Molluscum Contagiosum Virus in Adult Human Skin Cultures

An Electron Microscopic Study

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MOLLUSCUM CONTAGIOSUM (MC) VIRUS, a deoxyribovirus, has the structural characteristics of a poxvirus and is so classified. However, relatively little is known about the biologic behavior of this virus, since it has yet to be propagated in tissue culture,[1,2] and attempts to transmit it to other than human hosts have failed.[3]

It is known that when MC virus from human lesion extracts is inoculated into a number of different cell cultures, a cytopathic effect is observed.[1,2,4] However, the latter does not indicate replication of MC virus, since the cytopathic effect is not prevented by 5-iodo-2'-deoxyuridine,[5,6] and it is not observed beyond four to five serial passages.[2,5,6]

MC virus inoculated into some cell cultures causes interference with plaque formation by several unrelated viruses.[7,8] Such interference is mediated by interferon induced by MC virus.[8] Through these interference studies, evidence for the adsorption of MC virus to mouse embryo [9] and chick embryo cells [8] has been obtained.

An electron microscopic study of chick embryo cell cultures inoculated with MC virus showed that after adsorption to these cells, mature virus particles were phagocytosed and entered the cytoplasm in a partially uncoated state (first-stage uncoating).[10] The intracytoplasmic viral cores were never fully uncoated (second-stage uncoating) presumably because the "uncoating protein" was not elaborated. Replication of the virus did not occur.

The mechanism of second-stage uncoating is the subject of controversy. Joklik [11] believed that no RNA could be transcribed from viral cores containing DNA in a state inaccessible to DNase. Therefore, he concluded that second-stage uncoating enzymes were coded for by host

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cells. More recently, Kates and McAuslan [12] have shown that viral messenger RNA is transcribed from coated poxviruses' genomes. These authors tentatively proposed that the RNA released from the core probably codes for a variety of early proteins, including those required for second-stage uncoating.

The present electron microscopic study was undertaken to elucidate this problem. Since, to date, MC virus has been shown to replicate only in the epidermal cells of human skin—ie, in the lesions of MC: (1) explants of diseased skin from patients with MC were grown in tissue culture and (2) organ cultures of healthy adult human skin showing good epidermal outgrowths were inoculated with extracts from MC lesions. It is the purpose of this paper to show that (1) MC virus probably replicated within the molluscum bodies of the explanted MC lesions, but viral release and infection of the epidermal cells in the outgrowth were not observed; and (2) MC virus inoculated into organ cultures of healthy human skin was adsorbed to, phagocytosed, and partially uncoated by the epidermal cells in the outgrowth, but second-stage uncoating (ie, loss of viral capsid and release of viral DNA) and viral replication did not take place.

Materials and Methods

Preparation of MC Extract

An extract of MC lesions from each of 7 patients with clinically characteristic (and histologically confirmed) lesions was prepared separately and stored for up to 2 years in a manner previously described in detail.[8]

Preparation of Negatively Stained Virus

Grids coated with Formvar and carbon were used. A drop of MC extract was placed on a grid for 2 min. The excess fluid was adsorbed with filter paper. While still moist, the grid then was covered with a drop of 2% sodium phosphotungstate, pH 7.4, for 2 min, and the excess fluid adsorbed with filter paper. After drying, the grids were examined in a Siemens Elmiskop I.

Preparation of Organ Cultures of Diseased Skin from Patients with MC

Four biopsy specimens, each 0.5 cm in diameter, of split-thickness skin containing the MC lesion were excised from each of 2 patients. These specimens were explanted into the culture medium.[13] The epidermal outgrowth spread laterally from the explants during the length of these experiments, which lasted 32 and 48 days, respectively.

Preparation of Organ Cultures of Split-Thickness, Healthy Adult Human Skin

The method here employed is one that has been described previously.[13] Specimens of healthy skin from two patients were used in these experiments. Epidermal outgrowths were observed first on the fourth and ninth days, respectively. The out-

growths survived and extended laterally from the explant for the duration of the experiments, 50 and 52 days, respectively. Some epidermal cells underwent keratinization.

Inoculation of Organ Cultures of Healthy Adult Skin

At 4–9 days after a good epidermal outgrowth had formed, the cultures were inoculated with 0.1–0.4 ml of (undiluted or diluted 1:3) stored MC extract. Adsorption proceeded for 2 hr. These cultures were then washed free of unadsorbed virus, fed with 2.5 ml of the culture medium, and incubated at 37° C in a humidified atmosphere of 5% CO_2 in air for intervals varying from 1 hr to 48 days.

Preparation for Electron Microscopy

After washing twice in cold phosphate-buffered saline (PBS), the cultures were fixed in cold 3% glutaraldehyde in phosphate buffer for 1–12 hr. The cultures were postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr, passed through graded alcohols, and embedded in Epon. Since propylene oxide buckled the plastic dishes in which the biopsy specimens of skin were grown, it was omitted from the embedding procedure. A shearing force was used to remove the plastic dish from the polymerized Epon. Pieces of the embedded specimens were mounted on Epon blocks so that the specimens could be sectioned parallel to the surface of the culture.

Thin sections were cut with glass or diamond knives on a Porter-Blum MT-2 microtome, mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I. Micrographs were taken at original magnifications varying from 4,000 to 32,000.

