OBSERVATIONS ON THE GROWTH OF PSITTACOSIS VIRUS IN CHORIOALLANTOIC MEMBRANES BY ELECTRON MICROSCOPE

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It is the purpose of this paper to present observations obtained with the aid of the electron microscope on the development of psittacosis virus in the embryonated egg. The first phase of the problem was to study the adsorption of virus on the cell surface and its subsequent penetration into the cell. The development of the infectious process was then pursued by studying infected tissue disrupted by sonic vibration and infected allantoic fluids harvested after the growth cycle was completed.

METHODS AND MATERIALS

The virus studied was the 6BC strain of psittacosis virus, originally isolated by Dr. K. F. Meyer and adapted by repeated passage to grow well in the allantoic cavity of embryonated eggs. Harvests of allantoic fluid were made after 5 to 6 days' incubation and the virus was purified by centrifugation.

Titration for infectivity of virus samples was performed by the single-dilution method (Golub, 1948), in which the LD₅₀ end point is calculated from the average day of death of a group of eggs inoculated with one dilution by the yolk-sac route.

Chorioallantoic membranes of 9-day-old eggs were infected by inoculation on the surface through a hole in the side of the egg where a false air sac had been formed. The inoculum consisted of 0.5 ml of a 10 X concentrated, purified suspension of allantoic fluid virus or, in the case of controls, of 0.5 ml of sterile buffer solution. The shell holes were sealed with a cellulose-acetate mixture and the eggs incubated at 37 C. At the intervals of harvest the shell was broken away to the edges of the false air sac and the exposed portion of the chorioallantoic membrane removed with the aid of forceps and scissors.

Antisera were prepared in rabbits either by 1 inoculation of partially purified, living yolk-sac virus or 5 inoculations of active mouse liver and spleen virus by the intraperitoneal route. The rabbits were bled 2 weeks after the last inoculation and the serum was stored in a dry ice chest.

The surfaces of normal and infected chorioallantoic membranes, dried on the formvar-coated glass slides, were studied by the silica replica method. A description of the technique for replica preparation and of the method for rotary shadow-casting employed will be published elsewhere.

The chorioallantoic membranes were placed in a solution of 0.10 M NaCl and 0.02 M phosphate buffer at pH 7.0 and subjected to sonic vibration. After 15 minutes most of the suspension was removed and salt-buffer solution was added. The remaining fragments of the membrane were then vibrated another 15

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minutes at higher intensity. This procedure was adapted in order to avoid extensive disintegration of the more fragile structural elements and, at the same time, break up some larger aggregates too dense for microscopic studies. After vibration the suspension was given two cycles of centrifugation for 45 minutes at 4,000 rpm, and the sediment was finally freed from large particles and aggregates by being centrifuged 10 minutes at 500 rpm.

All fractions were investigated in the electron microscope, but the final supernatant fluid contained most of the viruslike particles and was the principal object studied. The RCA electron microscope, type A, with objective aperture, was used in this study. Specimens were prepared in the usual manner by placing a drop of suspension on the collodion membrane, removing the excess, and washing the dried screen in distilled water. Most specimens were shadow-cast by the rotary shadow-casting method. This method enables one to increase particle contrast without introducing visible shadows, which often obscure particle definition. In some cases, for spatial information, customary stationary shadow-casting was added.

Several other methods were tried for breaking up membranes but were found to be less satisfactory than sonic vibration. The grinding of membranes in the wet or dried state left too many heavy aggregates, and the relatively few single particles present were covered with dense layers of intracellular substances. This made observation of the configuration and morphology of particles difficult. Sonic vibration seems to free the particles from the loosely combined proteinlike materials and, at the same time, if carefully performed, does not disintegrate individual virus particles. Vibration experiments with pure virus samples did not reveal any visible changes in morphology.

