Studies on Measles Virus in Tissue Culture

(II) Electron Microscopic Study of Measles Virus Infected Cells and Localization of Virus Antigen Examined by Ferritin-Conjugated Antibody Method

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In the previous report (1), the author described the morphological changes of measles virus infected cells in vitro with particular references to the nature of inclusion bodies and also to interrelationship between histochemical changes and mechanism of viral reproduction. The results obtained by fluorescent antibody technique and acridine orange staining suggested possible participation of the cytoplasmic inclusion bodies in the synthesis of viral nucleic acid. However, the exact nature of inclusion bodies, especially those in the nucleus, and their role in replication of virus particles could not be clarified.

Although there are many reports regarding the general morphological changes of cultured cells infectied with measles virus, electron microscopic studies are relatively scanty. The fine structure of cellular inclusions in experimental measles has been reported by Kallman et al. (2), Baker et al. (3) and Tawara et al. (4). Their interpretation on the significance of inclusion bodies are not identical and their description are chiefly concerned with cellular inclusions and devoid of general morphology of the infected cells.

In the present paper, detailed studies not only on morphological changes of cultured cells infected with measles virus but on the fine structure of inclusion bodies, both in the nucleus and cytoplasm, have been undertaken using electron microscope. Another purpose of this paper is to demonstrate the localization of viral antigen by means of ferritin-conjugated antibody technique and furthermore to speculate on the mechanism of viral replication.

MATERIAL AND METHOD

VERO cells (green monkey kidney cell line) were grown in stationary bottles in a growth medium consisting of Hanks' balanced salt solution containing 0.5

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per cent lactalbumin hydrolysate and 5 per cent calf serum. When monolayer was obtained in 3 to 4 days, the fluids were replaced with 20 ml of maintenance medium composed of 99 per cent Earle's basal medium and one per cent calf serum. The cultures were infected with Edmonston strain of measles virus by adding 0.5 ml of virus fluid which had approximate titer of 10^4 TCID 50/0.2 ml. At the same time coverslip cultures were likewise infected.

Between the fifth and seventh day following innoculation, coverslip cultures were fixed with cold ethanol and stained with hematoxylin and eosin. At that time, giant cell and numerous inclusion bodies both in the nucleus and cytoplasm were readily observed. After the maintenance medium was removed from culture bottles, the cells were washed with phosphate buffered saline and were fixed with osmium tetroxide, which was buffered with veronal acetate at pH 7.4, on the glass wall in a ice box for ten to fifteen minutes. The cells were then scraped from the bottle wall with a rubber rod, washed with physiological saline solution and centrifuged for ten minutes at 800r . p. m.. Pellets were dehydrated in gradeed thanol and embedded in Epon 812. Thin sections were cut on a Porter-Blum ultramicrotome employing a glass knife and doubly stained with uranyl acetate and lead citrate. Then they were examined with a Japan Electron Optics LAB. JEM-5 HS electron microscope. Uninfected cultures, which were likewise sectioned and stained, served as control.

Ferritin-Conjugated Antibody Method :

Antiserum was produced in rabbits by repeated subcutaneous injection of virus fluid (Tanabe strain of measles virus) treated with potassium alum. The globulin fraction was separated from antiserum by ammonium sulfate precipitation and was conjugated with horse spleen ferritin* by using toluen 2,4-diisocyanate according to the method described by Singer (5). The ferritin conjugated globulin was absorbed with VERO cells and clarified by low speed centrifugation.

VERO cells infected with measles virus 5 days previously were washed in phosphate buffered saline and flooded with ferritin-conjugated antibody for 15 minutes at room temperature. Then the cells were washed again in phosphate buffered saline for several times, fixed in osmium tetroxide, dehydrated, embedded in Epon 812 and ultrathin sections were made. Thin sections were stained with uranyl acetate and examined by electron microscope. Uninfected VERO cells which were likewise flooded with ferritin-conjugated antibody served as control.

RESULTS

1) Uninfected VERO Cells

Fig. 1 shows uninfected VERO cell with somewhat round nucleus. Chromatin

* Nutritional Biochemicals Co. Cleveland 28, Ohio, U. S. A.

particles are evenly distributed and the nucleolus appears to be composed of fine granules closely packed in dense anastomosing strands forming irregular network. In the cytoplasm, moderate number of mitochondria are seen, but the endoplasmic reticulum is poorly developed. The cell surface is almost smooth though small cytoplasmic processes are occasionally seen.

2) Fine Structure of Infected VERO Cells

Fig. 2 shows small inclusion body in the nucleus. In the center of inclusion area, filamentous structures are partially arrayed in a parallel fashion and at the periphery they are randomly arrayed. The nucleolus at the vicinity of the inclusion area reveals no remarkable morphological changes. This picture would indicate an early stage of the development of nuclear inclusion body. The intranuclear inclusion body shown in Fig. 3 appears as almost round clear area because of remarkable decrease in chromatin particles. In this clear area, randomly scattered filamentous structures are seen. At the near right of the inclusion body, the nucleolus is visible, which shows no morphological alteration. Fig. 4 illustrates a large intranuclear inclusion body. Filamentous structures are more numerous than are recognized in Fig. 3 and they are characteristically arrayed in parallel fashion and look like finger print. At the lower left and upper right fields, the filamentous structures which have almost similar morphological appearence to those in the nuclear inclusion body are seen in the cytoplasm.

