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Morphogenesis of Rabbit Fibroma Virus

Correlation with Pathogenesis of the Skin Lesion

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Rabbit fibroma virus injected into the dermis of adult rabbit skin evokes an inflammatory, then granulomatous and finally proliferative or tumoral response. About 1 week after injection, the grossly visible nodular lesion reaches its maximum size and regresses, becoming hemorrhagic and necrotic. Unlike vaccinia, the morphogenesis of the RFV has not been validated satisfactorily. The present study shows that RFV-infected cells contain all the evolutive forms that have been identified during the course of vaccinia virus replication. In addition, long, twisting, intracytoplasmic lamellated inclusions were found in infected cells. These lamellae were composed of linear arrays of elongated, electron-dense fibers. When the inclusion was sectioned in a plane perpendicular to the fiber, the latter was found to be covered by projections ~160 Å long, spaced at intervals of ~80–90 Å; when sectioned tangentially, the lamellae appeared to be composed of tubules. Evidence is presented showing the similarities between the subunit of the lamellated inclusion and the virus membrane. It seems likely, therefore, that the viral membrane is covered by closely packed tubules ~160 Å long. The lambellar inclusion is thought to represent abnormal synthesis or excessive formation of viral membranes. In addition to lamellae, which probably indicate some defect in virogenesis, some infected cells contained viral membranes partially or completely encircling the host's cell constituents, or fragments of viral membrane instead of viral matrix. Furthermore, structures resembling virus nucleoid were lying free in the viral or cytoplasmic matrix. The course of viral morphogenesis was correlated with viral multiplication and the kinetics of interferon production at the site of viral inoculation in the rabbit skin. (Amer J Path 64:467–482, 1971)

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THE RABBIT FIBROMA VIRUS (RFV), a deoxyribovirus, first described by Shope,[1,2] has the morphologic characteristics of a poxvirus. When inoculated intradermally into adult rabbits, the virus causes tissue proliferation at the injection site. The localized lesion, manifesting itself as a nodule, attains its maximum size about 7 days after the injection and then regresses. Lasting immunity against reinfection is conferred on the tumor-bearing animal.

On the other hand, when RFV is inoculated (1) into a rabbit with impaired immunologic response—*eg*, the newborn[3,4] or an adult treated with either cortisone[5] or total-body irradiation,[6] or (2) into an adult injected with a carcinogenic tar,[7] it evokes a widespread, lethal infection.

Since growth of both the benign and lethal lesions depends on the presence of the virus, and since the fibroma cells are incapable of autonomous growth, these lesions are considered to be inflammatory granulomas. In a few reported experiments,[8] permanent malignant transformation of the fibroma cells may have occurred, but these experiments were never repeated and therefore are difficult to evaluate.

Studies of the rabbit fibroma are of considerable interest since this experimental lesion, which is grossly visible and easily reproducible, may be useful in studies on interferon inducers, simplifying the search for and evaluation of such agents. Recently, it has been reported[9] that the rabbit fibroma is suppressed by an interferon inducer (synthetic, double-stranded polyinosinic acid–polycytidylic acid).

For the aforementioned reasons and because prior electron microscopic studies of the fibroma reported by others[10–12] were performed on isolated lesions at a time when technics of fixation, embedding and staining of tissues were much less developed than they are at present, the present investigators studied the pathogenesis of the rabbit fibroma in the electron microscope, and the results are reported herein. In addition, these findings will be correlated with those obtained by assaying the lesions for (1) infectious virus and (2) interferon.

Materials and Methods

Light and Electron Microscopy

Each of ten separate sites on the shaved dorsal skin of each of 3 albino rabbits, 4–6 weeks old, was inoculated with 50 ID_{50} of RFV; the visibly infiltrated area was encircled with black ink. At each of various intervals after injection (6 hours, 1, 2, 4, 5, 6, 7, 8, 9 and 12 days), an encircled site on each of the rabbits was anesthetized and excised. The biopsy specimens of skin were transected into slices, 1–2 mm thick, fixed in phosphate-buffered 3% glutaraldehyde, postfixed in 1% osmium tetroxide, passed through graded alcohols and embedded in Epon. For

light microscopy, thick sections were cut with a glass knife on a Porter-Blum MTl microtome and stained with 1% toluidine blue. A slice of a biopsy specimen of skin excised 7 days after inoculation of RFV was fixed in formaldehyde, and paraffin sections were stained with hematoxylin and eosin. Thin sections of Epon-embedded tissues were cut with a diamond knife, stained with uranyl acetate and then with lead citrate, and examined in a Siemens Elmiskop I electron microscope.

Assays for Infectious Virus

Each of 11 separate sites on the shaved dorsal skin of a rabbit was inoculated intradermally with 50 ID_{50} of RFV. On each of 11 days after inoculation, an injected site and surrounding skin, 1 cm in diameter, was excised. After trimming away the subcutaneous fat, the skin was minced, ground in sand and the homogenate diluted with 9 volumes of Eagle's minimal essential medium (MEM). The resultant suspension was clarified by centrifugation at 1500 *g* for 10 minutes. Serial tenfold dilutions of the supernatant were made and 0.4 ml of each was inoculated into primary rabbit kidney cell (RKC) cultures grown to confluency in 60-mm plastic Petri dishes containing MEM supplemented with 10% gamma-globulin-free calf serum (CS) and thereafter maintained in MEM–2% CS. The virus was adsorbed for 2 hours at 37 C, tilting the dish every 15 minutes. The infected cultures were maintained in MEM–2% CS supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin; the medium was changed every 3 days. The plates were observed daily for cytopathic effect. The titers were expressed as the highest dilution of the supernate causing infection in at least 50% of the culture dishes ($TCID_{50}$).