EXPERIMENTAL RESULTS

Replica studies of membranes. Prior to the examination of membranes infected *in vivo*, the appearance of the virus body replicas was studied on control membranes dried on the surface of glass slides and subsequently inoculated with psittacosis virus. Figure 1A shows an imprint of a particle about 270 m μ in diameter, and stationary shadow-casting reveals it to be relatively flat compared to an original virus particle or to its replica taken directly from the surface of a glass slide. Figure 1B shows the same type of particle as well as a larger and even flatter imprint.

Membrane replicas were observed at various intervals from 0 to 24 hours after inoculation with either virus suspension or sterile buffer solution. The time interval was a significant factor in the resulting concentration of visible viruslike imprints. In the earliest phases, i.e., less than 1 hour after inoculation, imprints of elevations predominated, but numerous holes and depressions appeared later, suggesting virus penetration into the membrane. Figure 1C shows a replica 3 hours after inoculation and the shadow-casting reveals imprints of holes or cavities whose diameters are in the same range as the virus particles. Our observations suggest that the membranes examined 3 hours after inoculation showed

the highest number of viruslike replicas. By 12 hours, the infected membranes had resumed their normal configuration, showing neither elevations nor holes.

Control membranes inoculated with buffer solutions *in vivo* show various types of structural surface irregularities but no imprints corresponding to those just described. Occasional holes or cavities can be observed in the normal membranes, but their size is dispersed over a wide range.

Adsorption studies by particle counting in the electron microscope. Supplementary to studies of virus adsorption on membranes by the replica method, studies were also made of direct virus particle counts of the inoculum after various time intervals. The experimental procedure was as follows: 0.5 ml of a purified virus suspension was introduced through an artificial air sac on the surface of the chorioallantoic membrane of 9-day chick embryos, and samples of the inoculum were removed after various time intervals; particle counts of these fluids were then performed in the electron microscope. Virus samples studied were in contact with the membranes from 5 minutes up to 24 hours. Presented in figure 2

Figures 1A, B. Control replica from a dried normal chorioallantoic membrane inoculated with psittacosis virus.

Figure 1C. Replica from an infected chorioallantoic membrane removed 3 hours after inoculation.

is the percentage of decrease of virus particles as a function of the time interval between the inoculation and the removal of the virus sample. The initial number of particles was obtained by introducing the virus onto the membrane and removing it immediately. The counting of particles was performed on a constant area of the specimen screen for all samples. The initial sample revealed approximately 1,000 viruslike bodies, and subsequent samples showed continued decreasing concentrations. As seen from the adsorption curve on figure 2A, the number of virus particles in the harvested inoculum decreases at a rapid rate, and after 60 minutes, only 4 per cent of the original number were present in the suspension. After 4 hours, this figure had decreased to 0.8 per cent; at 12 and 24 hours, the few isolated particles that could be observed were in a state of disintegration and could not definitely be identified as virus particles. The initial sample had two morphological forms of virus particles present (see figure 14). Both particle types were counted and the rate of decrease in particle count was approximately equal for both types.

Adsorption studies in vivo. *In vivo* tests for the disappearance of virus from

the allantoic fluid of eggs inoculated into the allantoic cavity also suggested a rapid adsorption of the virus onto the surrounding tissue. Eggs were inoculated

Figure 2A. Percentage of decrease in particle count of membrane surface inoculum at various intervals.

Figure 2B. Infectivity titer of allantoic fluid harvests at various intervals after inoculation with psittacosis virus.

with 10^{-2} , 10^{-3} , and 10^{-4} dilutions of a stock 6BC psittacosis virus and at intervals of 2, 6, 24, 48, and 72 hours pooled samples of fluids from five living eggs of

each group were tested for virus activity. The titer at zero time for each inoculum was calculated from the known titer of the virus and the estimated dilution factor involved. The results are shown in figure 2B.

It can be seen that the infectivity titer of the fluids dropped rapidly within a few hours of inoculation. Control experiments have shown that simple incubation of virus *in vitro* for short intervals does not result in comparable decreases in titer. The less rapid drop with increase in inoculum concentration would suggest that the saturation point of cells susceptible to virus attachment was being reached.