Fig. 5 shows high magnification of the filamentous structures which array in parallel fashion in the intranuclear inclusion area. The filamentous structures are moderately electron-dense, and the outer surface of them is denser than the inner portion suggesting tubular structure. Tubular structure is clearly visible in the cross section of these filaments as is revealed in Fig. 6, which shows the bundle-formation of the tubular structures. The length of these tubular structures are not measurable because of their three-dimentional array, but they are longer than 1 micron as far measurable as possible. The width is too small to be accurately measured, but estimated at 15 to 20 mμ.

Fig. 7 shows fine structure of the cytoplasmic inclusion body which also consists of randomly arrayed tubular structures with somewhat clear inclusion matrix. At the vicinity of this area, the cytoplasmic organellae are seen. The tubular material in the cytoplasmic inclusion is morphologically similar to that in the nuclear inclusion, but never shows parallel array. Fig. 8 illustrates high magnification of the cytoplasmic inclusion body. The length of the tubular structures cannot be measured because they are meandering irregularly and array three-dimentionally. The width is approximately two times larger than that of the tubular material in the nuclear inclusion and is estimated at 30 mμ. There is no findings which suggest the direct transformation of the tubular material in the nuclear inclusion to that in the cytoplasmic inclusion.

In Fig. 9, characteristic finding is visible; numerous cytoplasmic processes and spherical forms which possess similar surface configuration are seen on and outside the host cells. Some of them contain dense internal component which is morphologically the same as the tubular structure of the cytoplasmic inclusion body. And some of them appear to be in process of detachment and others are already outside the cell wall acquiring spherical shape. Occasionally characteristic branching of these processes is recognized. These cytoplasmic buddings and spherical particles remarkably vary in size, measuring from 200 to 700 mμ in diameter. Fig. 10 and 11 show high magnification of the cytoplasmic process and spherical forms near the cell surface. Most of them have dense and moderately thick outer coat. And some of them have dense inner component and others are devoid of it appearing less dense. In some particles, vesicular structure which is morphologically indistinguishable from the cytoplasmic vesicles is occasionally seen.

3) Ferritin-Conjugated Antibody Technique

Fig. 12 illustrtates uninfected VERO cells reacted with ferritin-conjugated antibody. The cell surface is smooth and no cytoplasmic budding is seen. Ferritin particles are hardly visible on the cell surface and only scatteringly seen in the intercellular space. There are some morphological changes in the cytoplasm due to exposure to ferritin-conjugated antibody without any previous fixation.

Fig. 13 shows infected VEPO cells reacted with ferritin-conjugated antibody. There are numerous spherical particles heavily tagged with ferritin-conjugated antibody on and outside the cell wall. Although they are almost spherical in shape, there are great variety in their size measuring from 200 to 500 mμ in diameter. The larger one has distinct outer membrane which is morphologically similar to the cytoplasmic membrane of the host cell and in general they are devoid of dense internal material. Furthermore, some of them contains vesicular structures which have morphological resemblance to those recognized in the cytoplasm. The smaller one, on the other hand, have approximate diameter of 200 to 300 mμ. Occasionally sharply defined outer membrane is hardly visible because they have dense inner component. A few ferritin granules are seen on the surface of the host cell, but no ferritin has penetrated the cell. Fig. 14 shows characteristic cytoplasmic buddings and spheres on the cell surface, the majority of which are specifically tagged with ferritin-conjugated antibody. Some have thread-like inner component and a few of them contain the cytoplasmic vesicles. Fig. 15 and 16 also illustrate spherical forms and cytoplasmic processes. In Fig. 15, a cytoplasmic process which contains the cytoplasmic vesicles is also tagged with ferritin granules. The surface of the infected cell also contains viral antigen as denoted by the presence of ferritin-conjugated antibody. In Fig. 16, which shows a part of Fig. 14 at high magnification, ferritin granules are specifically seen on the surface of spherical forms. Some of them have filamentous inner

structure, but the others are devoid of dense inner material. Two spherical particles at the upper right field contain relatively dense inner material and have approximate diameter of 300 mμ. No ferritin particle is seen in the cytoplasm of the host cell.

DISCUSSION

Measles virus is one of the viruses whose morphology and chemical composition remained unknown until recently. Though Cooper (6) placed measles virus in the deoxyvirus group, there is now considerable evidence which is contrary to his view. Toyoshima and his associates (7) regarded this virus to be ribovirus because of findings of acridine orange staining of cultured cells infected with measles virus. Lam and Atherton (8) observed no significant plaque reduction with measles virus in the presence of halogen derivatives of deoxyuridine, and they estimated that the internal component of measles virus might be a ribonucleoprotein.

Only recently, measles virus has been classified into the myxovirus group from the morphological and biochemical characteristics similar to those of other myxoviruses. The term "myxovirus" was first suggested by Andrewes, Bang and Burnet (9) for a group of virus with certain features in common. They are sensitive to ether, have an affinity for mucoproteins and give generally rise to respiratory infections. Nowadays, each member of this group is further subdivided into two subgroups: Influenza group and Newcastle disease virus group (10, 11, 12). Measles virus is generally placed in Newcastle disease virus group, the whole particles of which are more pleomorphic and larger than the influenza group. Though the fine structure of Newcastle disease virus has been intensively studied, there is only a few reports about the structural feature and development of measles virus, and the exact nature of cellular inclusions still remains unknown.