Assays for Interferon

Each of six separate sites on the shaved dorsal skin of a rabbit was inoculated with 50 ID_{50} of RFV. At 1, 2, 4, 6, 8 and 10 days after injection, an inoculated site with surrounding skin, 1 cm in diameter, was excised, ground, and the homogenate diluted and cleared as outlined above. The supernate was dialyzed against HCl–KCl buffer, pH 2.0, for 72 hours, and then neutralized by dialysis against phosphate-buffered saline (PBS), pH 7.4, for 48 hours. Twofold dilutions of the supernate were then prepared with MEM–2% CS supplemented with penicillin, streptomycin and amphotericin in the concentrations as stated above. Two milliliters of each dilution was placed into plastic Petri dishes containing monolayers of primary RKC and incubated for 21 hours; duplicate controls were established in concurrence. The cells were then washed twice with PBS and were inoculated with approximately 50 plaque-forming units (PFU) of bovine vesicular stomatitis (VSV). The virus was adsorbed on the cells for 1 hour, the cells were overlaid with agar without neutral red, and the agar was covered with MEM–2% CS containing penicillin, streptomycin and amphotericin. Two to three days after inoculation, the MEM–2% CS was decanted and a second overlay of agar containing neutral red (1:20,000) was added and the plaques were counted. The titers of interferon were expressed as reciprocals of the highest dilution of the tested fluid causing inhibition of at least 50% of the control number of plaques.[13]

Results

Light and Electron Microscopy

When biopsy specimens of skin were examined by light microscopy, the earliest changes were found in specimens excised 2 days after

inoculation with RFV. The papillae and adjacent corium contained dilated, congested vessels, a few swollen spindle cells resembling fibroblasts, and edema fluid; around the hair sheath as it traversed the midcorium were small aggregates of mononuclear cells. On the third and fourth days after injection, edema increased considerably, spreading to involve the upper and midcorium. The edematous region contained a sparse, scattered, mixed population of cells (*ie*, polymorphonuclear leukocytes, lymphocytes, mononuclear cells and spindle-shaped fibroblasts) which tended to aggregate about and cuff thin-walled vessels and hair sheaths. By the fifth day, the lesion assumed a tumoral rather than an inflammatory or granulomatous appearance. The corium was filled mainly with large, round cells with pale cytoplasm containing a centrally placed, round or kidney-shaped nucleus with finely dispersed chromatin. Interspersed among the round cells were a few widely dispersed collagen bundles, occasional fibroblasts, polymorphonuclear leukocytes, lymphocytes and plasma cells. One week after injection, the entire corium was replaced by elongated, spindle-shaped cells resembling fibroblasts (Fig 1), which in the midcorium were closely packed with their long axes parallel to the skin surface and formed the wall of dilated vascular spaces (Fig 2). In the upper and lower corium, these cells were less ordered and less aggregated, with their long axes frequently perpendicular to the surface. Occasional mitoses were observed (Fig 2). At the lateral edges of the fibroblastic proliferation, there were densely packed cells of mixed population similar to those described above; a few small aggregates of these cells were present among the fibroblasts. The lower edge of the lesion (*ie*, the subcutaneous fat and muscle) was infiltrated mainly by plasma cells. On subsequent days, the thinned epidermis ulcerated and increasing numbers of focal hemorrhages and areas of necrosis were noted in the corium, which was now edematous and contained a moderate number of large, round cells with vesicular to pyknotic nucleus, polymorphonuclear leukocytes, lymphocytes and plasma cells.

When biopsy specimens of skin were examined in the electron microscope, minimal inflammatory changes were noted in specimens excised 6 hours after inoculation with RFV; the vessels in the upper corium were dilated and the surrounding tissue contained a few erythrocytes, polymorphonuclear leukocytes, some with extruded granules, and increased fluid. One day after injection, in addition to the above findings, small clumps of fibrin were seen in and around some small vessels. However, in specimens of skin excised 6 hours, 1 and 2 days after injection of RFV, neither immature nor mature virus was

found in any of the many sections from multiple blocks examined. From 4 to 8 days after inoculation, by examining many infected cells, all stages of the multiplication cycle of RFV could be observed, from phagocytosis of extracellular mature virus particles by fibroblasts (Fig 3) and macrophages to the release of fully replicated particles into large intracytoplasmic vacuoles. Virus was not found in the epidermis or skin adnexa. Subsequent to phagocytosis, mature virus particles were present in phagocytic vacuoles (Fig 3), where presumably they were partially uncoated (first-stage uncoating), and then entered the cytoplasmic matrix in the form of viral core (Fig 4). The viral core, which is round, oval or rectangular, depending on the plane of section, and approximately the size of the mature virus particle (*ie*, ~320 Å) is surrounded by a membrane that encompasses an electron-translucent space within which the electron-dense nucleoid is situated. The membrane has a smooth inner surface and an irregular outer surface showing radially arranged partitions spaced at intervals of ~80–90 Å. When the core is fully uncoated (second-stage uncoating), viral replication actually begins. Various-sized aggregates of fibrils having greater electron density than the cytoplasmic matrix (*ie*, viral matrix, or so-called viral factories) were found in the cytoplasm of infected cells (Fig 5 and 6). A few ribonucleoprotein (RNP) particles were found within or adjoining the viral matrix. Viral membrane, at first arc-shaped and later circular or oval, was formed focally on the circumference of the matrix (Fig 6). The viral membrane had an irregular outer surface covered by radial projections, ~160 Å in length, ~50 Å in width and spaced at intervals of ~80–90 Å. After the viral particles were pinched off the matrix, they underwent maturation and were seen to contain an eccentrically placed, electron-dense nucleoid composed of fibers (Fig 7), ~40 Å in width. Changing in shape from oval to rectangular, the virus particles developed lenticular-shaped lateral bodies situated between the capsule and the viral "core" which contained an electron-dense fibrillar nucleoid. The lenticular bodies compressed the nucleoid so that it was narrow at the waist and expanded at the ends. Virus release into intracytoplasmic vacuoles was observed (Fig 8).

Macrophages containing replicating virus were found in capillaries These vessels were situated in the edematous upper corium just below the epidermis.

