Structure and Development of Rabies Virus in Tissue Culture

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Structure and development of two fixed rabies virus strains in baby hamster kidney cells (BHK/21) were investigated by electron microscopy. The morphological development was correlated with fluorescent-antibody staining and infectivity titration. The uptake of virus was enhanced by addition of diethylaminoethyl dextran, and structural changes became apparent in the cytoplasm 8 to 9 hr after infection, when fluorescent-antibody staining was first discernible. These changes consisted of matrices containing fibers replacing normal cytoplasmic structures. Virus particles appeared at the edges of these matrices and inside them at 24 to 48 hr. This corresponded to significant rises in intracellular infectious virus. Formation of virus particles by budding from cell membranes was seen at 72 hr. Further incubation of the infected cells resulted in synthesis of bizarre structural elements. The complete virus particle was bullet-shaped with an average size of 180 by 75 m μ . It consisted of an inner core of filamentous material surrounded by two membranes of different densities. The surface showed a honeycomb arrangement with surface protrusions 60 to 70 A long having a knoblike structure at their distal end. These surface protrusions were absent at the flat end of the virus particle.

Rabies virus particles have been studied recently under the electron microscope by various investigators, who found the morphology of the virus to be variable. Working with either infected brain tissue (14, 17-21) or tissue culture preparations (1-4, 7), they observed, in addition to bullet-shaped particles, long filaments, round, hollow cores, and round forms resembling myxoviruses. This pleomorphism could have been ascribed to different morphological characteristics of various fixed and street virus strains used by these workers. It may also reflect, however, either the presence of artifacts created by excessive manipulation of the virus in the course of its partial purification, or the presence of contaminants such as *Mycoplasma*.

To elucidate this problem, the fine structure as well as the intracellular development of rabies virus were studied by electron microscopy (EM). The morphological observations were correlated with development of virus antigen, as tested by use of fluorescent antibodies (FA) and infectious virus.

The formation of virus-specific FA antigen can be greatly enhanced by the exposure of the cells to diethylaminoethyl (DEAE) dextran at the time of infection with rabies virus (22), and, therefore,

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the course of intracellular rabies infection was also followed in cells exposed to DEAE dextran.

The fine structure of the virus was studied by EM in thin sections as well as in negative-contrast

TABLE 1. *Rabies virus strains used in the experimental procedures*

Virus strains	Infectious tissue and LD ₅₀ titer ^a in mice			
	Chick embryo	Mouse brain	Rabbit brain	HDCS ^b
Flury high egg passage (15)	5.5	—	—	4.8 ^c
Pittmann-Moore (23)	—	—	5.2	5.5 ^d
Pasteur (23)	—	—	5.0	4.8 ^e
Challenge virus standard (23) . .	—	6.3	—	—

^a Expressed as log₁₀ per 0.03 ml.

^b HDCS = the WI-38 human diploid cell strain.

^c 16th passage in HDCS.

^d 54th passage in HDCS.

^e 28th passage in HDCS.

preparations. The latter method usually demands at least partial purification of the virus. To avoid observation of artifacts which may be created by somewhat drastic purification procedures, only cell-associated virus was investigated.

FIG. 1. Cytoplasm of a tissue culture cell 7 days after infection with the Flury (HEP) strain of rabies virus. The particles appear dispersed throughout the cytoplasm, occasionally forming closely packed arrays. They are sectioned in various planes, i.e. transversely (A), longitudinally (B), or obliquely (C). The mitochondrion (M) and the nucleus (N) are indicated. Thin section, $\times 40,000$. (Figures 1-14 were obtained on tissue culture cells 7 days after infection with the Flury (HEP) strain of rabies virus.)

MATERIALS AND METHODS

Virus strains. Strains of rabies virus used in the present study are shown in Table 1 (15, 23).