The nuclear inclusion bodies appear as a clear area, in which randomly or orderly arrayed tubular structures are characteristically visible. Such structures are morphologically similar to those previously reported by Kallman et al. (2) and Tawara et al. (4) in HeLa cells infected with Edmonston strain of measles virus. Both of them describe that these structures are fibrous or strand-like. In this experiment, however, the rectangular section of these structures clearly demonstrates the tubular structure possessing a hole in the center of the filaments. The accurate length of these structures is hardly measurable because of their random array, but is estimated to be longer than 1 μ as far measurable as possible. The width is too small to be correctly measured, but is approximately 15 to 20 mμ. Intranuclear inclusion bodies are occasionally located in the vicinity of the nucleoli. There is, however, no molphological change in the nucleoli which suggests their participation in the formation of the intranuclear inclusion material.

A cytoplasmic inclusion area is also consisted of randomly arrayed tubular structures. Although they have morphological resemblance to the tubular material in the nuclear inclusion, no direct continuation is found between them. The tubular structures in the cytoplasmic inclusion have an approximate diameter of 30 mμ. The length of them cannot be measured because they are more randomly arrayed and tangled each other, showing no ordered array anywhere. The regular crystalline-like arrangement, reported by Baker et al. (3) in the nuclear inclusion and also described by Tawara and his associates (4) in the cytoplasmic one, cannot be found in either inclusion bodies in this experiment. When relatively thick sections were examined by electron microscope, the ordered array of the tubular structures occasionally seemed as crystalline-like arrangement. Therefore, such difference in fine structure of the inclusion body may be probably due to the method of embedding, thin sectioning and staining, and not due to the difference of host cells.

Many of the infected cells containing the tubular material in the cytoplasmic inclusion area show characteristic cytoplasmic budding and spheres on and outside the cell wall. Some of them are in process of detachment and the others are outside the cell wall having no contact with the cell surface. Most of the spherical bodies outside the host cell have dense and moderately thick outer membrane. The spherical forms are moderately pleomorphic and vary in size. Some of them are as small as 200 mμ in diameter, but most of them are larger than that occasionally reaching about 1000 mμ. Generally, the small one has relatively dense inner component but the large one is devoid of it. Although the cytoplasmic processes and spherical bodies at the cell surface moderately vary in size and shape, it may be presumed that some of them are the viral particles at various stage of differentiation and release. Baker et al. (3) have demonstrated the particles which have double membrane and are about 120 mμ in diameter, and they have regarded these particles as mature measles virus. Benyesh et al. (13) estimated the diameter of measles virus particle to be 1400 A from the result obtained by ultrafiltration. Examining measles virus particle by electron microscope, Waterson et al. (14) have reported that its diameter is 1200 to 2500 A. If the diameter of measles virus particle is 150–200 mμ, the small spherical bodies with dense inner component may probably be mature virus particles.

The large spherical bodies, on the contrary, have almost the same surface structure as that of the small one, but they are generally devoid of inner component. Some of them contain the small vesicles which are morphologically indistinguishable from the cytoplasmic vesicles of the host cell. These spherical forms are far from being regarded to be mature virus particle and may probably be the cytoplasmic fragments derived from the host cell. Such a peculiar mixture would account for the pleomorphism of the spherical forms just as Morgan et al. (15)

reported in the case of influenza virus.

Much of the surface of both small and large spherical bodies contain viral antigen as is denoted by the presence of ferritin-conjugated antibody. As a result of infection, the surface of host cell is transformed so as to contain viral antigen, therefore it will be natural that such detached fragments of the cytoplasm also possess viral antigen and exhibit the characteristic of incomplete virus. The presence of ferritin-conjugated antibody only at the surface of the spherical forms and partially on the surface of the host cell would lead to the following two assumptions. One is that the antibody used in this study is specific only for the outer coat of measles virus particle. The other is that ferritin granules are too large to penetrate the cell wall, so it is impossible to decide whether the antibody can react with the internal component of spherical body. As to clarify these matters, antisera specific either for the internal nucleoprotein of viral particle or for their outer coat must be used. Furthermore, the infected cell must be treated so that the ferritin-globulin complex gains entrance, while structure and antigenic composition of virus are preserved (16).

In conclusion, it is tempting to speculate that (a) the tubular structures in both intranuclear and cytoplasmic inclusion bodies may be consisted largely of ribonucleoprotein; (b) ribonucleoprotein could reach the cytoplasm near the surface of the cell and thus become incorporated into the protrusion; (c) the surface structure of the spherical forms is acquired during moving out through the cell wall; and (d) the cytoplasmic fragments, which are more pleomorphic and devoid of the inner constituent, are formed almost the same manner whereby the complete virus is released.

SUMMARY

The fine structure of inclusion body and morphological changes in VERO cells infected with Edmonston strain of measles virus has been examined in the electron microscope. Localization of virus antigen has been studied by means of ferritin-conjugated antibody method. And the following results are obtained.

1) The fine structure of the nuclear inclusion material is found to consist mainly of randomly arrayed tubular structures with frequent ordered array. The width of these tubular structures is estimated at 15–20 mμ.

2) The cytoplasmic inclusion is also consisted of an aggregate of randomly arrayed tubular material,which has the morphological resemblance to that found in the nuclear inclusion but is wider than that having approximate diameter of 30 mμ. Ordered array of the tubular structure is never revealed in the cytoplasmic inclusion area.