In addition to the above, there were findings that suggested defective virogenesis. Several infected cells contained aggregates of electron-dense fibers (some of which were arranged in the form of a double helix)that resembled viral nucleoid and were lying free in the viral or

cytoplasmic matrix (Fig 7). Some viral membranes formed arcs or circles, partially or completely encompassing spaces that were of the same electron translucency as the surrounding cytoplasm or contained coiled fragments of viral membrane, RNP particles or fragments of cytoplasmic membranes (Fig 7 and 8). Lastly, lamellated inclusions, up to ~6 μ in length and ~40 mμ in width, were found within or adjacent to viral matrix (Fig 6, 8 and 9) and rarely, in the cytoplasm, associated with mature virus particles (Fig 9). These inclusions were composed of linear arrays of elongated electron-dense fibers that were present in the matrix or appeared to emerge from its circumference: one surface of the fiber was smooth; the other, irregular with radial projections ~160 Å in length, ~50 Å in width and spaced at intervals of ~80–90 Å. When these fibers were found in the vicinity of viral membranes, the similarity between the two was striking (Fig 6). Some of the lamellated inclusions changed direction during their course and thus showed sudden changes in width, or assumed shapes resembling a check mark (Fig 9), the letter V or a triangle. As the lamellae changed direction, the plane of section through them varied. Therefore, in addition to the linear arrays described above, there were areas within the length of the inclusion that resembled sectioned closely-packed tubules with a very orderly arrangement (Fig 9).

Assays For Infectious Virus

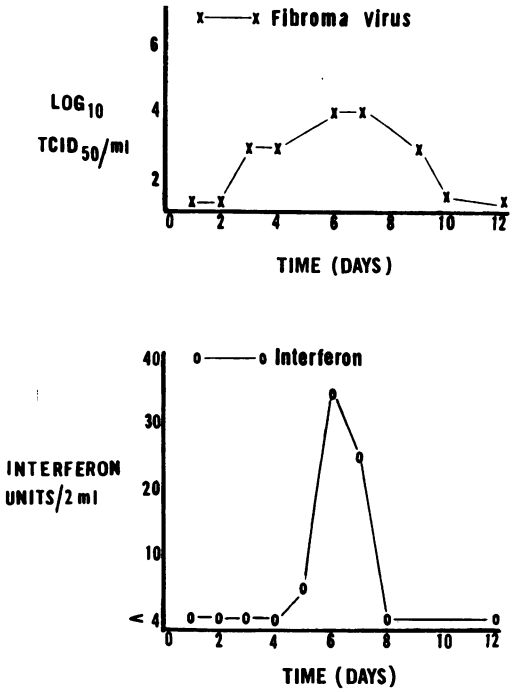
When extracts of skin that had been excised 1 and 2 days after injection with RFV were inoculated into primary RKC cultures, no cytopathic effects were observed. Skin that had been injected with RFV 3 days prior to extraction contained 10^3 $TCID_{50}$/ml of extract. The titer attained its maximum on the sixth and seventh day after injection, reaching 10^4 $TCID_{50}$/ml. On subsequent days, the titer dropped and on day 11 after injection with RFV, the skin contained no detectable virus (Text-fig 1).

Assays for Interferon

Skin extracted 1–4 days after RFV was injected failed to show detectable interferon production. Low levels of interferon became detectable in skin injected with RFV 5 days prior to extraction; it was maximal, 32 units/2 ml of extract, on day 6 after injection; and it disappeared 8 days after injection (Text-fig 1).

Discussion

The clinical evolution of the rabbit fibroma as herein reported is similar to that described by others [14,15]; reported differences in the interval



TEXT-FIG 1—Curves showing infectious virus and interferon titers obtained from skin that had been injected with 50 ID_{50} of RFV. The time is in days after intradermal injection of virus. Each point on the curves represents the titer obtained from an injection site with surrounding skin, 1 cm in diameter.

between injection of the virus and appearance of the tumor,[16] and in maximum size of the fibroma are consistent with variations in the route of injection (intradremal or subcutaneous) and in the number of infectious particles injected.

Serial biopsies of the rabbit fibroma studied by light microscopy resulted in findings similar to those already reported.[14,15] RFV inoculated into skin evokes, at first, an inflammatory response that subsequently appears granulomatous and then, prior to resolution, proliferative or tumoral. Resolution of the lesion is accompanied by hemorrhage and necrosis. Finally, the skin is restored to a healthy state. During the 5 days after inoculation of the virus into the skin, the predominant cell in the infiltrate is large and round with pale cytoplasm containing a centrally placed, round or kidney-shaped nucleus. These round cells probably represent primitive mesenchymal cells capable of differentiating into fibrocytes, histiocytes with a similar potential [17] and macrophages. During the proliferative phase, the cells in the lesion are spindle-shaped fibroblasts. During resolution, the predominant cell is once again large and round, but it shows degenerative changes as evidenced by nuclear hyperchromatism or pyknosis.

Unexpectedly, we failed to find any virus in the early fibroma lesions

examined in the electron microscope (*ie,* in those specimens of skin that had been inoculated less than 4 days prior to excision). Similarly, no virus was detected when early fibromas were assayed for infectious particles; the earliest lesion containing quantifiable particles was one excised 3 days after injection with RFV. On the other hand, in electron microscopic studies of tissue cultures infected with RFV,[18] beginning virus replication (viral matrix) and newly formed, mature particles were found 5 and 8 hours after inoculation, respectively. Our failure to find virus particles in the early fibroma lesions may be due in large part to the following: (1) the low dose of the inoculum, 50 ID_{50} of RFV, injected into each skin site; (2) some of the virus particles, both injected and replicated, were probably transported from the injection site to the circulation since in our study virus-infected macrophages were found in small vessels, and Duran-Reynals [4] has shown that for 8 days after an injection of virus into adult rabbit skin, RFV can be extracted from some of the viscera; (3) replicated fibroma virus is released inefficiently from infected cells, limiting the spread of infection to adjacent cells during the early phase of infection (*eg,* Appleyard and Westwood [19] reported that during the first 24 hours after inoculating RFV into cell cultures, 90% of the replicated particles were intracellular in location, and Febvre *et al,*[18] after inoculating tissue cultures with virus, could find no virus in the culture medium for 3 days); (4) the sampling problem in electron microscopy is such that the injected virus and presumably the few cells infected in the first days after injection were missed; and finally, (5) the virus assay used in these experiments when applied to lesion extracts (extraction presumably results in a loss of some particles) is probably not sensitive enough to detect the number of particles present in the skin during early stages of RFV infections.