Results

Negatively Stained MC Virus

The negatively stained virus particles were arranged singly or in clumps. The majority were rectangular in shape (C forms) and measured $\sim$ 320 $\times$ 230 mμ (Fig 1). These particles showed a capsule $\sim$ 230 Å in width, surrounding a smooth inner body. The outer surface of the capsule was somewhat indistinct; and the inner, smooth. The inner portion of the capsule was subdivided by radially arranged partitions with intervals of 80–90 Å. Rarely, C forms were found which showed only a capsule and no smooth inner body. M (mulberry) forms (Fig 2), were less numerous; they were less electron dense and smaller than the C forms and measured $\sim$ 230 $\times$ 215 mμ. The surface of the M form was covered by a meshwork of threads, 100 Å wide, which occasionally were arranged in an orderly fashion. The threads appeared to be composed of filaments, $\sim$ 35 Å thick, which formed a double helix. Winding around the particle, the threads gave the edge a toothed appearance. The internal structure of some M forms was vaguely discernible (Fig 3); it appeared that the threads were applied more closely to the particle. Rarely, a virus particle was surrounded by a very loosely applied single membrane. A few particles, elliptical in shape, were seen in profile. The

biconcave viral core contained a moderately electron-dense nucleoid. At either end the nucleoid was covered by the capsule; and laterally, by the lateral body and capsule (Fig 3).

Cultured Explants of Diseased Skin from Patients with MC

The strata germinativum, spinulosum, and granulosum were necrotic. The corium of the explant was covered by the stratum corneum. The latter contained molluscum bodies—ie, large anuclear cells with a moderately electron-dense, fairly homogeneous cytoplasmic matrix containing many MC virus particles of varied maturity (Fig 4) and small aggregates of fibrils showing greater electron density than the cytoplasmic matrix—ie, viral matrix or so-called viral factories. Viral membrane was formed focally on the circumference of these aggregates; at first it was arc-shaped, and later, circular or oval. The viral membrane packaged small amounts of viral matrix into immature viral particles (Fig 5). After the viral particles were pinched off the viral matrix, they underwent maturation. As the particles matured, their shape changed from oval to rectangular (Fig 6). In front view, the mature virus particles were composed of a grey (moderately dense) capsule and a central black (electron-dense) fibrillar nucleoid. The central nucleoid was missing from some of the viral particles (Fig 6). In profile, some of the viral particles showed lenticular-shaped lateral bodies situated between the outer viral membrane and the viral core, which contained an electron-dense fibrillar nucleoid. The lateral bodies compressed the viral core and the nucleoid so that they assumed an hourglass or figure-of-eight appearance (Fig 6). Viral release was not observed. The flattened cornified cells adjacent to the large infected cells contained no virus particles. The cells in the epidermal outgrowth surrounding the explant were not infected.

Healthy Adult Human Skin Cultures Inoculated with MC Virus

The epidermis covering the explant did not contain virus particles. The latter were seen only in the epidermal outgrowth. Here, epidermal cells were efficient in removing mature virus particles from the medium; 48 hr after the cultures were inoculated with MC virus, either very few or no mature virus particles were found in the intercellular spaces (Fig 7). Removal of the particles by these cells started between 1 and 2 hr after inoculation when mature virus particles were adsorbed to and engulfed by epidermal cells (Fig 8). Shortly thereafter, these particles, singly or in large numbers, were located within phagocytic vacuoles (Fig 9) where they underwent varied degrees of degradation. Thus, in

some vacuoles viral cores were found—ie, the lateral bodies and outer part of the capsule had been stripped from the mature particle (first-stage uncoating); in older cultures, large phagocytic vacuoles contained uniformly stained granules, membranes, and vaguely outlined virus particles. In cultures fixed within 3–4 hr after inoculation, viral cores had escaped from the phagocytic vacuoles and had entered the cytoplasm (Fig 10 and 11). These cores were round, oval, or rectangular, depending on the plane of section. Their size approached that of the mature particle. They were surrounded by a capsule with smooth inner and indistinct outer surfaces. When the plane of section was propitious, the capsule showed radially arranged partitions ~ 100 Å in length and spaced at intervals of ~ 75 Å (Fig 11). The capsule surrounded an electron-translucent space within which the electron-dense nucleoid was situated. The latter was composed of curved and twisted fibers, presumably representing fibrous DNA, ~ 60 Å in width. Occasionally some of the fibers made contact with the capsule. In a section of an infected cell, the number of intracytoplasmic cores varied from a few to approximately 50 (Fig 12). These viral cores persisted within the cytoplasm of infected cells for the duration of these experiments—ie, 52 days following the inoculation of the cultures with MC virus. Both nonkeratinized and keratinized cells in the epidermal outgrowth contained viral cores (Fig 13). Rupture of the core with release of the viral genome was not observed. Replication of the virus did not occur.

Discussion

After negative staining, the examination of MC extracts used in the experiments reported herein showed that the extracts contained structurally mature virus particles. The latter were of two types, M and C forms;[14] they were similar in appearance to negatively stained MC virus [15,16] and vaccinia virus [14,17] as described by others. Westwood *et al* [14] have shown that the M and C forms are interconvertible. The ratio of M to C forms in vaccinia preparations is in part due to the rate of drying of the stained virus particles, and transition to C forms is rendered complete by detergent treatment.[17]

When the outer viral membrane and lateral bodies are stripped from mature vaccinia particles, the M form is revealed.[17] The latter has a thin lipoprotein membrane or layer which covers the C form.[14] When the structural integrity of this membrane is damaged, the virus particle is more permeable to the stain, revealing the more electron-dense C form, which exhibits an internal structure. The inner, partitioned portion of the capsule becomes the outer wall of the viral core.[17]

Our studies of cultured specimens of diseased skin from patients with MC showed that the infection remained limited to the MC lesion and did not involve the epidermal outgrowth. In somewhat analagous experiments, Mitchell [18] grafted an MC nodule onto the chorioallantoic membrane of embryonated hens' eggs; the chorioallantois was not infected by the virus.