3) Many of the infected cells which contain tubular structure in the cytoplasmic inclusion show the characteristic cytoplasmic processes or spherical

forms on and outside the cell wall. The spherical forms might be divided into two groups; one is the small one which has dense internal component and the other is the large one which is devoid of internal structure. Although the small one may be mature viral particle, the larger one, on the contrary, could not be estimated as viral particle but as the cytoplasmic fragment though it has the same surface appearance as that of the small one.

4) Measles virus antigen has been demonstrated on the surface of both the small and large spherical particles, and also occasionally on the surface of the host cell.

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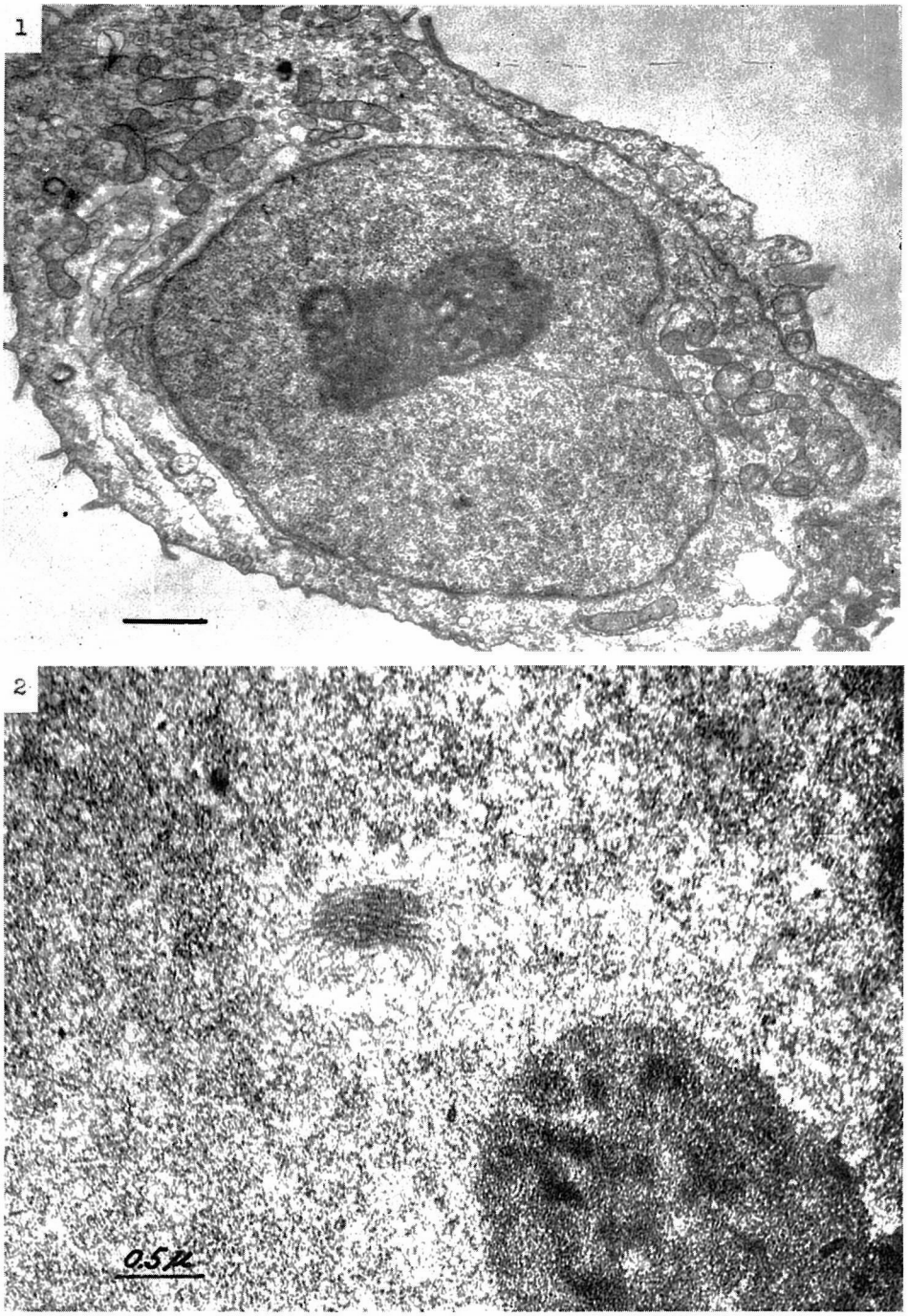
use of ferritin-conjugated antibody. *Virology*, **14** : 292, 1961

EXPLANATION OF PLATE 1.

Fig. 1. Control VERO cell. The nucleus is round with evenly distributed chromatin granules, and the nucleolus appears to consist of a mass of tiny granules organized in irregular rows. In the cytoplasm, a moderate number of mitochondria are seen, but the endoplasmic reticulum is poorly developed. Tiny cytoplasmic processes are occasionally seen.

Fig. 2. A small inclusion body is seen at the vicinity of the nucleolus which shows no morphological changes. In the center of inclusion area, parallel array of the filamentous structures is visible, but they are randomly arrayed at the periphery. This micrograph may illustrate an early stage of the development of a nuclear inclusion body.