In agreement with other investigators,[2,4] no virus particles were found in the regressing fibroma (*ie,* on day 9 after injection of virus, in electron microscopic studies, and on day 11, in virus assays of lesion extracts).

Contrary to findings in other poxviruses (*eg,* vaccinia virus [20]), the sequential arrangement of the evolutive forms of replicating RFV is uncertain due to the observed asynchronism of viral replication in infected cells. This was not unexpected since the multiplicity of infection was low (50 ID_{50} of RFV per injection site), the cells in the corium divide asynchronously, and lastly, we were unable to find virus particles in the skin during the early days of infection. Nevertheless, in a composite of many infected cells, we were able to observe evolutive

stages in replication that were similar to those found in cultured cells inoculated with vaccinia virus.

Some infected cells contained long, twisting, intracytoplasmic lamellated inclusions similar to those described by other investigators reporting on the rabbit fibroma,[10] tissue cultures infected with RFV [21] and other pox virus infections, (*eg*, fowlpox,[22] ectromelia [23] and Yaba monkey tumor [24]). Only de Haven and Yohn [24] noted the perfect resemblance between the subunit of the lamellae and the viral membrane around the immature particle, an observation with which we concur. While Scherrer [21] saw the resemblance between the two structures, it was not absolute in the sections he examined. Nevertheless, he felt that the lamellated inclusions were related to membranogenesis since (1) they were found only in infected cells; (2) both the lamellae and virus capsule (derived from viral membrane) were relatively resistant to pronase, suggesting the possibility that both structures are lipoprotein in composition; and lastly (3) the viral membrane had some similarities to the inner part of the virus capsule of negatively stained RFV, which is subdivided by radially arranged partitions with intervals of ~85 Å. It is of interest that in some poxvirus infections (*ie*, vaccinia and molluscum contagiosum) similar lamellated intracytoplasmic inclusions have never been found.

If viral membrane is indeed related to the lamellar inclusion, then by correlating the morphologic features of lamellae sectioned in planes perpendicular and tangential to its elongated fibers, it appears that the radiating projections on the membrane represent closely packed tubules ~160 Å in length which are placed on the membrane at intervals of ~80–90 Å.

Lamellar formation is, in our opinion, an indication of defective and inefficient virogenesis. Some of the viral DNA may code for "nonsense" viral membranes which aggregate into lamellae. In addition, some viral membranes either partially or completely encircle the host's cytoplasmic constituents (*eg*, RNP particles and cytoplasmic membranes) and/or fragments of viral membrane. Another indication of defective virogenesis is the finding of viral nucleoid lying free in the virus matrix or cytoplasm.

It appears likely that the lamellar inclusion is a manifestation of abnormal synthesis or of excessive formation of viral membrane. It seems less likely, as has been suggested,[24] that the lamellae contribute viral membranes for encapsulation of viral matrix, since in the present study lamellae have been found in cells containing only mature particles and no immature viral structures.

Although it is well known that normal rabbits recover spontaneously from rabbit fibroma infection within approximately 3 weeks, the relative contribution of various mechanisms of host resistance (*eg*, cellular and humoral immunity, and interferon) is not understood. We have explored the possible role of interferon in the recovery process by comparing the time of appearance of both virus and interferon in the inoculation site. Since the appearance of interferon preceded the decrease in the titer of infectious virus, it is conceivable that interferon contributes to the resolution of infection.

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[Illustrations follow]

Legends for All Figures

Fig 1 and Fig 2 represent photomicrographs of rabbit skin obtained 7 days after injection of 50 ID_{50} of RFV. The section was stained with H&E.

Fig 1—The corium, replaced mainly by spindle-shaped fibroblasts, contains thin remnants of skin adnexa *(arrows)*, dilated vessels (area enclosed in frame is shown in Fig 2), and occasional aggregates of chronic inflammatory cells *(X)*. Zones of chronic inflammation are seen at the lateral edges of the photomicrograph. The subcutaneous muscle *(M)* is shown (× 20).

Fig 2—In the area enclosed by the frame in Fig 1, closely packed and loosely arranged, spindle shaped fibroblasts, dilated vessels, a cell with mitotic figure (arrow), and occasional chronic inflammatory cells are seen (× 350).

Fig 3–9 represent electron micrographs of rabbit skin obtained 7 days after 50 ID_{50} of RFV were injected. All of the material was fixed in glutaraldehyde followed by osmium, embedded in Epon and stained with uranyl acetate and lead citrate.

Fig 3—Part of a fibroblast showing nucleus *(N)*, mature virus particles *(arrows)* in the vicinity of the outer cytoplasmic membrane being surrounded by cytoplasmic processes, and mature virus particles in an autophagic vacuole *(A)* (× 22,400).

Fig 4—Part of a fibroblast showing virus cores *(c)* within the cytoplasmic matrix. Virus membrane with radial projections *(arrow)* surrounds the core (× 50,100).

