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SUBJECT OF INVESTIGATION

ON THE GROWTH MECHANISM

OF

PSITTACOSIS-TRACHOMA VIRUSES

IN

TISSUE CULTURES

RESPONSIBLE INVESTIGATOR

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Kyoto University (Japan)
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ON THE GROWTH MECHANISM OF PSITTACOSIS - TRACHOMA VIRUSES IN TISSUE CULTURE

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Abstract

Trachoma virus has been successfully adapted to HeLa cells. The virus passes through eclipse phase during the multiplication and seems to arise from the matrix material, therefore there is no immediate morphological continuity between the infected particles and the new generations of the virus. There is no evidence that trachoma virus multiply by binary fission which had been supposed so far.

It was found that the preparations of meningopneumonitis virus (MPV) purified by a combination of differential centrifugation, treatments by nucleases and trypsin, and sucrose density gradient centrifugation consisted mostly of elementary bodies of the virus. Chemical analysis of the elementary body showed that it contained approximately equal amount of ribo- and deoxyribonucleic acid.

CONTENT

1. A statement of the problem

The growth mechanism of meningopneumonitis virus (MPV), a typical member of the psittacosis-lymphogranuloma group, has been made clear by us. However, in case of trachoma virus, the developmental cycle including virus entrance into host cells, the fate of infecting virus particles and the emergence of the new generation has little been known.

In spite of large number of investigations, the purification of meningopneumonitis virus has not been successful, therefore it has been uncertain that the virus contains both type of nucleic acids or not.

2. Analysis of the problem

a. Trachoma virus: The studies with trachoma virus-HeLa cell system were carried out under a systematic combination of growth curve experiments of virus determined by infectivity titrations and morphological examinations with the optical and electron microscope.

The growth cycle experiments may show whether virus pass through non infectious phase, and the morphological examinations may indicate the mechanism of viral entrance into host cells, fate of the infecting virus and the emergence of the new virus.

b. MPV: Partially purified preparation by enzymatic digestion and differential centrifugation has been found to contain two types of particle: one type is 0.25 - 0.3 μ in diameter and the other is 0.5 - 1.0 μ. Only the small particle (elementary body) is infective. By a combination of differential centrifugation, nuclease and trypsin treatment, and sucrose density gradient centrifugation, the large particles (reticulated bodies) were nearly completely eliminated from the preparations. The study aims at the analysis of nucleic acid of the small particle.

3. Outline of experimental procedure

Virus

MPV used was a Cal 10 strain that had been transferred many times in the yolk sac of embryonated eggs. It was subjected to serial passages in strain L cells. The inocula used in the studies were fluid virus stocks that were peepared as follows. Fliud from L cell suspension cultures infected with the virus was harvested 40 hours later, adjusted to pH

7.2 and partially purified and concentrated by two cycles of differential centrifugation. Tests were made with thioglycolate broth to exclude the presence of bacteria. Stock virus so prepared had titers of the order of 2×10^8 PFU (plaque forming unit) per ml.

Trachoma virus used was a T'ang strain (TE-55) which had been cultivated in eggs.

Cells and medium

Cells used were strain L cells and HeLa cells which were grown in (0.1%) yeast extract and (0.5%) lactalbumin hydrolysate in Earle's balanced salt solution with the addition of 10% bovine serum, 200 µg of streptomycin, and with 200 units of penicillin per ml in case of MPV, and without penicillin in case of trachoma. Cell maintenance medium was the same as the growth medium except that the serum concentration was decreased to 2% and penicillin was omitted.

P^{32} medium: Lactalbumin hydrolyzate 5g, streptomycin 0.2g, glutamine 0.1g, and eight vitamins (Eagle, 1955) were dissolved in 1 liter Earle's balanced salt solution without phosphate compounds, containing 2% bovine serum, and 1 µc P^{32} (carrier free) per milliliter.

MPV infection of L cell suspension and trachoma infection of HeLa cells

Suspension cultures were grown at 37 in 100-ml. volumes in Pyrex bottles and agitated continuously at a speed 30 times per minute. Cell concentration was kept at between 5×10^5 and 7×10^5 cells per ml. by dilution with growth medium every 2 days. Sufficient stock virus suspended in maintenance medium was added to the cells to give a multiplicity of infection of 0.06 to 50.0 PFU per cell. After 1 hour of adsorption at 37°, infected cells were washed three times with cold PBS and one time with cold maintenace medium; finally they were diluted to about 3 to 5×10^5 cells per ml. with maintenance medium and incubated at 37° with continuous agitation.