Studies on sonic-wave-vibrated normal chorioallantoic membranes. Electron microscopic studies on vibrated normal membranes revealed many structural components of the cell and gave the impression of the very complex nature of the cellular organization. Comparative studies on normal and infected membranes were made, and only those structural elements that seemed to be associated with the development of the psittacosis virus were extensively investigated. The significance of the comparative study of normal and infected membranes became apparent after repeated observations disclosed some modes of virus development appearing to be integrated with normal growth of the intracellular elements. For this reason, a few examples of structural elements of the normal membranes following sonic vibration are presented.

Electron microscopic observations on vibrated normal membrane suspensions reveal a large number of dense particles having a broad size distribution. A striking feature is the large number of fibers interconnecting various bodies and often originating in large masses from such bodies. In figure 3A is shown an example of dense intracellular particles, suggestive of nuclear material. Figure 3B shows a more irregular particle with a less dense border area, and in figure 3C are examples of the frequent association of particles with bundles of fibers. Individual fibers exhibit transverse striations and some variation in their dimensions. A few fibers were measured and were found to be in the range of 34 m μ to 56 m μ ; the distance between the striations was, on the average, 64 m μ .

Fibers were also observed in suspensions of ground membranes and therefore cannot be considered as a phenomenon resulting from vibration. Furthermore, replica studies on membranes, where a layer of tissue was pulled off by an attached plastic film, revealed that such fibers are normally present in the cell.

Studies on sonic-wave-vibrated infected chorioallantoic membranes. Electron microscopic studies were performed on vibrated membranes at 6, 12, 24, 48, and 72 hours after infection, a period considered to cover the complete range of virus development. At the same time, studies were also performed on corresponding control membranes that had been inoculated with sterile buffer solution in place of the virus suspension.

Six-hour infected membranes did not show any visual difference in structural elements from the controls. The 12-hour infected samples showed some isolated particles that closely resembled the typical virus particle. Twenty-four, 48-, and 72-hour membranes showed increasing numbers of viruslike particles and aggregates not seen in the controls.

Both infected and normal samples contained a large number of forms of vary-

ing density and broad size distribution. In infected membranes, however, an abnormally heavy concentration of particles was seen in the range between ap-

Figure 3. Structures observed in sonic-wave-vibrated, normal chorioallantoic membranes.

proximately $300\text{ m}\mu$ and $900\text{ m}\mu$. Considerable numbers of apparently dividing forms were visible, similar to those appearing in virus samples prepared from

allantoic fluid. In addition, infected membrane samples showed various dense bodies and aggregates to which many particles, apparently psittacosis virus, were attached. Figure 4 shows various single particles from infected membranes, demonstrating the size variation and the elevated, inner dense material of irregular shape. The particle shown on figure 4C has an approximate height of 170 $m\mu$. In the smaller forms, the dense central portion fills the body more completely than in those forms of larger diameter. Figure 5A, B, C shows examples of larger particles and, with increasing size, there seems to be a rearrangement or dispersion of the dense central material. The bodies seen in figure 5A, B appear to be some intermediate form between the relatively dense bodies seen in figure 4 and the flat form shown in figure 5C. The approximate height of the particle in figure 5A is 70 $m\mu$.

The simplest grouping of particles observed is a paired combination as seen in figure 6. In figure 6A both particles of a pair seem to have the same general morphology, but one of the two is often of greater diameter. On the other hand, figure 6B, C shows groupings in which a smaller particle is combined with a larger form of different structure.

More complex types of particle groupings are shown in figure 7. An irregular pattern of particle combination is seen in figure 7A, and figure 7B reveals a combination of two large bodies with a smaller, centrally dense particle. In figure 7A four particles appear in a row, with two larger particles having a less dense border region whereas the remaining two are completely dense. A different type of combination is presented in figure 8, in which two flat bodies are seen attached to intracellular fibers.