Tissue culture. The BHK/21 cells (16) were grown in milk dilution bottles or in Falcon plastic bottles (75-cm² area) in modified Eagle's basal medium and Earle's balanced salt solution, supplemented with 10% inactivated calf serum, and containing 1.8 g of sodium bicarbonate and 5×10^4 μ g of chlortetracycline per liter.

Infection of tissue culture. To study adsorption and penetration of the virus under the electron microscope, three samples of 25×10^5 to 30×10^6 BHK/21 cells were dispersed in 2 ml of maintenance medium containing $2.0 \times 10^{7.3}$ mouse LD₅₀ of the HEP high(egg passage) strain [material passaged in the WI-38 human diploid cell strain (HDCS)] and 50 μ g of DEAE dextran per ml. The suspensions were stirred constantly at 37 C for 15, 30, and 60 min, respectively. After each time interval, one of the cell suspensions was mixed with an equal amount of buffered osmic acid, and the fixed cells were pelleted and processed for EM.

For study of intracellular growth of rabies virus, monolayers of BHK/21 cells grown in milk dilution bottles for 24 to 48 hr and containing 4×10^6 cells per bottle were exposed to 2 ml of virus inoculum without preceding ultraviolet (UV) treatment (22). Various rabies virus strains (Table 1) were used in these experiments at an aimed input multiplicity of 2 to 5 mouse LD₅₀ per cell. The virus, in either the presence or absence of 50 μ g of DEAE dextran per ml, was allowed to adsorb for 1 hr at 37 C; subsequently, the cells were washed with phosphate-buffered saline (PBS), and fresh medium was added to them.

Immunofluorescent-antibody staining and infectivity titrations. The techniques described by Wiktor et al. (22) were employed.

Electron microscopy. For thin sectioning, infected as well as control cell cultures were harvested, fixed in phosphate-buffered osmic acid, dehydrated, and embedded in epoxy resin as previously described (12).

The technique used for negative staining of cell-associated virus in these experiments was the same as described before (13).

RESULTS

Monolayers of BHK/21 cells were infected with the virus strains shown in Table 1 and were harvested 7 days later. Although virus particles were seen under the electron microscope in all preparations, the greatest number was found in cells infected with the Flury and Pitmann-Moore (PM) strains. Since the basic structure of virus particles seen in all preparations did not differ, the Flury and PM strains were used in all subsequent experiments.

Thin sections. For morphological studies of rabies virus in thin sections, BHK/21 cell cultures infected with the Flury strain were harvested 7 days after infection. Virus progeny was found exclusively in the cytoplasm, except for occasional virus particles seen between the nuclear membranes (see below). The appearance of an infected cell at this time can be seen in Fig. 1.

The elongated particles appeared singly, in groups, and at times in closely packed arrays. They

FIG. 2. Virus particles (VP), sectioned either transversely or longitudinally, located between the nuclear membranes (indicated by arrows). Nucleus (N) is indicated. Thin section, $\times 40,000$.

were sectioned at various planes and, therefore, were seen as round particles when sectioned transversely, and as rods when cut along the axis. Oblique sections resulted in intermediate forms. These areas are indicated in Fig. 1 as A, B, and C. In Fig. 2, virus particles, indicated by arrows, can be seen between the nuclear membranes.

The majority of the particles were observed free in the cytoplasm, as in Fig. 1, but they were also found in cytoplasmic vacuoles, sometimes budding into them and, less frequently, budding from the cell surface (Fig. 3 and 4).

In cross sections, the virus appeared to consist of a core surrounded by a dense membrane. This core, with an average diameter of $40\text{ m}\mu$, most often appeared empty, but occasionally some material could be found inside. The dense membrane was always surrounded by a fringe of surface projections. The diameter of the total particle appeared to be $80\text{ m}\mu$. The details of a transversely sectioned particle can be seen in Fig. 5, indicated by arrow A.

The complete virus in longitudinal section resembled a bullet. The dense membrane did not cover the flat end but seemed to be continuous around the convex part and was visible when properly sectioned. The diameter of these particles was the same as that obtained for transverse sections. The length, on the other hand, was more variable. Complete, bullet-shaped virus particles measured about $180\text{ m}\mu$ in length. Such longitudinal sections are demonstrated in Fig. 5 (arrow B).