Trachoma infection followed almost the same except that monosheet cell cultures were used.

Virus assay

A plaque assay technique was used for MPV and inclusion forming unit was used for trachoma.

Chemical fractionation

Cold trichloroacetic acid (TCA) was added to the sample to be analyzed to a final concentration of 10%. After standing for 15 minutes at 0°, the TCA-insoluble material was centrifuged and the precipitate was washed three times with cold 10% TCA; the supernatants were pooled to make up the acid-soluble fraction.

The TCA precipitate was washed once with 95% alcohol at room temperature and extracted three times with a mixture of absolute alcohol-ether (3:1) at 60°, for 15 minutes. The extracts were pooled to form the lipid fraction. The precipitate was hydrolyzed according to the method of Schmidt-Thannhauser (1945); 2 ml of 1 N KOH was added to each sample, and the samples were incubated for 18 hours at 37°, chilled in ice water, neutralized with 6 N HCl, and precipitated with perchloric acid (final concentration, 5%). The precipitates were separated by centrifugation and washed once in ice-cold 5% perchloric acid. The supernatant was part of the RNA fraction. The precipitates were resuspended in 5% perchloric acid and hydrolyzed for 20 minutes at 70°, chilled in ice water, centrifuged and washed again with 5% perchloric acid. The supernatant was designated as the DNA fraction and the pellets were dissolved in 1 N NaOH (residual fraction).

Each fraction obtained, namely, acid soluble, lipid, RNA, DNA, and residual, was assayed in a Geiger counter for radioactivity of P^{32}.

Protein was measured by the method of Lowry et al.(1951), using Folin phenol reagent. Crystalline bovine serum albumin was used as a standard.

Electron microscopy of HeLa cells infected with trachoma virus

A number of electron microscopy of infected cells was carried out every 2 hous after infection, parallel with the virus growth & experiments and optical microscopy of the infected cells. Infected cells were fixed in acetate-Veronal buffered osmium tetroxide, pH 7.4. Cells were embedded in epoxy resin by the methods of Luft (1961). Thin sections were cut with the LKB Ultrotome: A JEM-60 machine was used.

4. An explanation of controls used

Electron microscopic examinations of uninfected control HeLa cells which were cultivated under the same conditions were done to see the normal structural elements.

5. Explanation of instrumentation employed

The LKB Ultrotom Ultramicrotome was used for the preparation of thin sections of the infected cells for electron microscopy, and the sections obtained were examined with the JEM type 6C electron microscope. Cell homogenate was prepared by the use of Macro Homogenizer (Vir Tis).

6. Results obtained

a. Trachoma virus

A T'ang strain(TE-55) of the trachoma virus which had been cultivated in embryonated eggs has been successfully adapted to the HeLa cell cultures. It has been maintained in the 30th passages, showing characteristic cytopathogenic effects on the third to fifth day of incubation (Fig. 1).

Growth curve experiments of the trachoma virus in HeLa cell cultures with multiplicity of five showed the lag period up to twenty four hours. The amount of cell associated virus during the first twenty four hours after infection dropped extremely below the number of the infective centers, showing that inclusion forming units of the cell associated virus per infected cell during the lag period were as low as 10^{-3}. After twenty four hours of lag period, cell associated virus increased exponentially until fourty eight hours after infection.

Electron microscopy of cells 3 to 5 hours after infection showed that the elementary bodies entered the cells by the mechanism of viropexis. Intracellular changes of the bodies after entrance was as follows. The invaded bodies succeesingly flocked around the Golgi apparatus, and eventually ceased to exist as such. The unusual bodies quite different from the infected elementary bodies in size, shape and structure were first seen 15 hours following infection. They were occurred within the intracytoplasmic matrix areas composed of reticulated material (Figs. 2 and 3). They were irregular in shape varying in size, usually larger than the elementary bodies and made up of rather homogeneous reticulated material. Some of them possessed well defined limiting membrane, but others were partially enclosed by the membrane. They were designated as reticulated bodies. Succeedingly in the reticulated bodies sometimes there developed two or daughter reticulated bodies (Figs. 4 and 5). Repeated observations created the impression that the characteristic particles seen in cells during the last 2/3 lag phase were confined to the reticulated bodies.