In the stratum corneum of the explanted MC lesions, the infected cells resembled both a factory and a storehouse for virus particles. However, viral release from these infected cells was not observed. Within the cytoplasm of these cells, viral matrix and particles ranging from the very immature to the mature were seen. These findings were similar to those of Middlekamp and Munger,[19] who studied MC lesions fixed immediately after excision. In addition, the viral matrix and particles in the MC lesion resembled those described in studies on the development of vaccinia virus [20,21] and fowl pox.[20]

It is presumed that the infected cells in the cultured MC lesions synthesized viral DNA for an undetermined length of time. Tanigaki and Kato [22] have shown that when MC lesions are cultured for 1–3 hr (the duration of their experiments), ^{3}H-thymidine is incorporated into the cytoplasm of infected cells with inclusions up to $\sim$ 30 μ in diameter. In cells showing intracytoplasmic (viral) DNA synthesis, these authors found less or no nuclear DNA synthesis. They concluded that the MC nodule was due to the proliferation of cells in the strata basale and spinosum, which subsequently were infected, rather than the division of virus-producing cells.

When organ cultures of healthy adult skin were inoculated with MC virus, the sequence of events that followed was similar in many respects to that observed in chick embryo cell cultures similarly inoculated.[10] The mature particles in the inoculum quickly were adsorbed to and phagocytosed by the epidermal cells in the outgrowth. When compared with chick embryo cells, epidermal cells appeared to be more efficient in removing virus particles from the inoculum. The mature virus particles were degraded partially to viral cores in phagocytic vacuoles, presumably due to the action of lysosomal enzymes. The cores entered the cytoplasm of the infected cells and remained virtually unchanged for the duration of the experiments. The intracytoplasmic location of these virus cores is of some interest, since it is known that when vaccinia virus is inactivated by heat or antibody, the normal transfer of cores from phagocytic vacuoles is blocked.[23]

Second-stage uncoating—ie, the release of the viral genome from ruptured cores—and viral replication were not observed in either chick

embryo cell cultures [10] or organ cultures of healthy human skin. In these systems, MC virus apparently failed to induce the synthesis of "uncoating protein." Supporting data for this conclusion is derived from the results of experiments reported by others [24]—ie, that heat-inactivated vaccinia virus in cell culture is not reactivated by superinfection with live MC virus, whereas superinfection with another live poxvirus results in reactivation.

The inoculum used in the experiments reported herein contained structurally mature MC virus particles. Although these particles replicate in the epidermis of intact skin, they fail to do so in organ cultures of healthy human skin. We do not know why MC virus behaves so differently in these two situations. Nevertheless, we have given consideration to some of the mechanisms that may account for this difference. While the morphologic similarities between the epidermis in biopsy specimens of healthy skin and the epidermis of cultured healthy human skin are striking,[25] we can assume that the nutritional requirements and the biologic potential of intact and cultured human epidermis differ. Such differences are found commonly when mammalian cell populations are compared with intact animals.[26] As a corollary, it would appear that intact skin contains some factor or factors necessary for the elaboration of the uncoating protein; these factors are not present in cultured skin. This does not exclude the possibility that the intracytoplasmic cores code for viral DNA-dependent RNA and some enzymes. On the other hand, it is conceivable that the uncoating protein is not synthesized because some metabolic product of cultured cells inhibits the expression of viral DNA.

Summary

Extracts from molluscum contagiosum lesions were stained with sodium phosphotungstate. They contained structurally mature virus particles, both C and M forms, similar in appearance to many other poxviruses.

Diseased skin from patients with MC were grown in organ cultures. Well preserved molluscum bodies were found in the stratum corneum covering the explant. These bodies contained viral matrix and immature and mature virus particles. It is presumed that for a limited period of time the virus replicated within the molluscum body. However, viral release was not observed and the epidermal cells in the outgrowth surrounding the explant were not infected.

Organ cultures of healthy skin showing good epidermal outgrowths were inoculated with MC lesion extracts. Mature virus particles adhered

to and were efficiently phagocytosed by the epidermal cells in the outgrowth. These particles were converted to viral cores within phagocytic vacuoles. The cores left the vacuoles and entered the cytoplasm where they remained virtually unchanged for the duration of these experiments. Second-stage uncoating and viral replication were not observed.

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[*Illustrations follow*]