Apart from these presumably complete particles, rods of varying length were also observed. They had basically the same structure and same diameter as the virus particles, but the dense membrane did not cover either end (Fig. 5, arrow C). These rods seemed to form by breaks in larger, often branched structures (Fig. 6A), but the breaks could have also resulted in structures resembling virus particles (Fig. 6B).

Negative contrast. A more detailed analysis of virus structures was obtained by the negative-staining technique, which was performed on cell-associated virus by use of phosphotungstic acid (PTA). In these preparations, a variety of structures became apparent which could be correlated to those seen intracellularly by use of thin sectioning. Two intact bullet-shaped particles and one partially disintegrated particle can be seen in Fig. 7. The intact particles measured 180 by $75\text{ m}\mu$ and showed a fine fringe of surface "spikes." At the flat end of the virus, the fringe was missing. An indentation, similar to the neck of an arrow, was evident. The surface of the intact particles appeared as a dense, irregular mass, and it was difficult to discern any regular surface structure.

FIG. 3. Virus particles located free in cytoplasmic vacuoles, and budding into them. Thin section, $\times 40,000$.

FIG. 4. Virus particles forming on the cell membranes by budding. Thin section, $\times 40,000$.

On occasion, however, particles were found that had a distinct surface arrangement (Fig. 8). Since the diameter of this particle was larger than that of the viruses shown in Fig. 7, it may be assumed that its surface was stretched, revealing an underlying symmetry in the middle part. Both ends showed signs of disarrangement. Where a symmetry of the surface structure could be seen, as in the area indicated, it appeared that this part of the surface was arranged in hexagons forming a honeycomb. Surface extensions, 60 to 70 A long, with a knoblike structure at the distal end, were clearly resolved.

Disintegration of the structure, presumably by surface tension caused by the drying process on the specimen support, resulted in collapse of the hexagonal arrangements, leaving only diagonal bands visible (Fig. 9).

The internal structure of the rabies virus can

FIG. 5. Details of virus manifestations in the infected cell. In transverse sections (A), the virus consists of an inner core surrounded by a dense membrane with surface projections. Longitudinal sections reveal bullet-shaped particles (B) or long rods flat at both ends (C). Thin section, $\times 80,000$.

FIG. 6. Long and branched structures breaking into parts resembling rods (A) or virus particles (B). Thin section, $\times 80,000$.

be seen in detail in Fig. 10. The end-on view of these particles in negative contrast corresponded to that of transverse sections. For direct comparison, such a section is shown in the inset of Fig. 10 in a reverse negative image and enlarged to the

FIG. 7. Virus particles in negative-contrast preparation. Two complete particles show the characteristic shape with the rounded end and the notch in the flat end. A fine fringe of surface extension, or spikes, is visible. The particle on the right is partially disintegrated. $\times 200,000$.

same magnification as the negatively stained particles.

The fine structure of the viruses showed some differences, depending on the techniques of preparation employed. In the negative-contrast prep-

FIG. 8. Virus particle at higher magnification. The middle part of its surface shows a structural arrangement resembling a honeycomb. The spikes have a knob-like protrusion at their distal end. Negative contrast, $\times 400,000$.

FIG. 9. Virus particle showing breakdown of the surface structure. Remaining parallel strands give a ribbed appearance. Negative contrast, $\times 400,000$.

eration, the surface fringe was narrow and not prominent, but two continuous membranes were clearly visible underneath. The dense membrane on the outside was separated by an interspace from the inner membrane which appeared less dense. The core seemed to be filled with convoluted material. On the other hand, in the transversely sectioned particle, a very prominent fringe and only a single membrane were evident. The core of the virus was also filled with material, presumably virus nucleoprotein. The core in the section was smaller in diameter than that in the particle in negative contrast. This difference in size was probably caused, on the one hand, by expansion due to increase in surface tension in the course of negative staining and, on the other hand, by contraction of the particle due to the dehydration. Thus, the single membrane seen in the thin section may actually represent a fusion of two distinct membranes seen in PTA preparations.