At the next stage, reticulated bodies came to maturity to form elementary bodies with a nucleoid through the sequence of stepwise maturation; becoming increasingly smaller without showing any evidence for fission, showing a denser marginal portion and much less dense internal portion (indicated by arrows in Figs. 4 and 5) and tending toward nucleoid differentiation (arrows with circles in Fig. 5). The earliest emergence of the elementary bodies corresponded well to the sharp increase in virus titer (24 hours after infection). At this stage, reticulated bodies were still seen, however, with time elementary bodies increased in number and reticulated bodies decreased in number. This is another evidence that reticulated bodies come to maturity to form elementary bodies.

b. Meningopneumonitis virus

The preparations of the meningopneumonitis virus purified

partically by combination of enzymatic digestion and differential centrifugation contained two major types of the virus, they appeared as small dense-centered bodies about 0.3 micron in diameter and as larger bodies 0.5 to 1.0 micron in diameter, without a dense central structure. The former referres to elementary bodies and the latter reticulated bodies. The two morphological types have been separated by sucrose density gradient centrifugation. Fig. 6 shows the purifed elementary bodies. Table 1 shows recovery of infectivity and protein at each step of purification procedure.

TABLE 1

Recovery of Infectivity and Protein at Each Step of Purification Procedure

Treatment	Total ml	PFU/ml	Total PFU	Protein (g/ml)	Total protein (mg)	PFU/mg protein
Starting material	500	8.4×10^6	4.2×10^9	11500	5750	7.30×10^5
After two-cycle high-low speed centrifugation (Step 1)	50	2.2×10^7	1.1×10^9	2160	108	1.02×10^7
After enzyme treatment (Step 2)	1.5	4.8×10^8	7.2×10^8	-	-	-
After sucrose gradient centrifugation (Step 3)	50	1.3×10^7	6.5×10^8	109	5.45	1.19×10^8

TABLE 2

Chemical Distribution of P^{32} in a Purified MPV Preparation

Fraction	P^{32} content	
	Total cpm	%
Whole virus	48500	-
Acid soluble	3500	7.22
Lipid	15490	31.5
RNA	13300	27.4
DNA	12900	26.6
Residuals	1980	4.07
Recovery	47170	97.0

Suspensions of a single type of elementary bodies have been analyzed using the isotopic techniques. Table 2 shows that the elementary bodies contained about equal amount of DNA and RNA.

7. Discussion and Conclusion

a. Trachoma virus

A study of the cultivation of a Chinese trachoma strain in HeLa cell cultures has been carried out, and it was found that the virus could be transferred serially, accompanied by the characteristic cytopathy and the presence of the cytoplasmic inclusions as early as fourteen hours after infection.

As in the case of MPV, the trachoma virus becomes non-infective in the sense that the infected cells during the lage period do not contain infective virus, showing the evidence for a noninfective form of the virus.

The electron microscope experiments indicated that the trachoma virus elementary bodies were incorporated into the cells studied by phagocytosis and that after entering into the cells they ceased to exist as such. The virus seemed to arise independently of the other from the matrix material of reticulous structure which was newly formed in the cytoplasm in response to the infection. The formation of the reticulated bodies, which would correspond to the large blue-stained initial bodies of the virus in the optical microscope in the same specimen at the same stage, can be represented as a differentiation of the matrix material in the sense of condensation, progressive formation of limiting membrane (see Figs. 2 and 3). There was no immediate morphological continuity between the infected elementary bodies and the reticulated bodies and there was no evidence that the reticulated forms multiphy by binary fission which had been supposed so far.

b. Meningopneumonitis virus

Two independent criteria suggest that pure suspensions of the elementary bodies of the MPV are obtained by the purification procedure described. First, in sucrose density gradients, the samples give single sharp bands as characterized by number of infectious units, and optical density at 260m . Second, in electron micrographs, the preparations are not only free from particulate or amorphous matter of recognizable host origin, but also freefrom the large reticulated bodies(see Fig. 6).

Emphasis must be given to the fact that the purified preparations contain exclusively elementary bodies, in contrast to the preparations of Jenkin (1960) and Moulder (1962). Moulder (1962) mentioned that purified MPV suspensions nearly

always contained two large reticulated bodies for each small elementary bodies, and that suspensions of a single type of virus particle have not yet been analyzed.

