routine scanning on the fluorescent screen was at $\times 20,000$. Particular scrutiny was made of the cell periphery in that portion of the study dealing with virus entry, with scanning at $\times 40,000$.

RESULTS

Assays of virus yield showed typical one-step growth curves, with the 20-hr samples indicating yields between 400 to 800 PFU/cell. Cells were also examined by light and electron microscopy for cytopathic effects; after 12 hr of incubation, almost all of them showed typical signs of structural degeneration.

Virus entry. About 400 cell sections of the amnion, Vero, and KB cells fixed at 2, 5, and 10 min postinoculation were examined. They exhibited a total of 34 particles identified as poliovirus in locations exterior to the plasma membrane, with similar findings for each of the three culture types (Fig. 1-3). Identification was based upon the similarity in size and shape of the extramembrane particles to those seen in similarly prepared sections of pellets of purified poliovirus (made available by F. L. Schaffer, University of California, Berkeley). No particles exterior to the plasma membrane were found in about 300 cell sections taken from samples that had been incubated for 30 min, 1 hr and 2 hr.

The 1,200 or so cell sections referred to above exhibited 48 particles identified as poliovirus interior to the plasma membrane (Fig. 4-8) with 27 of these appearing in the 2 min samples. Again, there was no discernible difference among the cell types. The particles were seen at various dis-

tances up to 150 nm from the inner lamella of the membrane. Although their morphology was generally similar to those in the extracellular space, their identification as poliovirus was based upon their absence in the approximately 400 cell sections from the control cells. They were distinguishable from ribosomes by their greater electron opacity and more sharply delineated profiles. Most of them differed in appearance from the extramembrane virions by being slightly swollen and misshapen by elongation along a line perpendicular to the plasma membrane, with the portion of the particle nearest the membrane appearing conical and of relatively lower density.

Some of the interiorly located particles were found adjacent to regions of the plasma membrane that clearly exhibited the bilamellar structure typical of transversely sectioned membranes, thus allowing an examination of the membrane for any disturbance of its structure. It was found that, regardless of the distance from particle to membrane, the latter had its normal structure. Although pinocytotic invaginations containing ferritin were seen, no particles resembling virions were found within any of them. No virus particles interior to the plasma membrane were found in sections of cells sampled at 30 min, 1 hr, and 2 hr.

Virus release. At 1 and 2 hr postinoculation, the structure of the inoculated cells appeared similar to that of the control cells. Particles of progeny virus were first seen at 3 hr. Subsequent morphologic changes in the cells were generally similar to those previously described (4, 20), except that intracellular virus crystals did not develop; however, regions appeared in which virus particles were aligned along filamentous structures [Fig. 9 (10, J. D. Levinthal, T. H. Dunnebacke, and R. C. Williams, *Virology*, *in press*)]. The chorion and amnion cells were notably more delayed in development of morphologic changes than were those of the other six culture types.

Virus-containing vesicles were commonly found (Fig. 10-14), increasing in number with time after inoculation. When the vesicles appeared circular in section, they were most commonly seen to enclose only one particle (Fig. 10 and 12), but when they were encountered in tubular, or convoluted form, they were occasionally found to enclose several (Fig. 11 and 13). A common appearance was like that shown in Fig. 12, where the contour of the vesicular membrane is seen to conform to the disposition of the enclosed particles. Such cisternae were found deep within the cytoplasm and near the cell margin, where they were frequently seen to be open to the extracellular space (Fig. 13-16).

FIG. 1-3. Poliovirus particles (arrows) exterior to a human amnion cell at 2 min (Fig. 1), a human amnion cell at 5 min (Fig. 2), and a KB cell at 10 min postinoculation (Fig. 3). Fig. 1 and 2, ferritin molecules (f) which had been added in the inoculum. $\times 100,000$, inserts $\times 300,000$.

DISCUSSION

Virus entry. Although particles seen near the plasma membrane seem to be identifiable as virions of poliovirus on the basis of their morphology and their absence in sections of control cells, the credibility of this identification can be checked by making a rough comparison of the number of particles per cell section seen with the

number anticipated. If the rate constant of poliovirus adsorption (22) for comparable inoculation conditions is taken, and if reasonable estimates are used for section thickness (500 Å), cell diameter (40 μm), and the fraction of each cell section available for examination in the electron microscope (0.5), the number of virions per cell section should be about 0.5 at 5 min postinoculation. The average number actually found was

FIG. 4 and 5. *Poliovirus particles (arrows) immediately subjacent to the plasma membranes of KB cells at 2 min (Fig. 4) and at 5 min (Fig. 5) postinoculation. $\times 100,000$, inserts $\times 300,000$.*

about 0.1 per cell section. Thus, some agreement exists between the numbers of virions anticipated and the number of particles found.

The observations made at the three earliest incubation times indicate that poliovirus, in relatively intact form, enters the host cell by direct penetration of the plasma membrane and without the intervention of any observable kind of pinocytosis. They also indicate that passage of the virion through the membrane occurs in a manner such that the membrane is able to heal what must have been a disturbed structure in less than 2 min. The former conclusion appears to disagree with the proposal by Mandel (18), and the supporting inference by Dales et al. (4) that successful inoculation is mediated by a pinocytotic event. But the disagreement may be only an apparent one. Mandel's experiments did not deal directly with the development of pinocytotic vesicles; indeed, his results bearing on the interactions between normal and neutralized virus with their host cells are equally compatible with either the notion of pinocytotic action or of direct membrane penetration. The cytologic investiga-

tions of Dales et al., in which virions were found within plasma membrane invaginations, led to the tentative interpretation that such particles had been pinocytosed from the inoculum. However, since the first specimens examined were at 3 hr of incubation, when progeny virus particles were also present in the cells, a distinction between them and the entering virions would be difficult to establish.