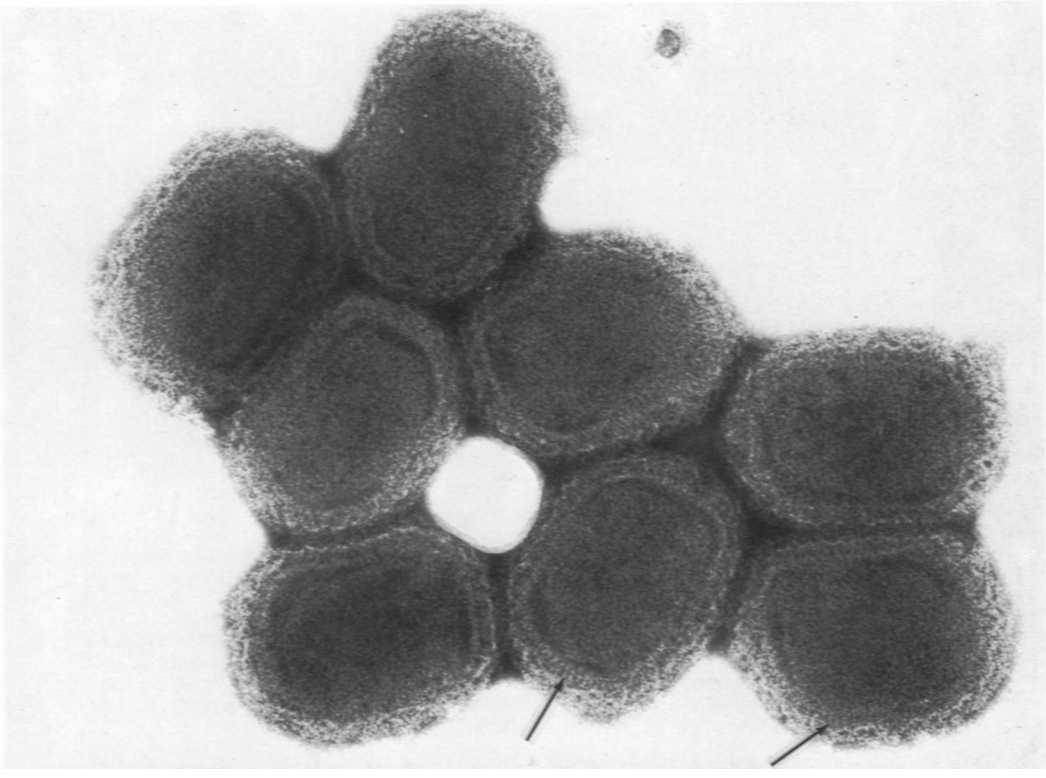
Legends for Figures

Extracts of molluscum contagiosum lesions were stained with 2% sodium phosphotungstate. All tissues prepared for electron microscopy were fixed in 3% glutaraldehyde and postfixed in 1% OsO_4. The illustrations are of sections stained with uranyl acetate and lead citrate.

Fig 1. Mature virus particles, C forms, in extract of MC lesion. Particles show a capsule surrounding a smooth inner body. Outer surface of capsule is indistinct; inner surface is smooth. Radially arranged partitions *(arrows)* subdivide inner portion of capsule. × 108,000.

Fig 2. Mature virus particles in extract of MC lesion. M forms *(M)* and C forms *(C)* are shown. Threads composed of filaments forming a double helix *(arrows)* are wound around the M form. × 133,000.

Fig 3. Mature virus particles in extract of MC lesion. M forms *(M)* show a more electron-dense center, suggesting an internal structure. Threads covering the surface appear to be more closely applied to the particle. Upper particles are C forms *(C)*. One particle, seen in side view, shows a biconcave nucleoid *(arrow)* surrounded by a capsule. × 80,000.



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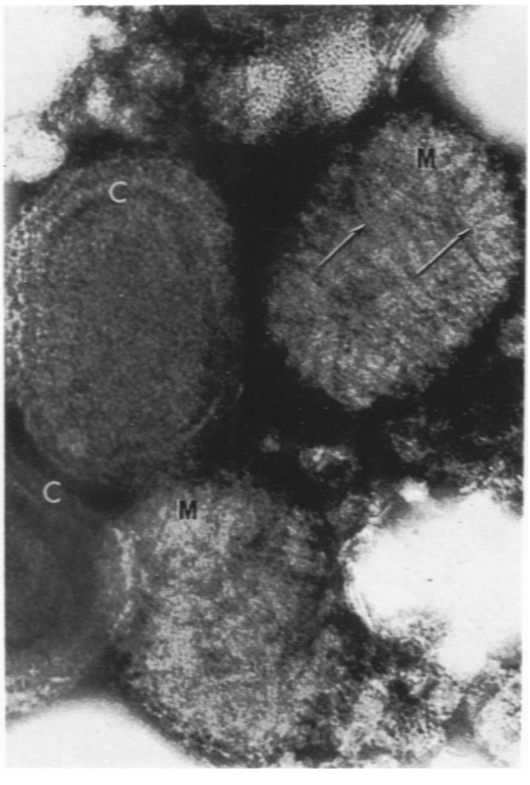

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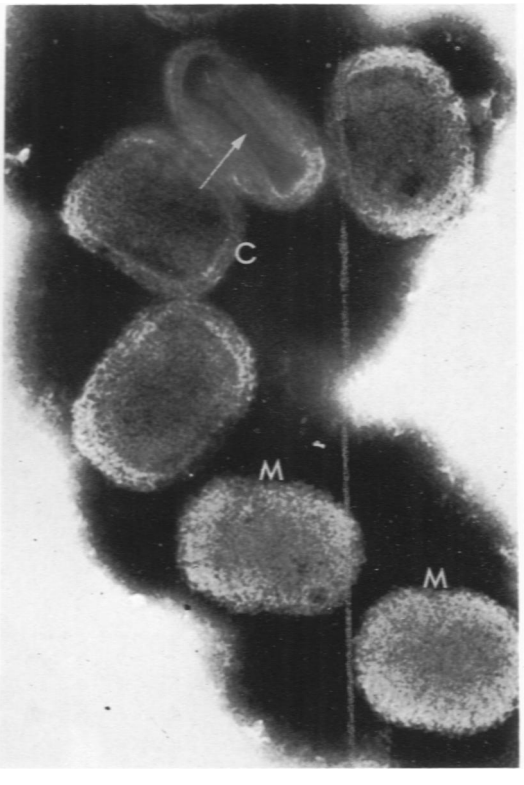