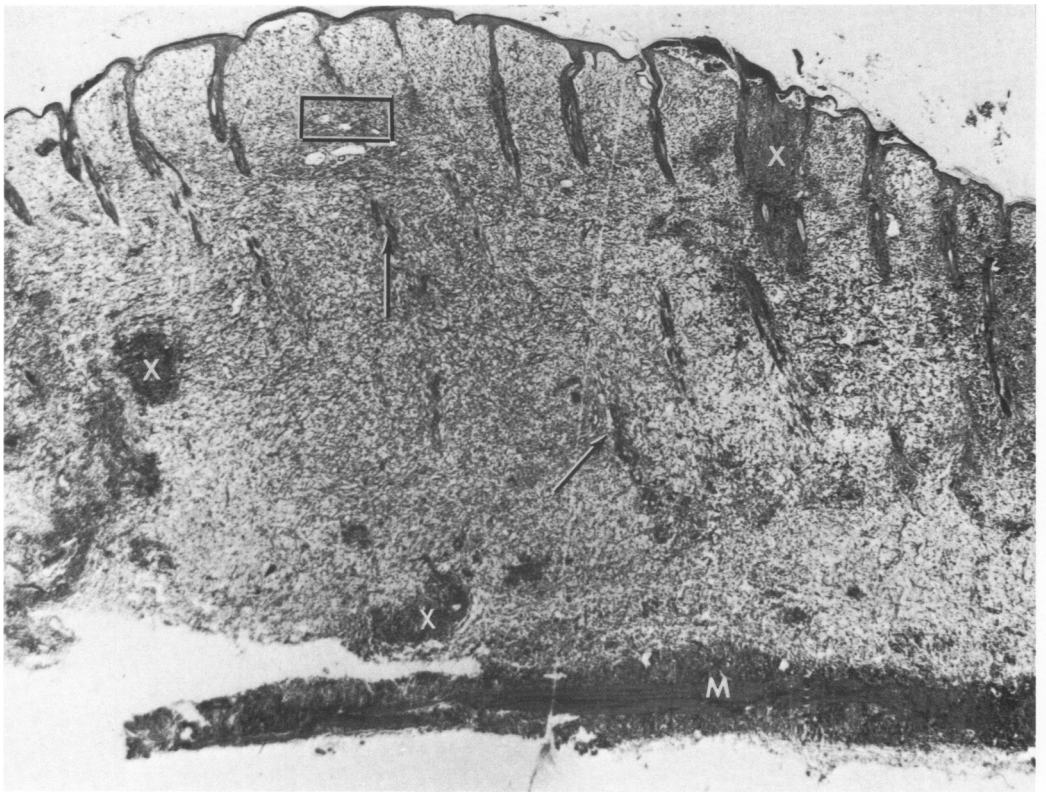
Fig 5—Several fibroblasts showing nucleus *(N)* and mature virus particles within the cytoplasm *(arrows)*. The cytoplasm of one cell is replaced in part by virus matrix *(V)*. Replicating, immature virus particles *(I)* have been segregated from the virus matrix by virus membrane. At the periphery of the virus matrix, mature replicated particles *(arrows)* are shown; they are oval or rectangular and electron-dense (× 18,000).

Fig 6—Part of a macrophage showing intracytoplasmic, fibrillar virus matrix *(V)* focally covered by arc-shaped virus membranes. Lamellated inclusions *(L)* are seen. At the mid and lower left of the electron micrograph, a lamella extends from the virus matrix to an inclusion. The arc-shaped virus membrane with projections at the upper left *(arrow)* is similar in appearance to the subunits forming the adjacent lamellated inclusion (× 62,000).

Fig 7—Part of a fibroblast showing immature, moderately electron-dense virus particles *(I)* surrounded by virus membrane. One of the particles contains an aggregation of electron-dense fibers, the virus nucleoid *(n)*. An aggregation of similar fibers *(arrow)* is shown lying free in the cytoplasm. Two particles in the upper center of the micrograph show virus membrane encircling an electron-translucent space containing fragments of virus and cytoplasmic membranes (× 104,000).

Fig 8—Part of a cell showing virus matrix *(V)*, an immature virus particle *(I)*, two arc-shaped virus membranes partially encircling cytoplasmic membranes and ribonucleoprotein particles *(r)*, and a long lamellated inclusion *(L)*. Electron-dense, replicated virus particles are present in the cytoplasm; one of these *(arrow)* projects into a large vacuole (× 26,000).

Fig 9—Part of a fibroblast showing lamellated inclusion *(L)*, virus core *(c)* and mature virus particles. In relation to the subunit, the twisted, check-shaped inclusion is sectioned in varied planes. One region of the inclusion has elements that are perpendicularly arranged *(arrow)*; another, in the vicinity of the frame (area enclosed by the frame is seen in accompanying inset), contains sectioned tubules (× 49,600). **Inset** shows area enclosed by the frame. Sectioned, closely packed tubules with very orderly arrangement are seen (× 131,200).



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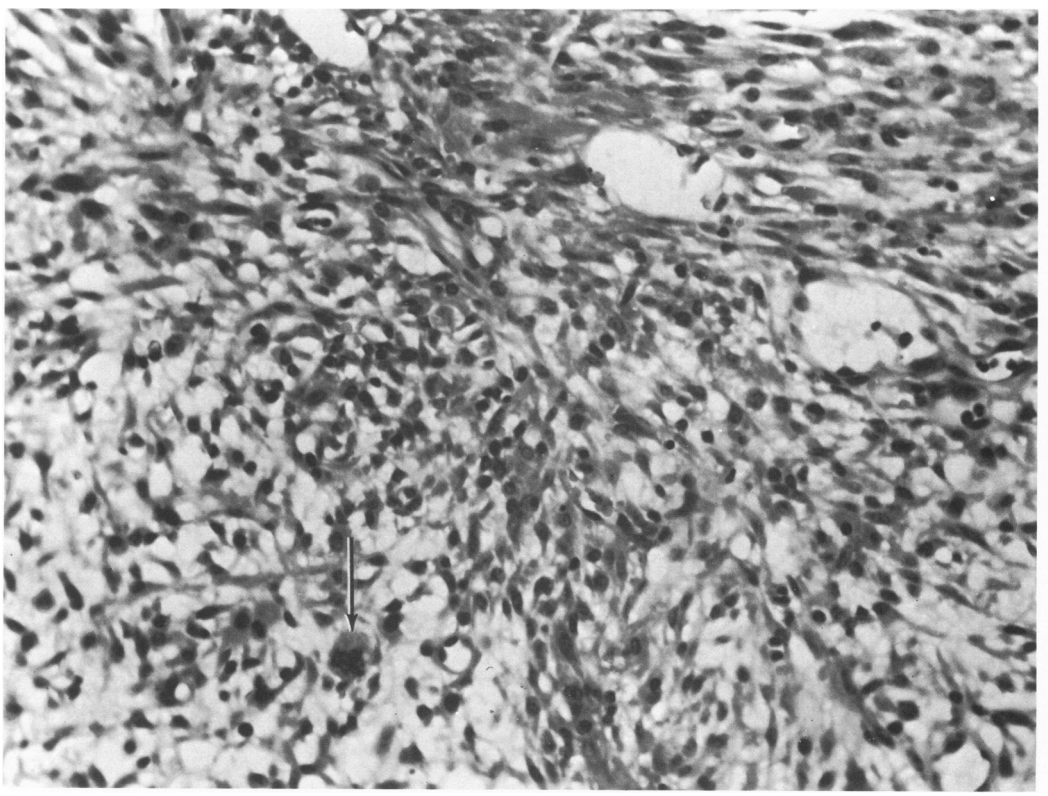