The electron microscopic finding that virions can enter the cell within 2 min after the application of the inoculum is compatible with the results of Holland (12) from experiments in which recovery of infectivity was measured. Whether the act of entry persists over a prolonged period is not determinable from the present experiments, owing to the obvious difficulty of discerning, with certainty, very small numbers of virus particles. All that can be reasonably asserted is that a pronounced diminution was found in the number of entering, and entered, virions after approximately 30 min of incubation.

The misshapen appearance of the virus particles found interior to the plasma membrane

FIG. 6-8. Poliovirus particles (arrows) in the peripheral cytoplasm of human amnion (Fig. 6 and 7) and Vero (Fig. 8) cells at 2 min postinoculation. $\times 100,000$, inserts $\times 300,000$.

suggests that they have begun the process of uncoating, leading to irreversible eclipse. If this suggestion is valid, it would seem that the disassembly process can begin very promptly after adsorption has taken place. But the electron microscopic evidence for uncoating is, at present, quite marginal, certainly not convincing enough to lead to such an interpretation if it were not known from other work (12, 13, 19) that uncoating does occur.

Other electron microscope studies have shown that viruses can enter inoculated cells by either direct penetration of the plasma membrane or by pinocytosis. Direct penetration has been reported for influenza virus (24), Sendai (14, 23) and Sindbis (J. D. Levinthal, *in preparation*). Pinocytosis has been shown for vaccinia virus (6), adenovirus (3), reovirus (28), all in cells examined prior to the development of progeny virus. Herpes simplex virus and vesicular stoma-

FIG. 9. Progeny virions aligned on cytoplasmic filaments in a HeLa cell at 12 hr postinoculation; pm, plasma membrane. $\times 80,000$.

titis have been reported to effect entry by both direct penetration (11, 25) and by pinocytosis (5, 29).

Virus release. The virus particles seen in vesicles within the cytoplasm must come from one or more of three sources: (i) the applied inoculum, (ii) progeny virus previously released from the cells and pinocytosed from the extracellular space, or (iii) progeny virus remaining within the cells. The first alternative is unlikely, since virus particles within vesicles did not appear until 3 hr postinoculation, long after the early indications of virus entry had ceased, and their numbers then increased with time. The second alternative is acceptable only under the unlikely presumption that poliovirus has two entry mechanisms separated in time, i.e., direct penetration for the initial inoculum and pinocytosis for re-entry of released virus. The third alternative, therefore, seems the most likely source of the virus particles contained within the vesicles.

Since virus-containing vesicles were frequently found near the cell surface and some of these were seen to be open to the extracellular space, it is believed that they mediate release of progeny virus. If the peripheral vesicles do persistently release virus particles from the cell, their activity might account for the results obtained by Dunnebacke and Williams (7) which indicated that about one-third of the cells in the monolayer

culture of FL cells continued to accumulate intracellular poliovirus during and after release of infective units. But such activity of the cisternae would not be compatible with the results of Lwoff et al. (17) and Howes (15), who found evidence that poliovirus is released from HeLa cells in a single "burst," unless it is assumed that continuous release through cisternae is quantitatively insignificant. Indeed, the results of the present investigation do not preclude the possibility that large quantities of virus are released discontinuously, perhaps by blebbing of virus-associated filamentous complexes (Levinthal, Dunnebacke, and Williams, *in press*) and by release of intracytoplasmic virus crystals such as those found by Stuart and Fogh (30) and by Dales et al. (4).

The appearance of the membranes of the virus-containing cisternae suggests that they may derive from the smooth endoplasmic reticulum (ER), inasmuch as they have a characteristic bilamellar structure and are not noticeably associated with ribosomes. Such cisternae are present in the vesicular or centrosphere regions of the cytoplasm where the virus appears to be assembled (Levinthal, Dunnebacke, and Williams, *in press*), and their function may be the isolation of newly made virus from the cytoplasmic matrix.

The occurrence of cisternae filled with virus particles has been reported in cultured cells infected with vesicular exanthema swine virus (31,

FIG. 10-12. Progeny virus within cisternae (arrows) at various depths in the cytoplasm and near the plasma membrane (pm) of a HeLa cell at 8 hr (Fig. 10) and human chorion cells at 20 hr (Fig. 11 and 12) postinoculation. $\times 80,000$.

FIG. 13-16. Progeny virus within cisternae in the cytoplasm (c), near the plasma membrane (pm), open to the exterior, and in the extracellular space (e) in chorion cells at 20 hr (Fig. 13 and 14) and in cisternae opening to the exterior in HeLa cells at 8 hr (Fig. 15) and 12 hr (Fig. 16) postinoculation. $\times 80,000$.

32) and with SV40 virus (16, 21, 26). In the former case, the cisternae are quite pronounced near the cell margins, and from their occasional opening into the extracellular space it has been suggested that they provide a mechanism of virus release. The cisternae enclosing the SV40 virus are more commonly found deep in the cytoplasm where they may provide a route by which completed virus is transported from the nucleus (16). Poliovirus within intact kidney tissue has been reported to occur within elongated tubules whose association with ribosomes has suggested their derivation from the rough ER (2).

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