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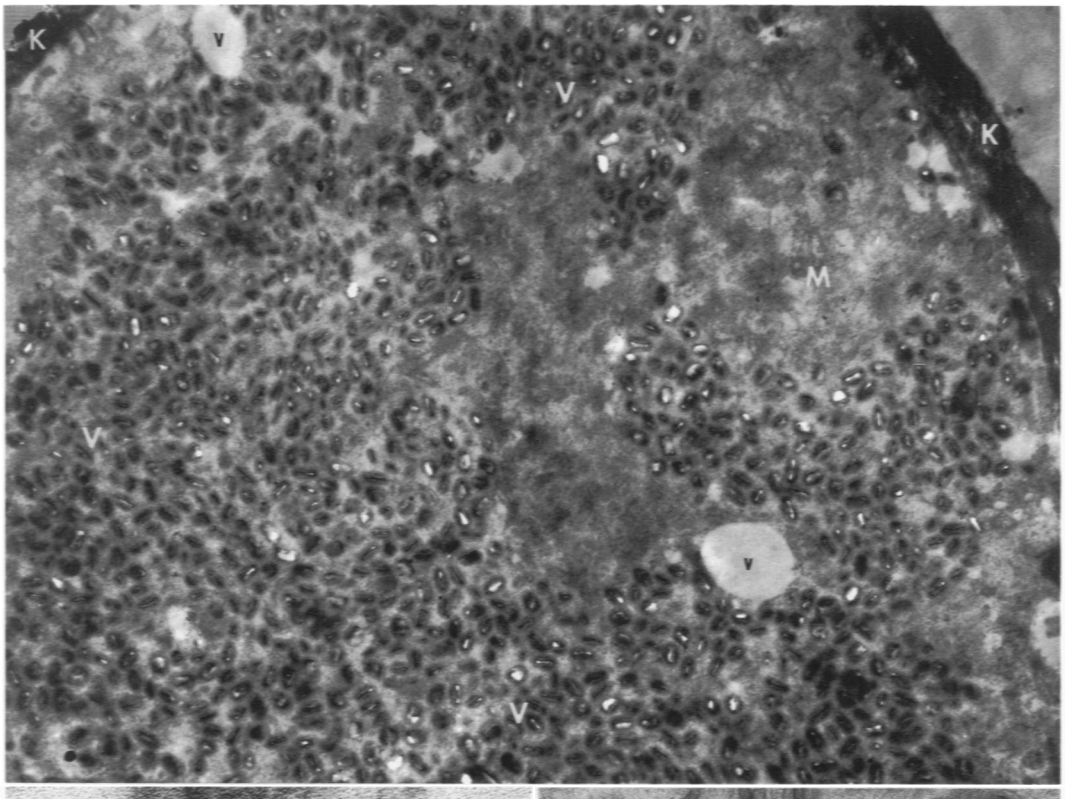
Fig 4. Part of molluscum body situated within stratum corneum covering explant of diseased skin from a patient with MC. The body, covered by a keratinized cell (*K*), contains numerous virus particles (*V*), viral matrix (*M*), and vacuoles (*v*). × 11,200.

Fig 5. Part of molluscum body in stratum corneum covering explant of diseased skin from a patient with MC. Circumference of fibrillar viral matrix (*M*) is focally covered by arc-shaped membrances (*arrows*). In upper part of photograph, viral matrix is encircled completely by a membrance (*crossed arrow*). Mature virus particle (*V*) has a dumb-bell-shaped electron-dense nucleoid. × 49,000.

Fig 6. Part of molluscum body in stratum corneum covering explant of diseased skin from a patient with MC. Virus particles of varied maturity are shown. Some, in front view, are oval, (*arrow*); others, more mature, are rectangular with electron-dense fibrillar nucleoids surrounded by a less dense capsule (*X*). In side view, the electron-dense biconcave nucleoid as surrounded by a less dense capsule. Lenticular lateral bodies (*crossed arrows*) are seen on either side of the capsule. A thin viral membrane surrounds the particle. × 42,000.



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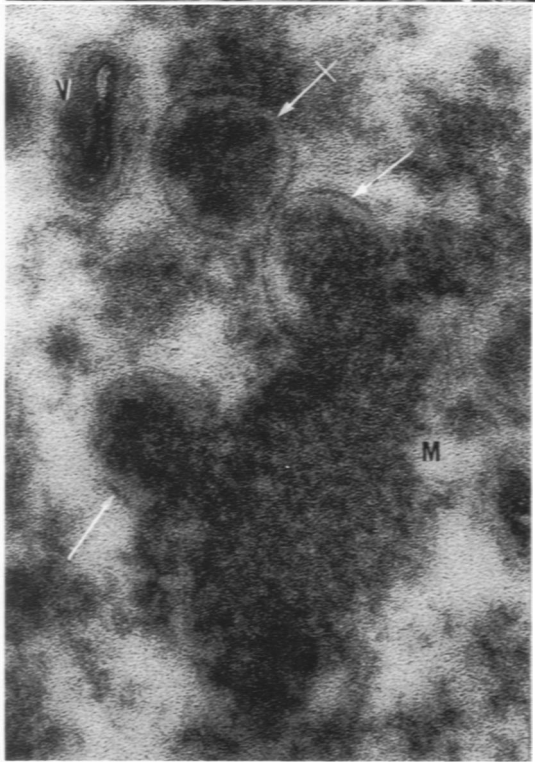

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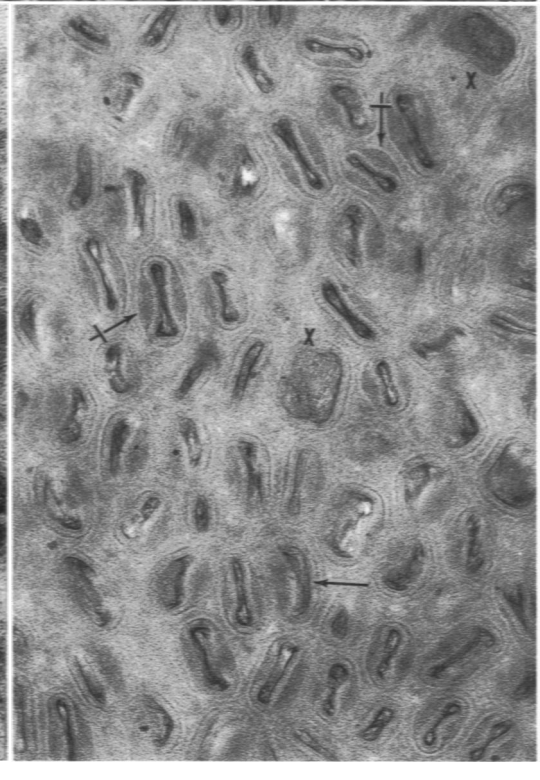