In addition to the virus particles of fairly uniform structure, other structures of different shapes and forms were observed in the infected cells.

Bullet-shaped particles, much shorter than the complete virus particles, are shown in Fig. 11a and b. In some of these, the less dense protruding area had the same width as the neck in complete

FIG. 10. End-on view of virus particles. The core contains convoluted material (arrow) and is surrounded by two membranes of different densities. The surface spikes are poorly resolved. The comparable appearance in a thin section is shown in the inset in a reverse negative image. Negative contrast, $\times 200,000$.

FIG. 11. Short forms of particles seen in the virus progeny. (a) Two particles with a hollow core; (b) one particle shows a tail-like appendix. Negative contrast, $\times 200,000$.

virus particles (Fig. 11A). Other types of short particles showed a tail-like appendage at one end, as in the one on the right in Fig. 11B.

The long rods seen in thin sections were also found in negative-contrast preparation, and their structure and diameter were similar to that of the virus particles. These rods were observed as a dense structure with the characteristic nock at one or both ends, and they were in various stages of disintegration (Fig. 12A-C). Two breaks, apparent in the rod structure in Fig. 12C, were surrounded by a membrane, presumably derived from the cell. Each of the three fragments into which the rod seemed to have separated measured about 200 $m\mu$ in length.

Even more bizarre structures were found, as shown in Fig. 13 and 14. The two branches in Fig. 13 closely resembled the fine structure of virus particles, as did the lower part of the particles shown in Fig. 14.

Growth cycle. The morphological study of rabies virus was conducted on cell cultures 7 days after infection, and the infected cells contained many virus particles and virus-associated structures. Because observation of infected cells at predetermined intervals after infection affords a more orderly insight into the importance of the

various structures in the developmental cycle of the virus, adsorption and penetration of the virus, as well as intracellular development of the viral progeny, were investigated, by use of the Flury and PM strains of rabies virus.

Adsorption and penetration. In the absence of DEAE dextran, few virus particles were seen either attached to, or inside, the cells; in the presence of DEAE dextran, a moderate number of virus particles were found, demonstrating the mode of attachment to the cell surface and the mode of penetration (Fig. 15A-E).

Virus particles adsorbed to the cell surface were attached to it by fine fibers; this attachment seemed to occur with any part of the particles except the flat end (Fig. 15 A and B). The particles passed into the cytoplasm by phagocytosis. Pino-cytotic vesicles were formed, enclosing single or several particles. The particles were found at these locations at 15 and 30 min after infection and can be seen in Fig. 15 C and D. At 60 min after infection, the virus-containing vesicles had moved deeper into the cytoplasm when intravesicular disintegration of particles was observed (Fig. 15E, arrow). No virus was found free in the cytoplasm at any time, but the input multiplicity of virus was probably too low to allow observation of all stages of adsorption and penetration.

Development of virus progeny. Appearance, and subsequent development of FA staining and infectivity corresponded closely to the data shown in Table 5 and Fig. 3 in the paper by Wiktor et al. (22).

In the presence of DEAE dextran, distinct and characteristic morphological changes were observed in the infected cells 8 to 9 hr after infection, when FA was first detected. The normal structures of the cytoplasm were replaced by a granular mass containing occasional fine fibers (Fig. 16). The ribosomes in the unaltered cytoplasm surrounding these areas showed distinct polyribosomal aggregates. The formation of these matrices, described before in tissue culture (3, 7) and mouse brain (14, 17, 18, 21), was considerably delayed in the absence of DEAE dextran. No further morphological changes were seen until virus particles became visible at the edges of some matrices (Fig. 17) at 24 to 48 hr, when infectious titers had shown a significant rise. This condition became uniform and was followed by condensation of the fibers into larger bundles, which often lined up in a parallel manner, and by formation of virus particles inside these areas (Fig. 18). The individual fiber, at this point, became thicker and showed an indication of periodicity.