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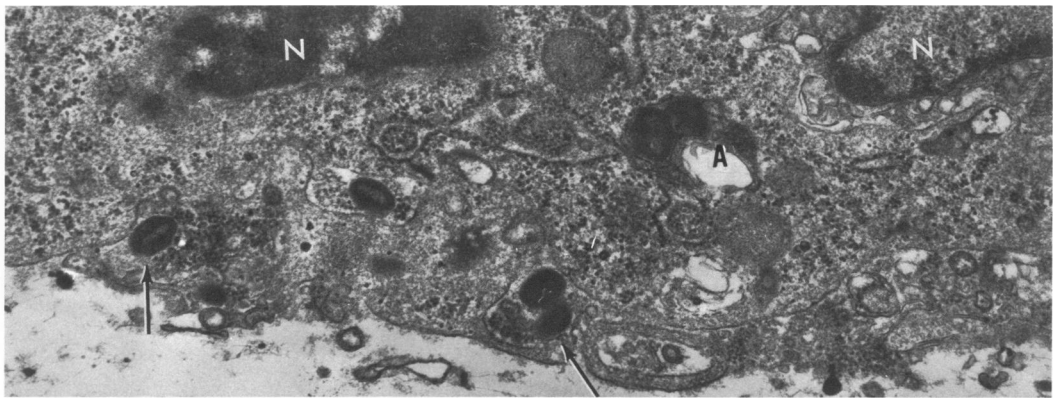

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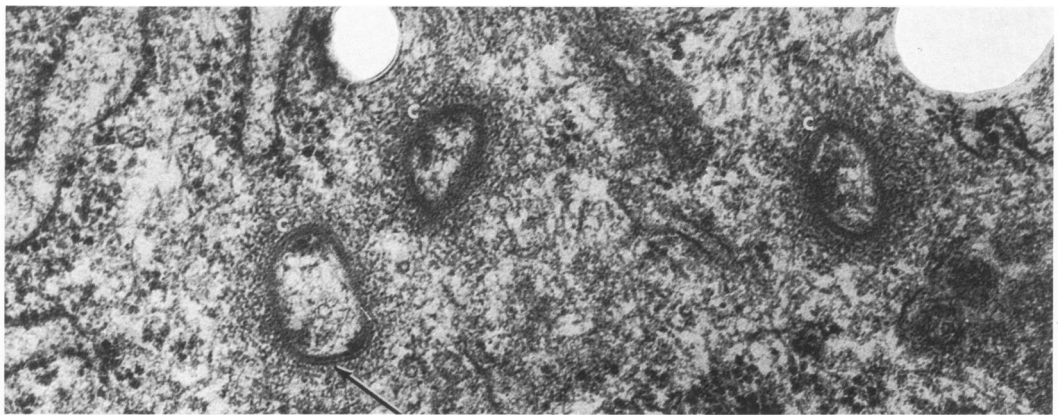

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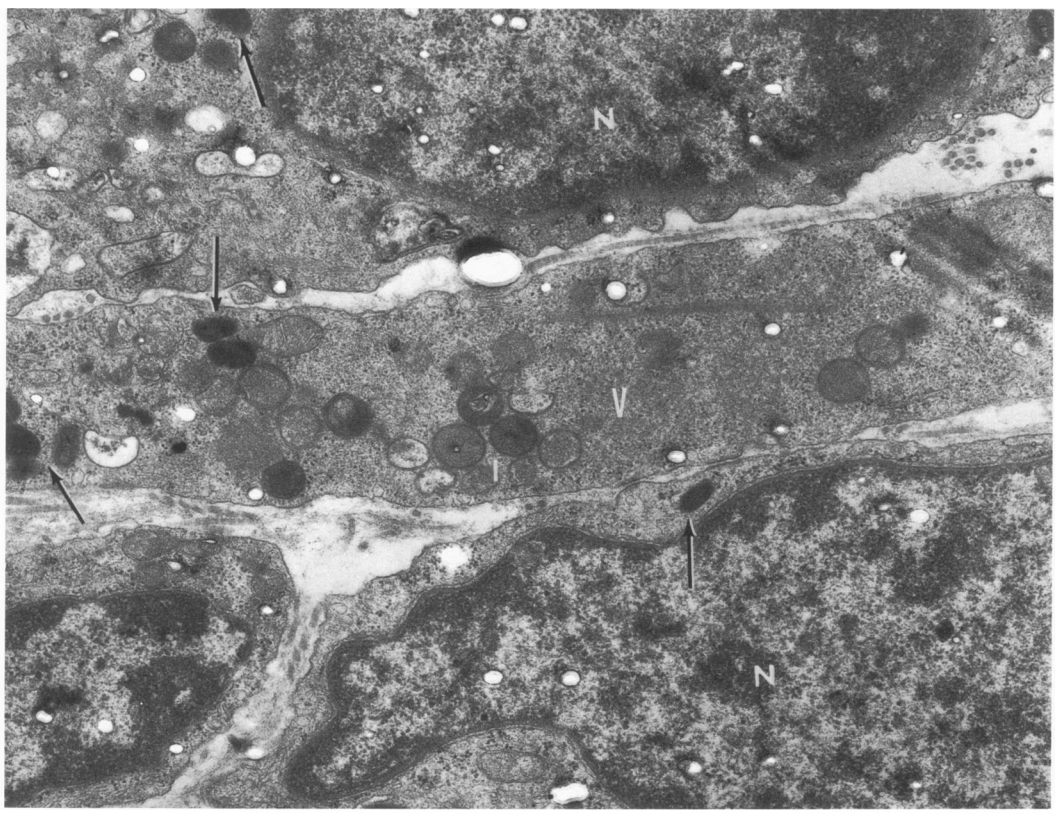