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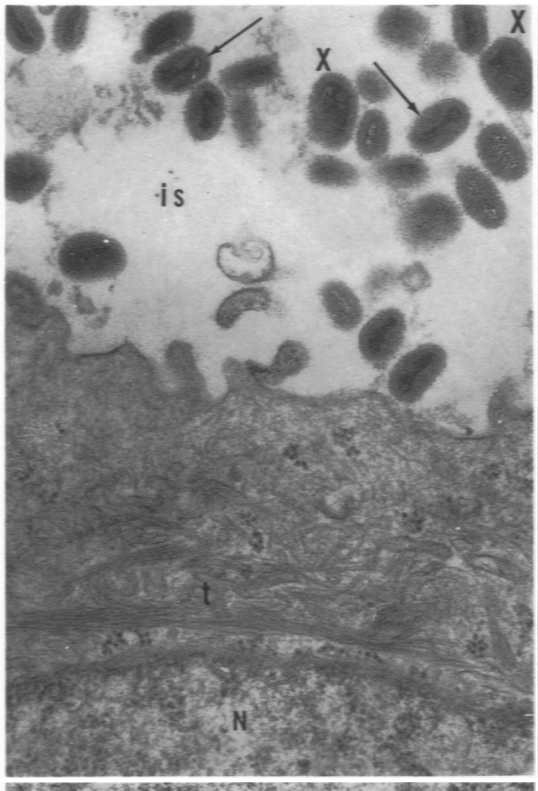
Fig 7. Part of epidermal cell in outgrowth from explant of healthy skin. Culture was inoculated with MC virus. Mature virus particles are present in intercellular space (*is*). Electron-dense nucleoid is biconcave in side view (*arrows*) and oval or rectangular in front view (*X*). A less dense capsule surrounds the nucleoid. Epidermal cell contains tonofilaments (*t*) and part of a nucleus (*N*). × 64,000.

Fig 8. Part of epidermal cell in outgrowth from explant of healthy skin. Intercellular space (*is*) contains mature virus particles which adhere to cell surface (*arrows*) and are encompassed partially by the cell. One particle (*X*) is engulfed completely by the cell. Part of cell nucleus (*N*) is shown. × 52,000.

Fig 9. Phagocytic vacuole in epidermal cell in outgrowth from explant of healthy skin. Vacuole is surrounded by a membrane (*arrows*) and contains virus particles (*V*) and black melanin granules. Part of nucleus (*N*), a lipid-filled vacuole (*l*), and tonofilaments (*t*) are shown. × 42,000.

Fig 10. Part of two epidermal cells in outgrowth from explant of healthy skin. Each cell contains part of a nucleus (*N*) and tonofilaments (*t*). In the upper cell a golgi apparatus (*g*), glycogen granules (*gl*), mitochondria (*m*), lipid-filled vacuoles (*l*), and viral cores (*arrows*) are shown. The latter are surrounded by a capsule which encircles an electron-translucent space containing the more electron-dense, filamentous nucleoid. a desmosome (*d*) interrupts the intercellular space (*is*). × 36,000.



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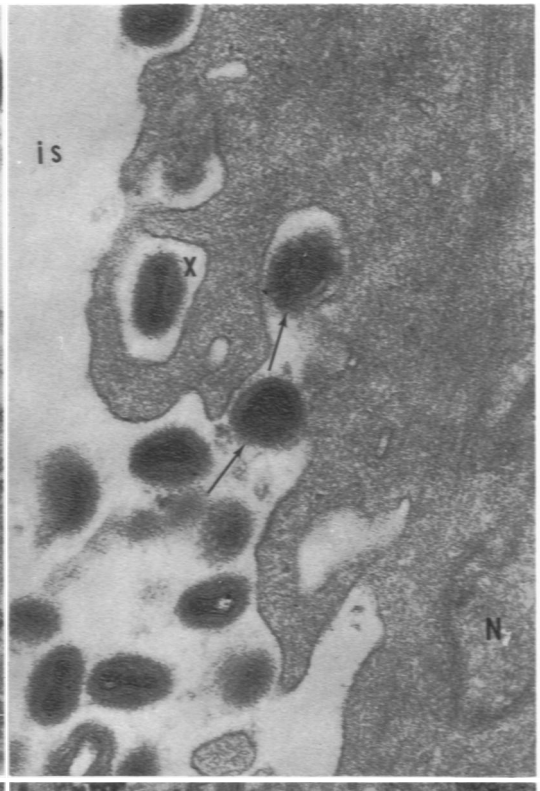

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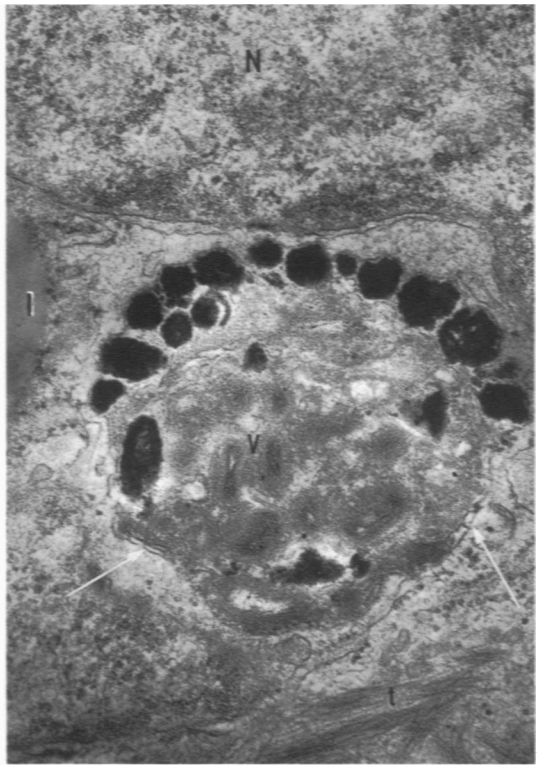