A similar type of combination of elements is presented in figure 9. In this series, viruslike particles are seen attached to intracellular fibers previously revealed (figure 3) as structural elements of normal cells. The combination between viruslike particles and fibers seems to be real. The large number of observations of this type rule out the possibility that such combinations are the result of accidental association only. In figure 9A, B the particles appear to be attached to a fiber, yet on figure 9C fibers seem to be terminating with the particle. This type of connection has been repeatedly observed, so that accidental combination of this type is also improbable. In figure 9D, E various particle forms are combined with heavier fiberlike structural elements of the cell.

A different type of combination observed is the close association of viruslike particles with dense intracellular substances. In figure 10 a viruslike particle is combined with a dense, elongated body with fiber attachments. Figure 10B, C shows, for the most part, a similar type of association.

In general, there exists a large number of combinations with varying degrees of complexity between intracellular elements and viruslike particles. Some of the more complex combinations are presented in figure 11, in which viruslike particles, dense intracellular bodies, and fibers are associated with one another. In figure 12 an interesting phenomenon is that viruslike particles are not only associated with fibers, but there seems to be a partially developed virus particle fused with the dense body. Dense massive aggregates can often be observed in which many viruslike particles appear to be incorporated; however, only those

Figures 4A-8.

Figures 4-13. Structures observed in sonic-wave-vibrated, infected chorioallantoic membranes.

Figures 9A-11 B.

Figures 11C-13.

Figure 14. Purified preparation of infected allantoic fluid, 5-day-old culture.

Figure 15. Psittacosis virus bodies agglutinated in the presence of specific immune serum.

Note: Stationary shadow-casting was performed with gold at an angle tangent $1/5$. Scale length under each group of electron micrographs is 1 micron. One-micron scale lengths for different magnifications are given directly on the print.

individual particles located in the border region of the aggregate are clearly visible, as demonstrated in figure 13. Repeated observations indicate that such aggregates are heavily loaded with virus particles.

Studies on virus bodies from allantoic fluid preparations. In figure 14 are shown the normal bodies in the varying morphology, as seen in many similar preparations of psittacosis partially purified from infected allantoic fluid. It was at first felt that the variations from the small, typical body were elementary bodies undergoing degradation. However, examinations of incubated samples for periods up to 10 days indicated that the proportion of the large, flat forms did not increase, although definitely visible morphological degradation of both forms

Figure 16. Size distribution of particles from infected allantoic fluid and chorioallantoic membranes.

was apparent. It is believed, therefore, that the large flat bodies are not direct degradation forms of the small elementary body.

To test the relationship of these large forms to the smaller elementary bodies, the purified virus suspension was examined after incubation for 3 hours with its specific immune serum. Figure 15 shows clumps of the bodies visible in this preparation, and it is seen that the large, flat cells were agglutinated along with the small, centrally dense bodies, indicating that the former are probably the actual virus in a morphologically different form. Strands of serum protein material are plainly evident and in some areas coat the virus aggregates so heavily as to mask completely the typical morphology of the individual particles.

Size distribution of virus particles. It was of interest to compare the size distributions of virus bodies originating from allantoic fluid with those obtained from the vibrated infected membranes. About 150 particles of each series were

measured and distribution curves calculated. The total count included both the small particles and the large flat bodies. Distribution data in graphical form are presented in figure 16.

The virus sample prepared from 5- to 6-day incubated allantoic fluid cultures shows two distinct size regions. The first maximum peak is in the range between 300 $m\mu$ to 440 $m\mu$ and represents particles with a dense central region and a diffuse border area. The second maximum is in the range of 650 $m\mu$ to 800 $m\mu$ and is made up largely of diffuse flat bodies. On the other hand, virus originating from 72-hour infected membranes shows only one distinct maximum peak, but there are more particles in the size range between the two peaks of the allantoic fluid material.

DISCUSSION AND SUMMARY

The initial phase of psittacosis virus infection of the host cell is in all probability an adsorption of the infectious particle on the cell surface. With regard to subsequent events, we have always assumed, actually without much direct evidence, that penetration of the cell membrane follows, in which internal environment the virus particle finds the proper conditions for its multiplication process.