PLATE 1

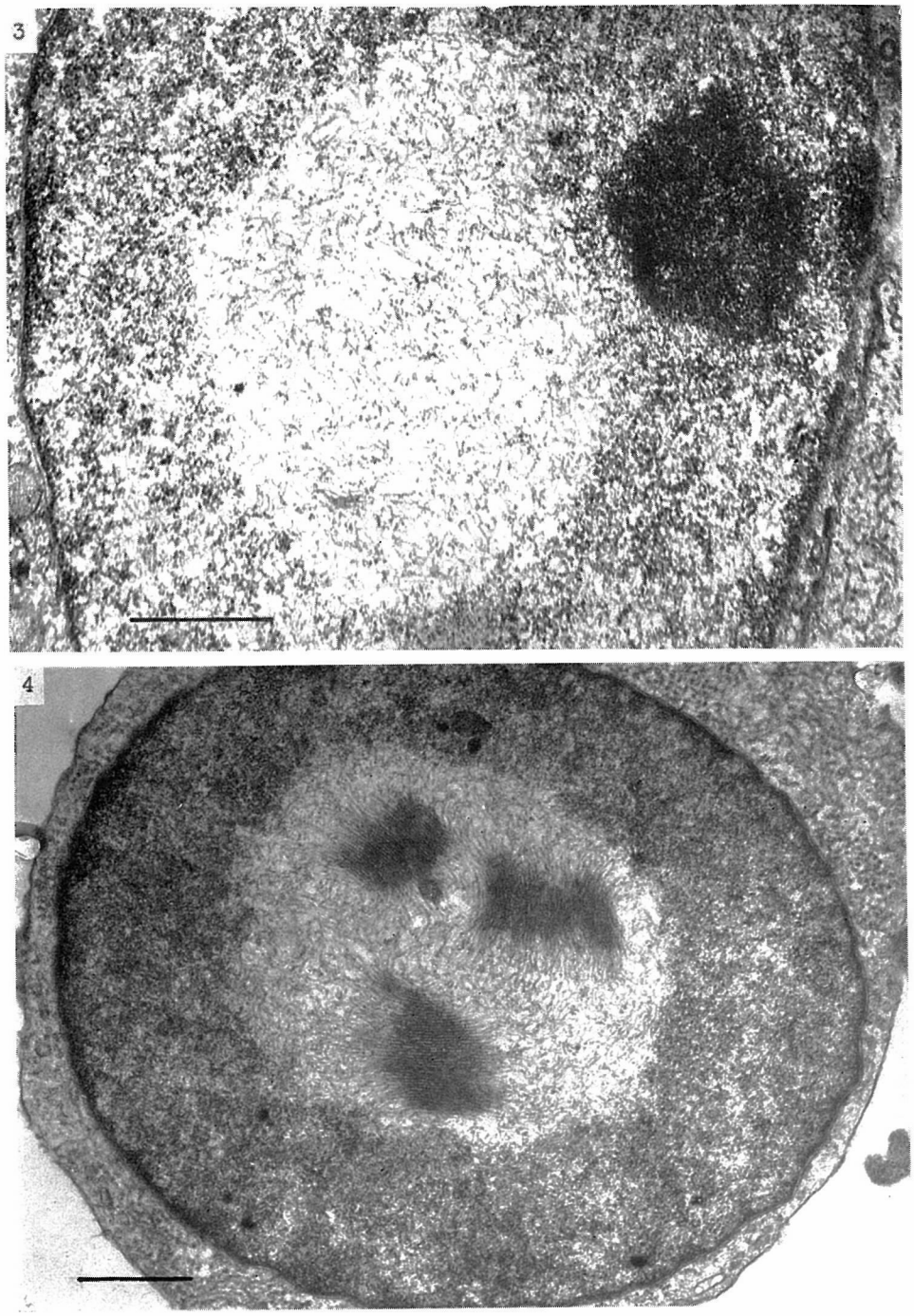


EXPLANATION OF PLATE 2.

Fig. 3. At the vicinity of the nucleolus, a large intranuclear inclusion which contains randomly arrayed filamentous material is seen. As a whole, inclusion body appears as a clear area because of complete disappearance of the chromatin granules.

Fig. 4. A round inclusion body occupies a large part of the nucleus. Three strands of ordered array are seen to extend continuously into the region of randomly arrayed filaments. Inclusion area is larger and contains more filamentous structures than is recognized in Fig. 2 and 3. This figure may show fully developed nuclear inclusion body. In the cytoplasm, randomly arrayed filamentous structures, which have morphological resemblance to the filaments in the nuclear inclusion but are larger than those, are clearly visible. There is, however, no direct continuity between the filamentous material in the nuclear inclusion and that in the cytoplasm.