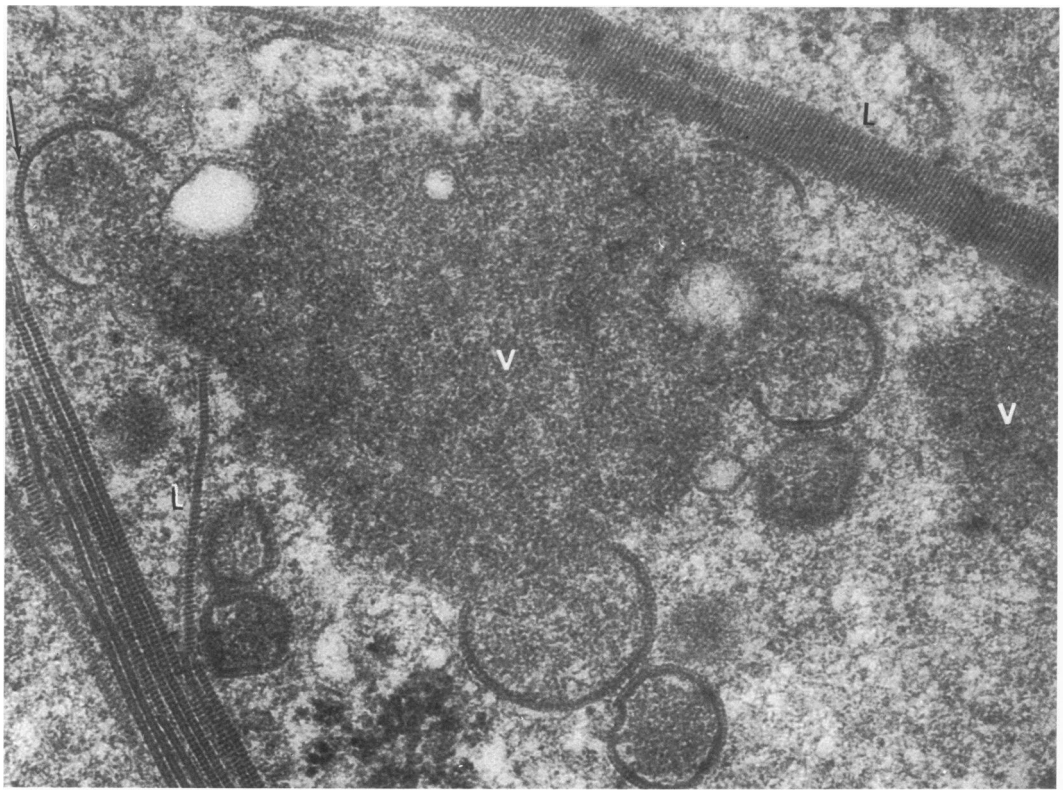

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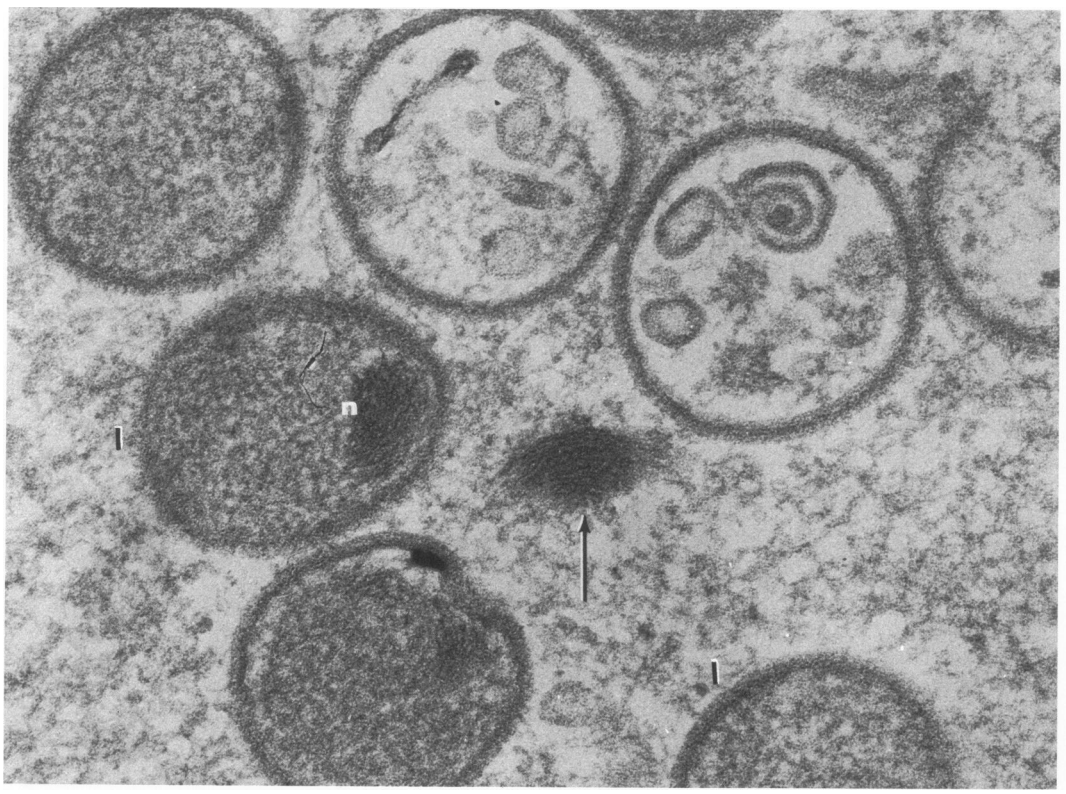