Zahler and Moulder (1953) first reported that the closely related feline pneumonitis virus contained both the RNA and DNA. Jenkin (1960) and Rappaport et al. (1960) also reported that both types of nucleic acids were present in purified preparations of the MPV. In their studies the two morphological types of the MPV contributed to the nucleic acids since the preparations contained both types of particles, elementary bodies and reticulated bodies. The study under project shows that the nucleic acids detectable by chemical analysis of P^{32} distribution are RNA and DNA, and our data preclude the presence of reticulated bodies in the purified preparations. Therefore, it is strongly suggest that both the RNA and the DNA of the purified preparations are intrinsic to smaller elementary bodies associated with infectivity.

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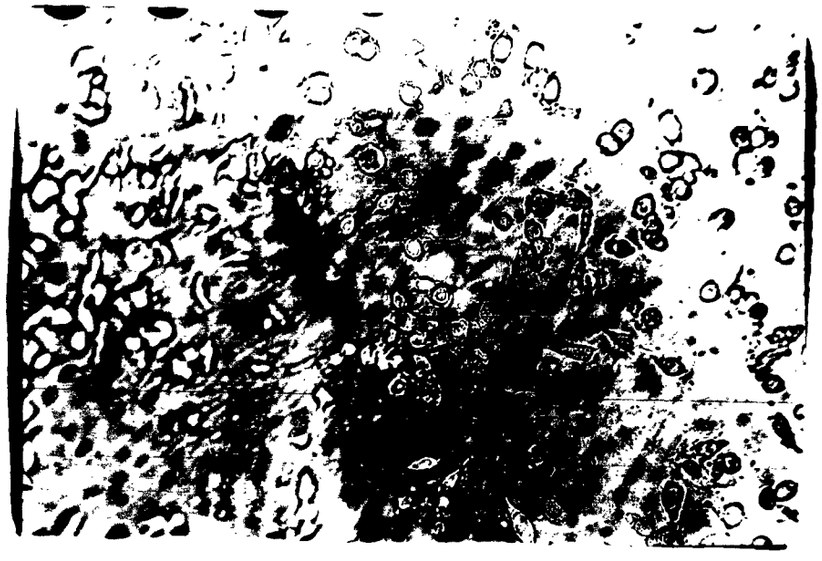

Fig. 1. Culture of Hela cells 5 days after inoculation of trachoma virus showing cytopathic degeneration.