Within 72 hr, virus particles formed on the cell

FIG. 12. Appearance of long rods in negative-contrast preparations. They are of varying lengths, with surface structures resembling those of virus particles. They show different degrees of disintegration (a and b). (c) A rod is breaking into three units of equal length. $\times 200,000$.

FIG. 13 and 14. *Bizarre structures seen in virus progeny. Parts of these structures resemble virus particles. Negative contrast, $\times 200,000$.*

surface by the process of budding. This occurred either in intracytoplasmic vacuoles or, to a lesser degree, on the cell surface (Fig. 19). At the same time, the synthesis of particles increased within the matrices (Fig. 20).

Further incubation of the infected cultures up to 7 days resulted in the appearance of bizarre structures in the cells. In Fig. 21 they appeared as long rods or bands, often branched. They were densely packed with no orderly grouping. These structures obviously were identical with those shown in negative contrast (Fig. 12 a-c), which were also derived from 7-day-old cultures. Although these rods still exhibited a close structural relationship to rabies virus, long filaments of varying width began to appear in the matrix (Fig. 22 and 23). These filaments were occasionally wide and diffuse (Fig. 22) or, more often, narrow and dense, with a definite substructure (Fig. 23).

Although all cells contained many virus particles and virus-related structures 7 days after infection, most cells were not disrupted and virus release by budding on the cell surface was minimal compared with the number of particles found intracellularly.

DISCUSSION

The distinct morphology of rabies virus indicates its close relationship to a number of agents.

Among these viruses are lettuce necrotic yellow virus (9), plantain virus (10), the sigma virus of drosophila (5), coccal virus (8), hemorrhagic septicemia virus of the rainbow trout (24), and vesicular stomatitis virus (11). They parasitize a wide variety of hosts, from plants to mammals. Little is known about antigenic relationships among them. In complement-fixation tests, coccal virus was found to be related to the Indiana strain of vesicular stomatitis but not to the New Jersey strain (8). No antigenic relationship was found by us in cross-neutralization tests between rabies and the Indiana strain of vesicular stomatitis.

The fixed rabies virus had a distinct and characteristic morphology. Unless an easily discernible concomitant infection with *Mycoplasma* was also present, myxovirus-like particles were never seen in the cell cultures of the experiments described above.

The particles appeared cylindrical with one rounded end, resembling a bullet. Their average diameter was 180 by 75 μ . Their surface extensions, or spikes, were 60 to 70 A in length with a knoblike structure at the distal end. The envelope seemed to show a periodicity of hexagons, in a honeycomb array. The capsids were labile, and, thus, the technique of specimen preparation greatly influenced their appearance. In the negative-contrast technique, characterized by changes

FIG. 15. (a) Virus particles (VP) adsorbed onto the cell membrane. They are sectioned either longitudinally or transversely. Fibrils (F) are discernible between the particle on the right and the plasmalemma. (Obtained 15 min after infection). (b) Beginning phagocytosis of a virus particle (see arrow). A second particle is attached to the plasmalemma with its convex end. (Obtained 15 min after infection). (c) Virus particle (VP) in forming pinocytotic vesicle. (Obtained 15 min after infection). (d) Four particles (VP) in pinocytotic vesicle in the cytoplasm. (Obtained 30 min after infection). (e) Two particles in pinocytotic vesicle deep in the cytoplasm. One particle (VPD) is disintegrating. (Obtained 60 min after infection). Thin section, $\times 80,000$. (Figures 15-23 were obtained at predetermined intervals after infection of tissue culture cells, as indicated.)

in surface tension during the drying process, viruses were seen in all stages of disintegration. Whether some morphological manifestations, such as the hollow core, the nock at the flat end found in most particles, or the short forms retaining the rounded end and thus resembling the S or T particles described for vesicular stomatitis (A. J. Hackett, S. H. Madin, and F. L. Schaffer, *Bacteriol. Proc.*, p. 123-124, 1966; A. S. Huang and R. R. Wagner, *Federation Proc.* **25**:491, 1966), are artifacts or integral parts of rabies virus populations remains to be investigated.