PLATE 2

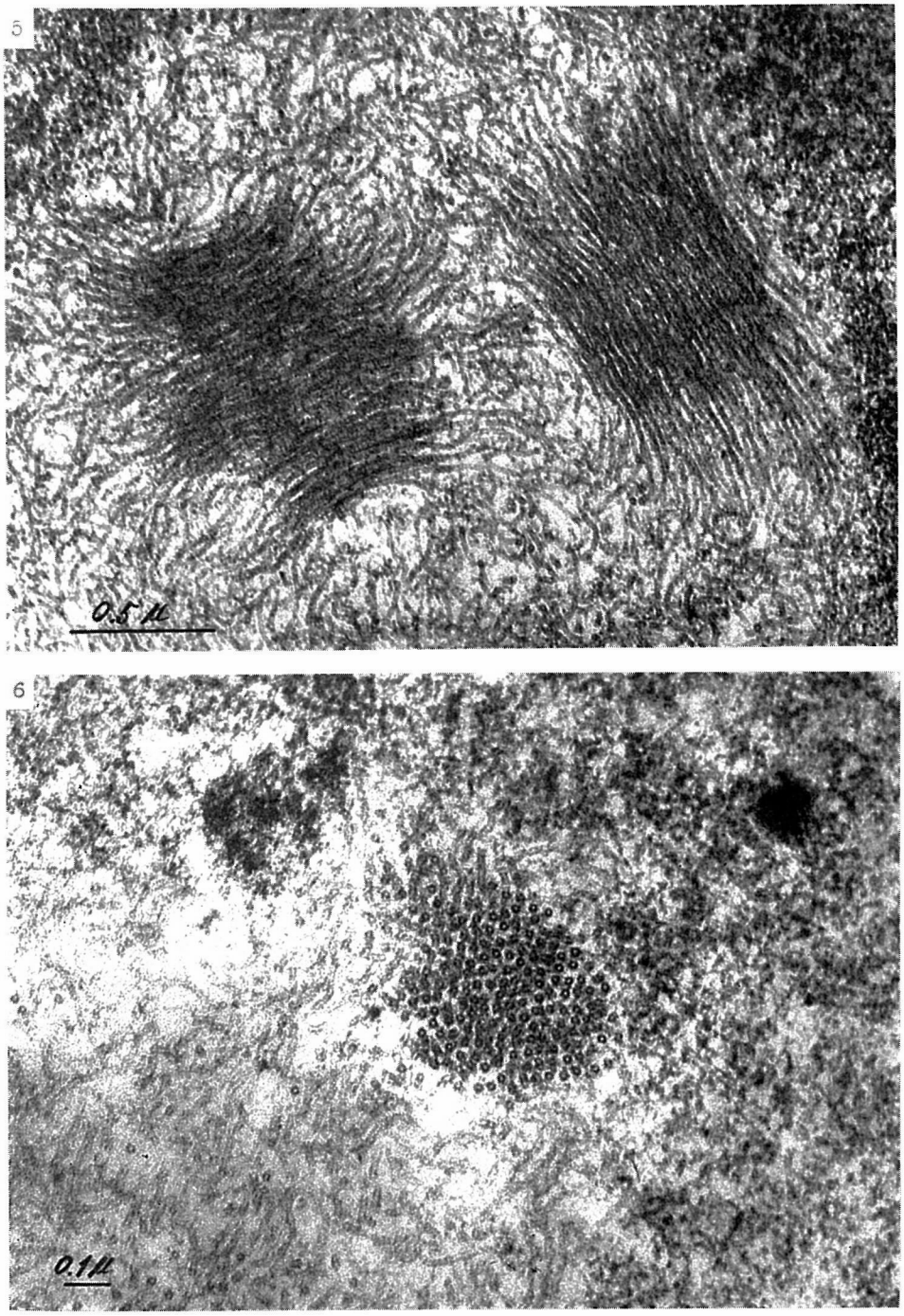


EXPLANATION OF PLATE 3.

Fig. 5. A higher magnification of a nuclear inclusion area, in which two strands of parallel array of filamentous structure is seen. The filamentous structure is moderately electron-dense and its outer surface is denser than the inner portion suggesting tubular structure.

Fig. 6. A cross section of a bundle of the filamentous structure in the nuclear inclusion reveals the tubular nature with a central hole.