Our observations on the disappearance of virus bodies from an inoculum on the surface of the chorioallantoic membrane, as measured by particle counts in the electron microscope, revealed that after 4 hours' contact with the tissue, only about 1 per cent of the original virus bodies remained suspended in the fluid, the rest presumably having formed an attachment to the membrane. The studies on the rapid decrease in activity titer of allantoic fluid inoculated with psittacosis virus *in vivo* confirm the rapid adsorption of the infective particles on the surface of susceptible tissue. Membrane replica studies of infected tissue, in which virus particle imprints practically disappeared 5 hours after inoculation of the membrane, suggest that the succession of events has progressed beyond the adsorption phase and that actual penetration is almost complete by this time.

It was revealed that the shape of the imprints of viruslike particles was actually changing from elevations to cavities and holes when observations were made at various intervals following inoculation, and this is construed as suggesting actual cell penetration. No visible differences were observed between normal membrane replicas and the 12-hour infected samples, further suggesting that the sites of virus invasion on the membrane surface had undergone repair by this time.

The fate of the virus particles after penetration of the cell membrane has been the subject of various speculations. The numerous light microscopic observations are not in complete agreement as to the multiplication process in all details, but one view is that the virus is capable of immediate multiplication without the formation of large bodies. This possibility is considered to be dependent upon intracellular environmental influences (Burnet and Rountree, 1935; Levinthal, 1935; Yanamura and Meyer, 1941). The more common and generally accepted alternative mode of psittacosis virus multiplication is the formation of large bodies from smaller particles and dispersion of the latter by a process of

disintegration resulting in the liberation of various-shaped smaller particles. The formation of a dense matrix around the seat of virus multiplication was considered to be an intracellular reaction of the host cell to virus invasion and constituted an accumulation of protoplasmic material (Bedson, 1933, 1934; Bland and Canti, 1935; Burnet and Rountree, 1935; Levinthal, 1935; Yanamura and Meyer, 1941). Observations on the multiplication of some other viruses reveal, in general, similar developmental modes. The observations of Tang and Wei (1937) on the multiplication of vaccinia virus suggest that this virus may possess two independent methods of multiplication. The first is by simple division, giving rise to new particles with varied morphology. The second process of multiplication is considered to be a combined action of the invading virus and the host cell, under which conditions large particles with accompanying matrix formation are observed. Somewhat similar observations were made by Rake and Jones (1942) on the development of lymphogranuloma venereum virus.

Our first observations with the electron microscope were performed on psittacosis virus purified from allantoic fluid. The two distinct morphological forms, one smaller, centrally dense particle, and another larger, flat body were revealed (figure 14). The size distribution curve had two distinct maxima (figure 16), and the ratio of the two particle types varied in the samples. Agglutination experiments indicated that both particles had antigenic factors in common. Our studies on viruslike particles originating from infected membranes reveal the same over-all size range as in virus from allantoic fluid, but the distribution of particle sizes is different. Morphologically, there is great similarity between particles seen in allantoic fluid (figure 14) and those obtained from vibrated infected membranes (figures 4 and 5). These findings suggest that we are probably dealing, in both cases, with the same type of virus particles, the principal difference being that in a 72-hour infected membrane the morphological development of the virus is, on the average, in an earlier phase than are those originating from 5- to 6-day cultures of allantoic fluid.

In our experiments virus particles from vibrated membranes were not apparent until 12 hours after infection. In spite of extensive observations only a few isolated particles were found that resembled the typical virus particle contained in the inoculum. The fact that the 6-hour infected membranes did not reveal any such virus particles indicated that the particles visible in subsequent samples were new growth and not original inoculum. Light microscopic and electron microscopic observations are apparently in agreement as to the time necessary for the first cycle of virus multiplication.

Our observations on later samples also revealed large, dense particulate aggregates, varying in size and density, on whose border areas were exposed many virus forms (figure 13) and which may correspond to a colony of virus. Many of the aggregates were probably distorted by the sonic vibration, but in general they manifested considerable resistance, indicating that the particles within the aggregates are firmly embedded.