On the basis of the presented developmental

studies, some aspects of rabies virus formation can be described; however, this description is based solely on two fixed virus strains and is limited to one host system.

Foci of synthesis become apparent in the cytoplasm, which presumably contains virus nucleoprotein, and virus membranes are formed *de novo* in or at the edges of these foci, or matrices. There is little doubt that the matrices contain virus-specific antigen, as evidenced by the close correlation of its appearance in the electron microscope with the development of positive fluorescent-antibody reactions. The organization of ribosomes

FIG. 16. *Appearance of the virus matrix (MA) in the cytoplasm. These areas are first seen 8 to 9 hr after infection in the presence of DEAE dextran, and 24 to 48 hr after infection in the absence of DEAE dextran. The mass is primarily granular, although fine fibers can also be seen. The nucleus (N) is indicated. Thin section, $\times 40,000$.*

FIG. 17. Formation of virus particles at the periphery of the matrix (MA) 24 to 48 hr after infection. Nucleus (N) is indicated. Thin section, $\times 40,000$.

into polyribosomal aggregates is pronounced when the matrices are apparent, indicating greatly accelerated nascent protein synthesis in the cells. Morphologically complete virus particles emerge in these cytoplasmic matrices, as has been described for vaccinia virus (6). Release of these particles to extracellular environment was not

observed. On the other hand, somewhat later in the growth cycle, a small number of particles are formed by budding on the cell surfaces with an obvious involvement of cell membranes.

During the early stages of infection, the morphology of the particles is surprisingly uniform. Long rods, often extensively branched, appear

FIG. 18. Some virus particles forming in the matrix (48 hr after infection). The fibers have thickened, lining up in a parallel manner in many areas and showing an indication of periodicity. Thin section, $\times 60,000$.

later. Since many of these rods seem to segment into smaller parts, Matsumoto (18) has suggested that virus particles are formed by this mechanism. This observation, however, pertained to studies of the virus at a late infectious stage in mouse brain. In tissue culture preparations, the production of disorderly components seems to occur only when the synthetic mechanism of the infected cell is producing virus at a high rate, thus interfering with an orderly process.

The origin of the extremely long filaments which appear late in the infectious process is obscure. They are found only in matrix material and seem to condense from it. They show a definite substructure and may consist of condensed, excess virus protein.

The infected cells, despite the fact that they contain numerous virus particles and other virus-associated material, retain much of their structural integrity. Virus release to the outside by budding on cell membranes is minimal, as compared with the amount of virus which is retained. The retention of most of the viruses by the cells, as observed *in vitro*, may have some bearing on

the slow spread of rabies virus in its natural hosts *in vivo*, as is evident from the irregular and often long incubation periods.

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FIG. 19. Formation of virus particles by budding on the plasmalemma (above right), and into cytoplasmic vacuoles (72 hr after infection). Nucleus (N) is indicated. Thin section, $\times 40,000$.

FIG. 20. *Increased synthesis of virus particles in the matrix (72 hr after infection). Thin section, $\times 40,000$.*

FIG. 21. *Appearance of long, and occasionally branched, rods (7 days after infection). Breaks of these rods into smaller units of regular size are indicated by the arrows. Thin section, $\times 40,000$.*

FIG. 22. Long filaments in the matrix (7 days after infection). They appear either wide and diffuse, as on the left, or narrower and more condensed, as on the right. Thin section, $\times 40,000$.

FIG. 23. Filaments are always confined to the matrix (M), assuming great lengths (7 days after infection). They show a regular structure in some areas, as can be seen in the inset. Thin section, $\times 40,000$; inset, $\times 80,000$.

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