PLATE 3

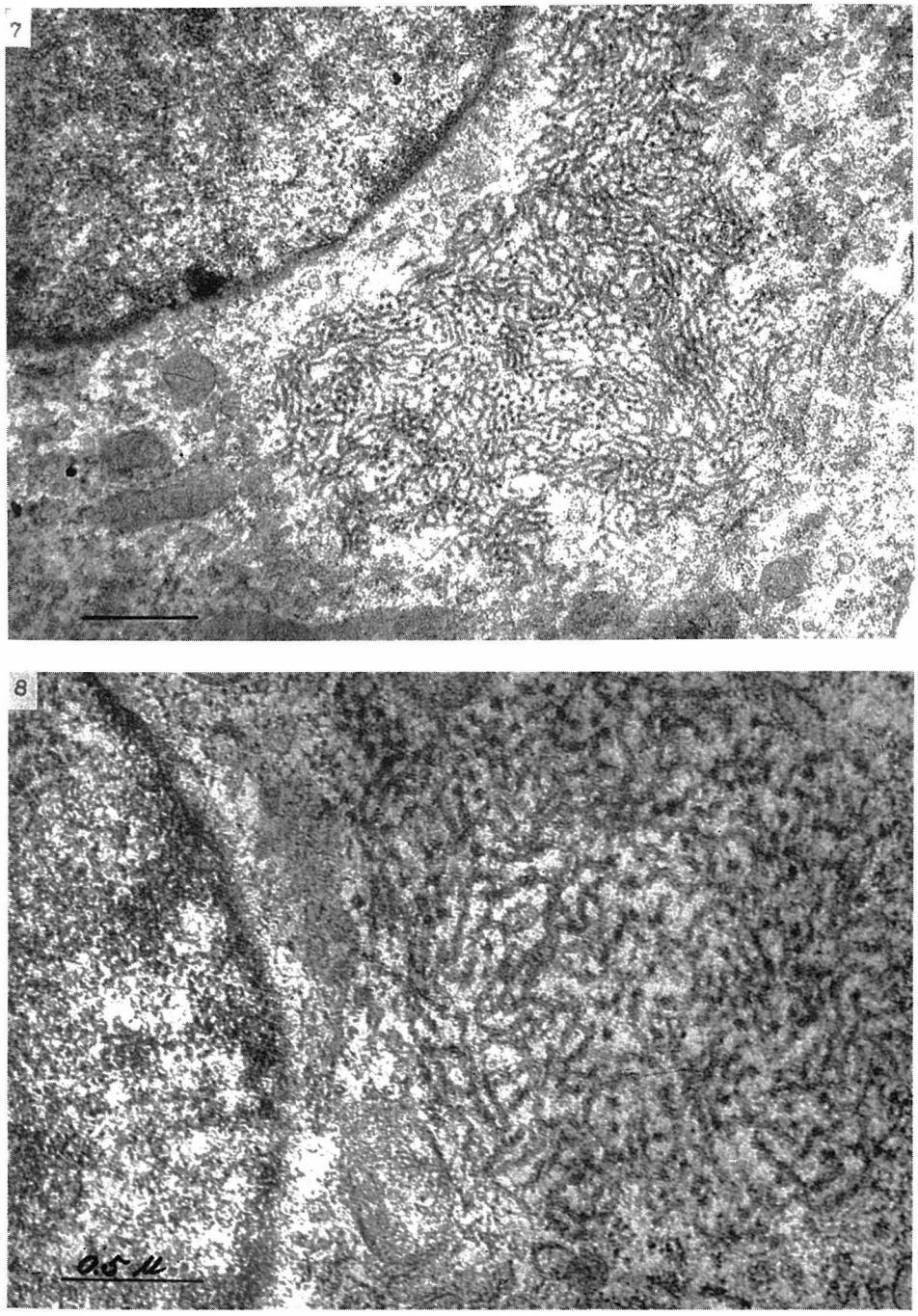


EXPLANATION OF PLATE 4.

Fig. 7. This micrograph shows a cytoplasmic inclusion body, which is consisted of randomly arrayed filamentous material. At the periphery of the inclusion area, electron-density is remarkably reduced because of the absence of the cytoplasmic organellae. No significant changes are seen in the mitochondria even at the vicinity of the inclusion area.

Fig. 8. A higher magnification of a cytoplasmic inclusion body also reveals the tubular nature of the filamentous material. The tubular material in the cytoplasmic inclusion body is more randomly arrayed showing no ordered array anywhere and is approximately twice larger than the filamentous structure in the nuclear inclusion body having approximate diameter of 30 mμ.

PLATE 4



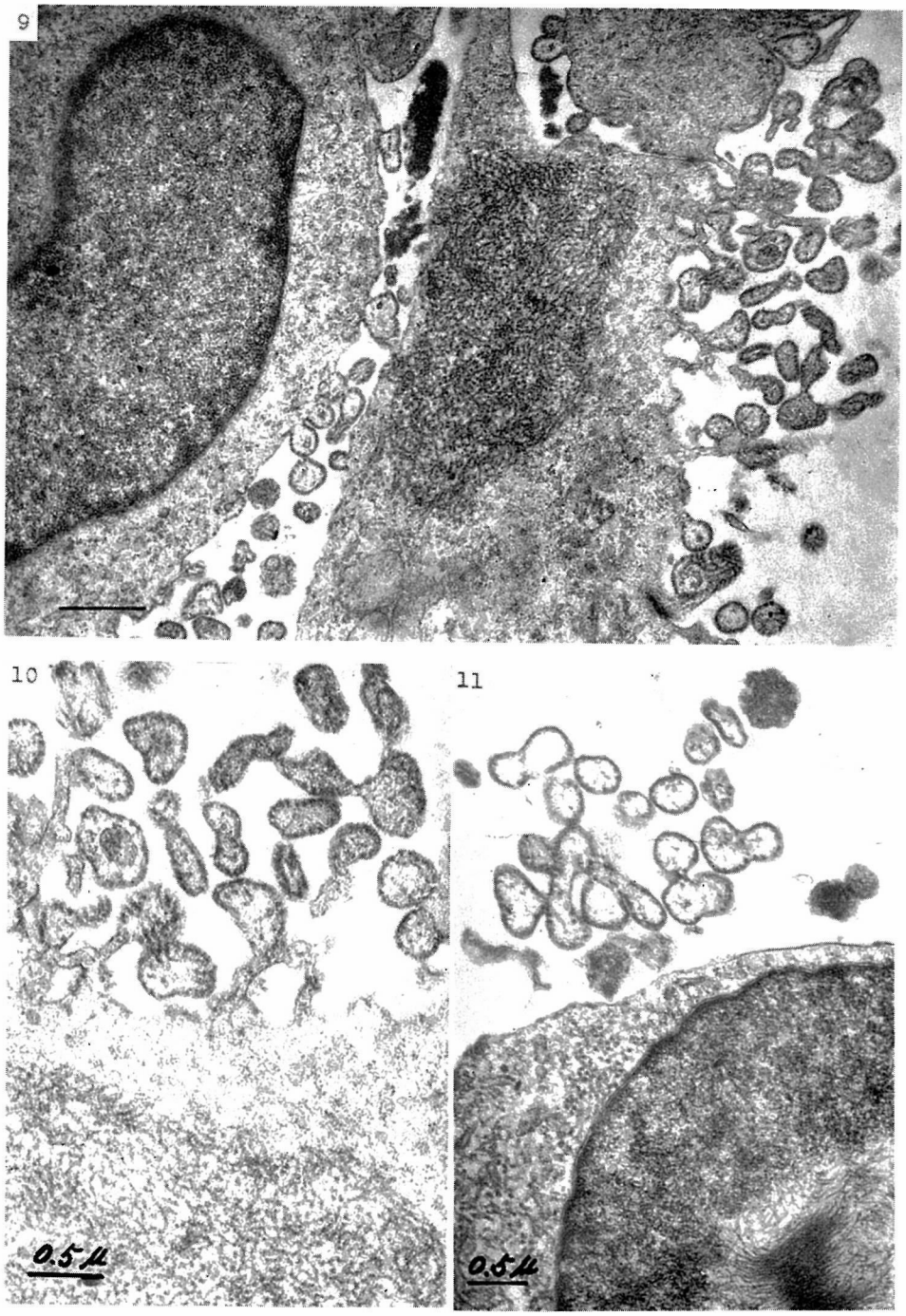
EXPLANATION OF PLATE 5.