It appeared to us that the virus in certain instances associates itself with various normal intracellular elements and possibly follows a pattern of development

similar to the normal reproduction of these components. Speculating along lines upon which others (Zinsser, 1937; Stanley, 1939; Stanley, 1941; Soule, 1940) have theorized, it may be that certain forms of the normal intracellular structure, once they have been in direct contact with an invading virus body, develop the potentiality themselves to produce virus at the seat of contact or induce virus growth in associated elements. This would then be merely an altered reproduction of the normal cellular constituents resulting from the new pattern induced by the virus. Virus growth as just described is simply an abnormally rapid multiplication process of modified normal intracellular structural elements within a certain limited size range. It may be akin to malignant tissue growth, wherein abnormal reproduction of apparently normal cells takes place. The stimulus for the latter is, in most instances, unknown, but in the case of the viruses would be the presence of living virus particles carrying the growth-inducing property. It will be recalled that even in virus infections one of the earliest manifestations is stimulation of cell growth in the vicinity of the affected area.

The question still remains as to whether direct virus multiplication can be initiated without association with some structural elements of the cell. Light microscopic observations, due to insufficient resolving power, can only indicate that multiplication proceeds without large particle formation. Electron microscopic observations, although of increased resolving power, have the disadvantage that they are performed on dried specimens and the objects of study are, of necessity, removed from their intracellular environment. Virus body combinations were seen in both vibrated membranes (figures 6, 7, and 11) and allantoic fluid preparations (figure 14), a fact which suggests that simple division occurs, although not necessarily equal division. Similar observations were made by Rake *et al.* (1946) on the development of lymphogranuloma venereum virus in the yolk sac. However, these forms may previously have been connected to large forms or to intracellular elements and become separated later. In most of these observations, the dense central material of the elementary body seems to play a significant role in the process.

Further uncertainty exists concerning the function and significance in virus multiplication of the two major type virus bodies, i.e., the small body with the dense center and the larger flat body. Repeated observations create the impression that there are usually one or several small dense particles associated with a large body and suggest that the flat body gives rise to smaller dense forms, following which it disintegrates (figure 14A, B). The earlier shape of free flat bodies is also uncertain, but our impression is that some type of the centrally dense particles (figure 4D, E, F) is a precursor of the flat forms. The phenomenon of rearrangement in the shape of the dense central material seems to be one step of virus multiplication, and further developmental forms, e.g., figure 10B, C, result in chain formation, as in figure 7C. Further suggestive evidence is seen in a comparison between the size distribution curves of particles originating from 5- to 6-day allantoic fluid preparations and the 72-hour infected tissue. The former has two distinct particle size maxima, but the latter shows more of the intermediate and smaller forms. This suggests that intermediate forms, after

further development and with rearrangement of the dense central portion, give rise to other centrally dense particles in the smallest size region, after which they appear as flat bodies, and thus might be considered as late stages of the so-called initial bodies. Our impression is that the "wrinkled pea" appearance (Rake *et al.*, 1946) of the elementary body with the irregular central mass is not an artifact due to the drying process, but is caused by the uneven distribution of this material in the virus body at certain stages in its development. The form of this material appears to change with the cycle from a rounded mass, almost filling the body, to a more irregular, ameboid-shaped form. In the later stages it becomes dispersed in scattered areas throughout the large body and finally disintegrates almost entirely, resulting in the flat forms.

In brief summary then, incorporating previous views, the following modes of psittacosis virus development in the egg are suggested: the virus bodies are adsorbed rapidly onto the cell surface and soon penetrate the cell wall, leaving holes in the membrane which are quickly repaired. Some of the virus particles may have the potentiality to multiply directly, provided the environmental conditions are suitable. This does not exclude the possibility, however, that the virus must contact certain normal structural elements of the cell before it acquires the ability to reproduce itself. It is suggested that one route of development may proceed by association with or incorporation into normal cellular constituents, following which either the normal cellular elements then produce the altered material recognized as virus or the virus itself gains therefrom the capacity to reproduce in kind.

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