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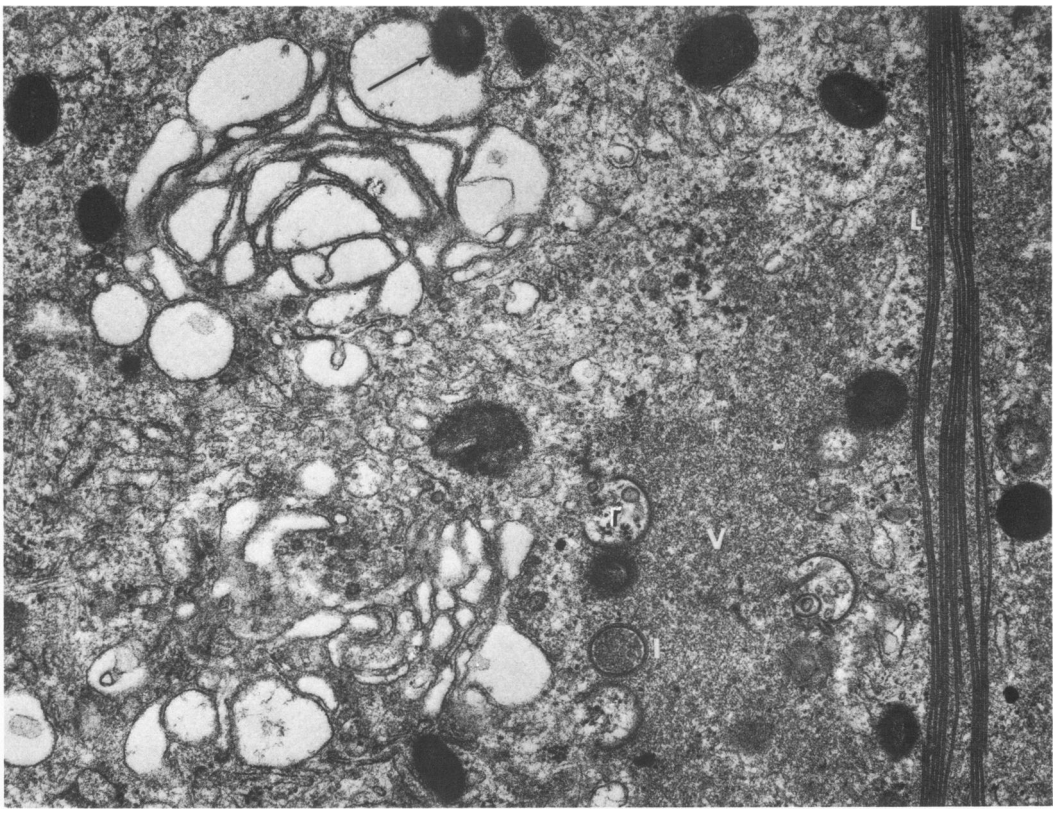

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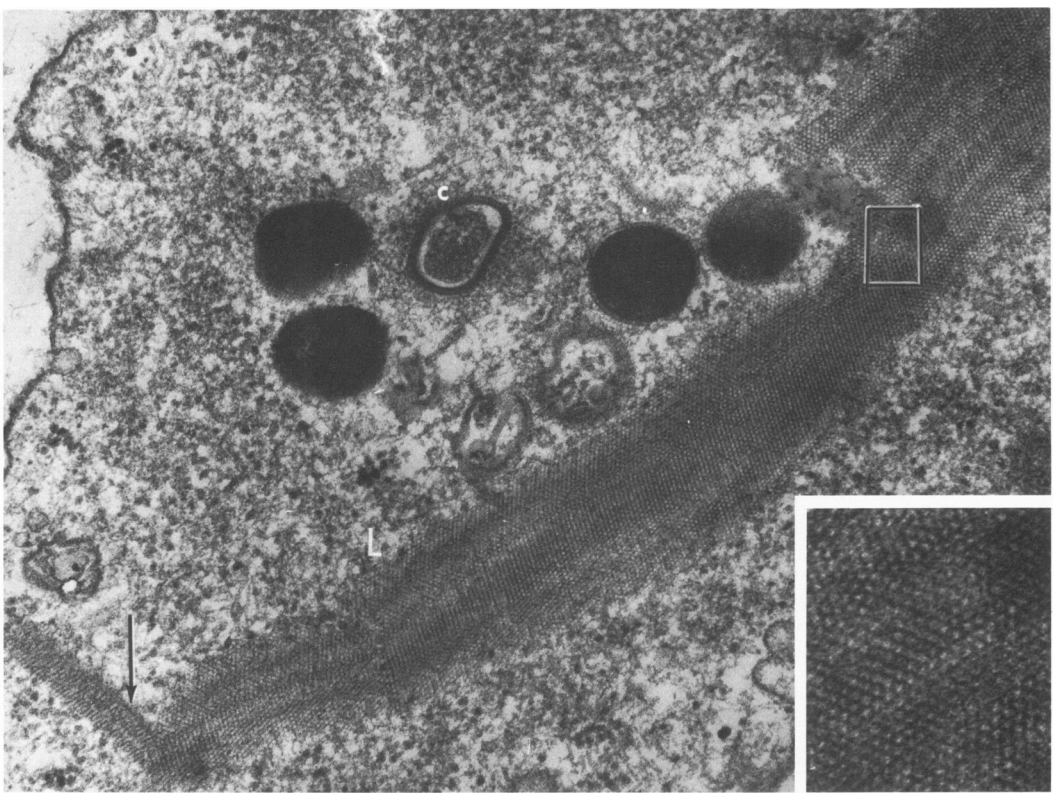

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