Fig. 9. A lot of cytoplasmic buddings and spherical forms are characteristically seen on and outside the surface of the host cells. Generally, they have dense outer coat which has morphological resemblance to the tubular structure in the cytoplasmic inclusion.

Fig. 10. This micrograph is a higher magnification of a part of Fig. 9. The sphrical forms at the surface of host cell vary in their size and shape, but some of them contain thread-like inner component which is morphologically similar to the tubular material in the cytoplasmic inclusion.

Fig. 11. Spherical bodies with dense outer coat are seen outside the host cell which contains both the nuclear and cytoplasmic inclusion bodies. Most of the spherical bodies are devoid of internal structure.

PLATE 5

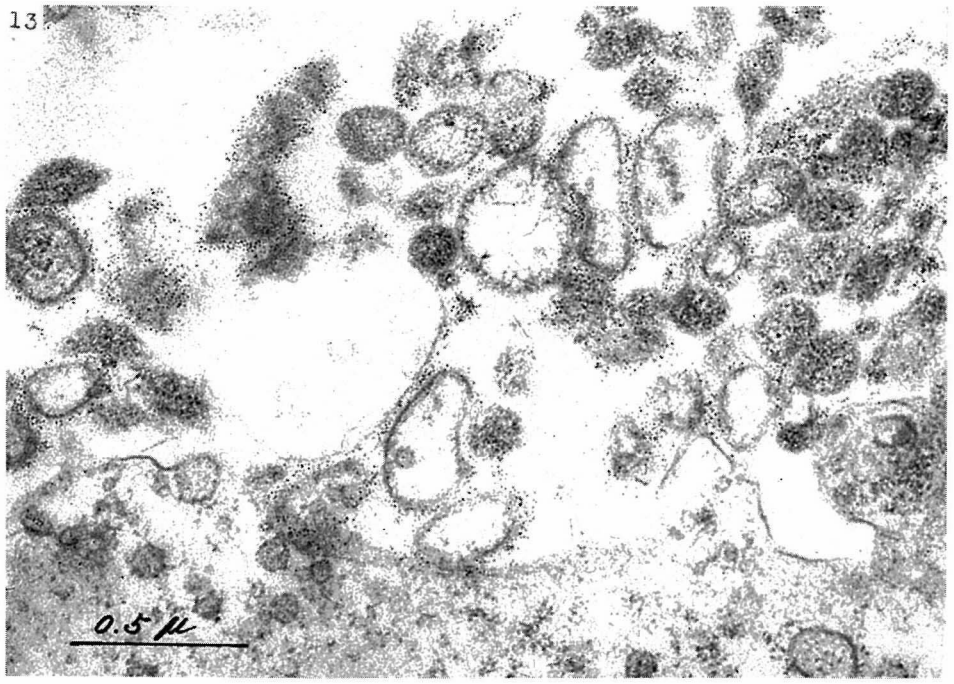


EXPLANATION OF PLATE 6.

Fig. 12. Unifected cells reacted with ferritin-conjugated antibody. The surface of the cells is almost smooth and ferritin granules are hardly visible on the cell surface. The cytoplasm shows some degenerative changes which will be probably due to exposure to ferritin-conjugated antibody without previous fixation.

Fig. 13. Infected VERO cell reacted with ferritin-conjugated antibody. Most of the spherical bodies are heavily tagged with ferritin particles. In general, the small spherical bodies have dense internal structure, but the large ones are devoid of it.

PLATE 6



EXPLANATION OF PLATE 7.

Fig. 14. Viral antigen is demonstrated on the surface of the spherical forms and cytoplasmic processes, as is shown by the presence of ferritin-conjugated antibody. In most of the spherical forms shown in this picture, tubular structures are scatteringly seen. However, a few of the spheres contain the vesicular structure which is similar to that seen in the cytoplasm.

Fig. 15. Spherical forms at the surface of the infected cell are specifically tagged with ferritin-conjugated antibody. Most of them shown in this figure are devoid of the internal structure though they have dense and moderately thick outer membrane. Some part of the cytoplasmic membrane is also tagged with ferritin particles.

Fig. 16. A higher magnification of a part of Fig. 14. It is clearly visible that some of the spherical forms contain tubular inner structures. Two spherical bodies at the upper right field contain relatively dense inner material and they have approximate diameter of 300 mμ. Such spherical bodies may probably be a mature viral particle. No penetration of ferritin granules into the cytoplasm is noted.

PLATE 7