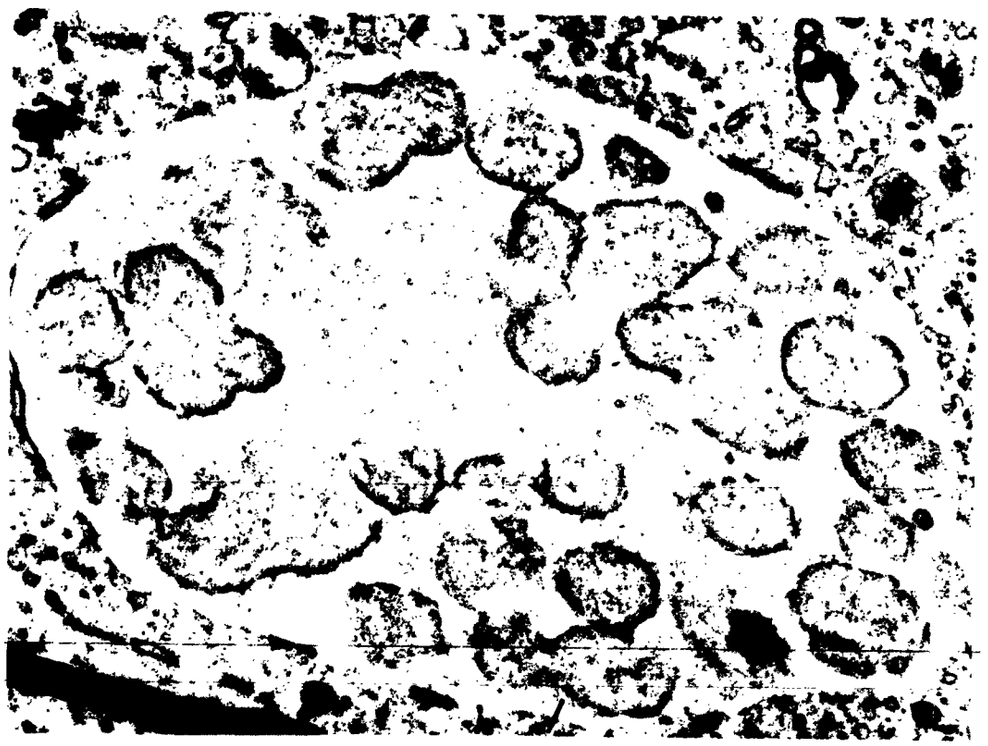

Fig. 2. Electron micrograph of emergence of reticulated bodies of trachoma virus within the matrix area formed 15 hours after infection. x 10,000.

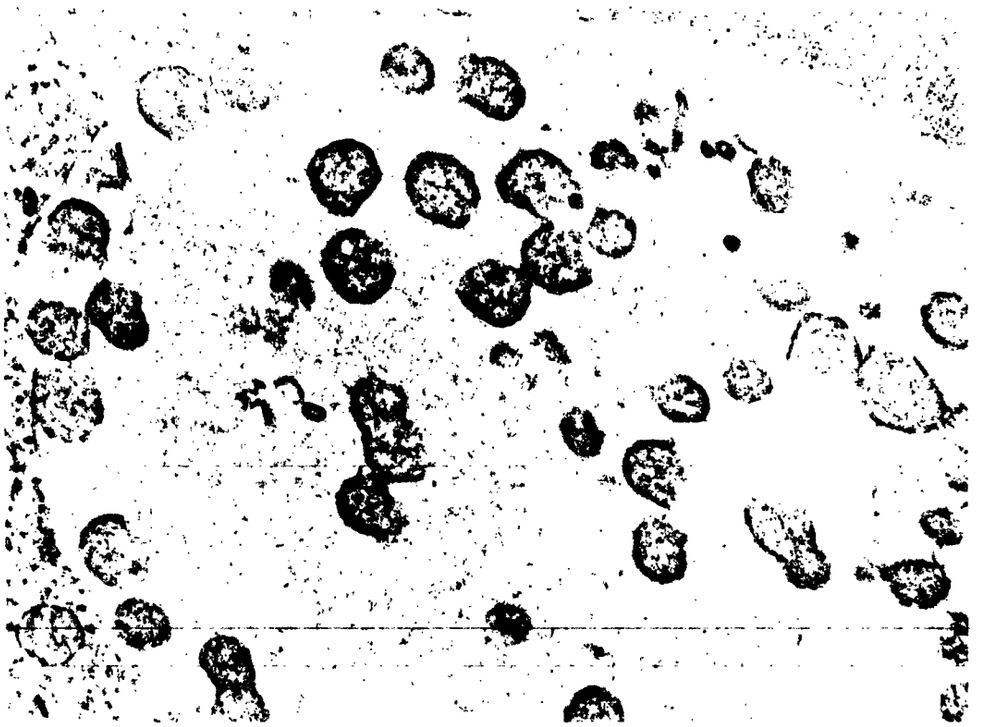

FIG. 3. Reticulate bodies of trachoma virus in the 15 hours after infection. × 17,000. microns