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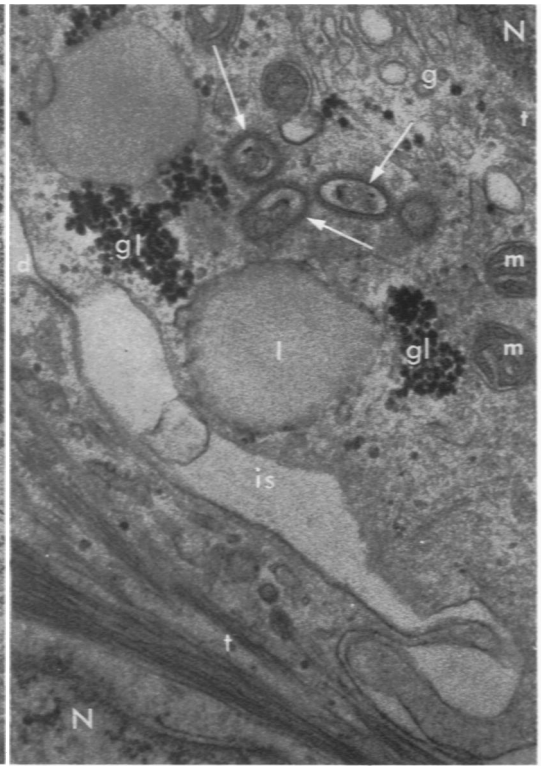

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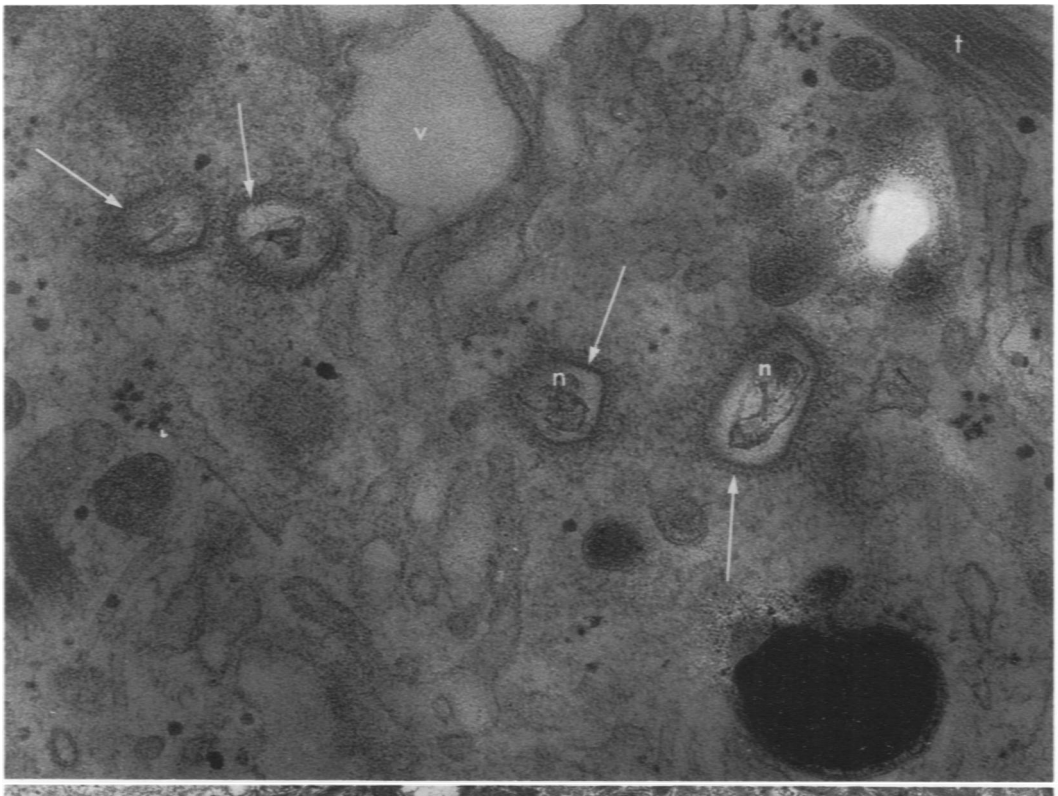
Fig 11. Part of epidermal cell in outgrowth from cultured explant of healthy skin, showing tonofilaments (*t*), vacules (*v*), and viral cores (*arrows*). The latter have a capsule surrounding an electron-translucent area containing filamentous nucleoid (*n*). At some points, the nucleoid is in contact with the capsule. In addition, lower pole of viral core, on the right, shows a capsule with radially arranged partitions. Membrane-bound black melanin granule is present in lower part of micrograph. × 78,400.

Fig 12. Part of two epidermal cells in outgrowth from cultured explant of healthy skin. One of these cells contains a nucleus (*N*) and a perinuclear "clear" zone containing many mitochondria (*m*) and large clusters of viral cores (*V*). Tonofilaments (*t*) are shown. The adjacent cell contains a viral core (*arrow*). × 13,600.