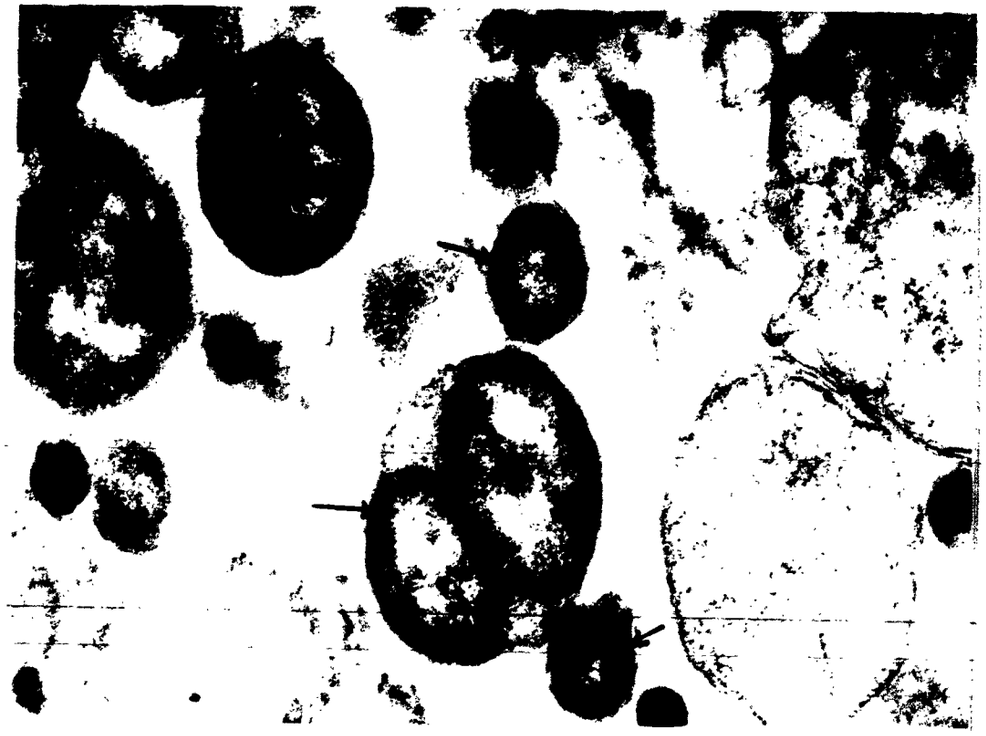

Fig. 4. Reticulated bodies showing marginal condensation (arrows). Two daughter bodies developing in mother bodies are seen in the lower middle part of the figure. x 36,000.

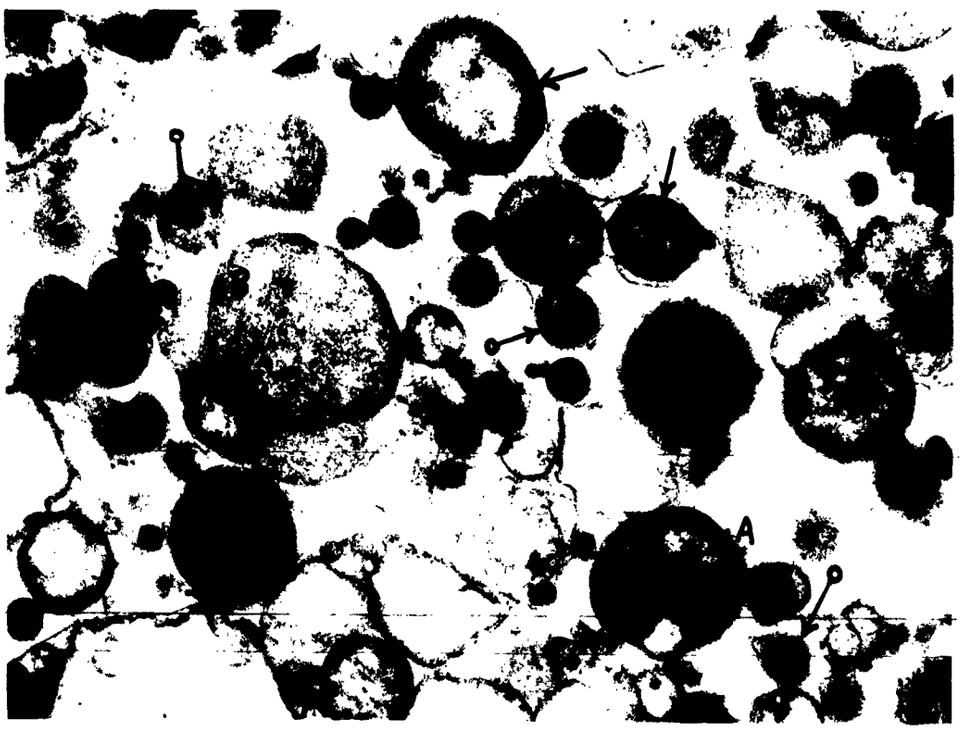

Fig. 7. A portion of HeLa cell 40 hours after infection, illustrating extensive virus development, a common finding at this stage of infection. Note the dense centered well maturated elementary bodies, reticulated bodies showing marginal condensation (arrows) and nucleoid differentiation (arrows with circle). In A and B there develop two daughter reticulated bodies in each mother body. X 20,000.