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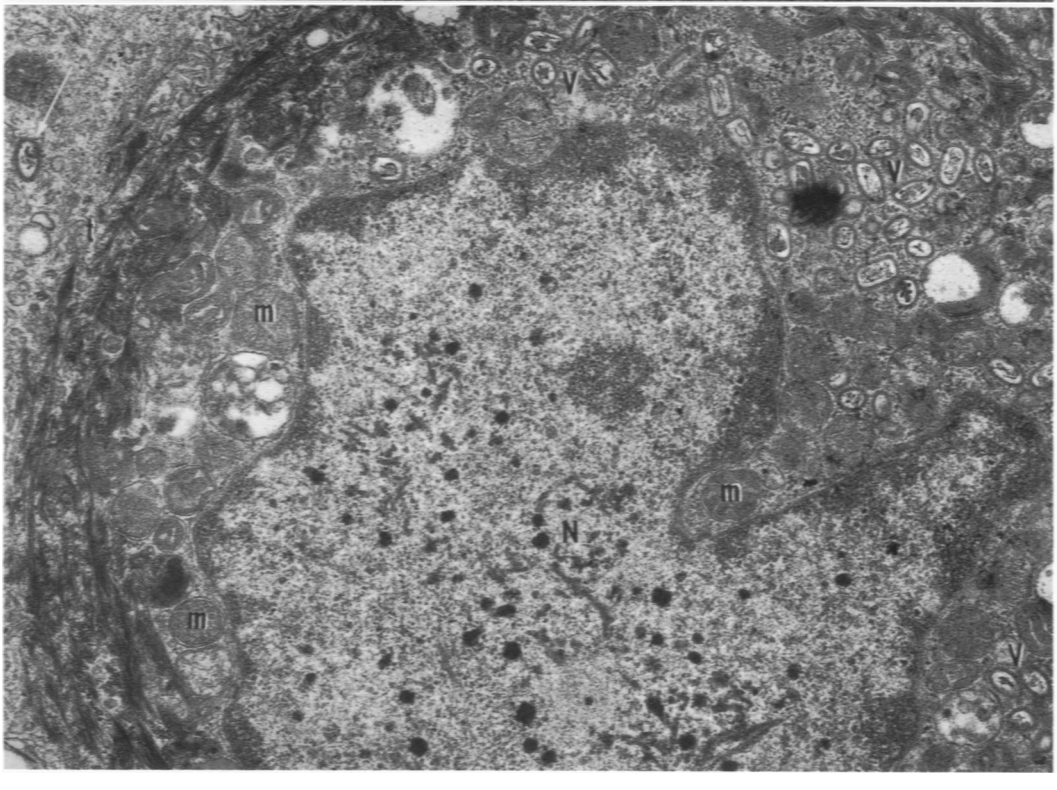

12

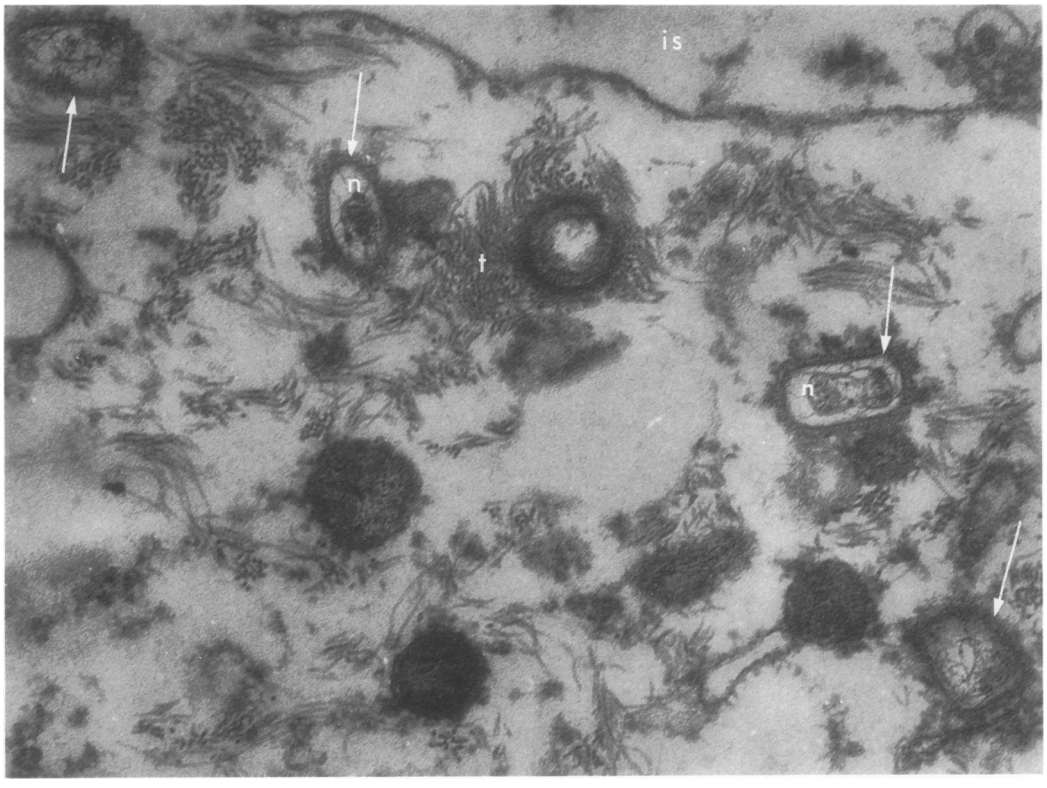

Fig 13. Part of keratinized cell in outgrowth from cultured explant of healthy skin. Cell contains viral cores (*arrows*) with central nucleoid (*n*), and many tonofilaments (*t*). Intercellular space (*is*) is shown. × 56,000.