MORPHOLOGY OF CYTOMEGALOVIRUS (SALIVARY GLAND VIRUS)

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ABSTRACT

SMITH, KENDALL O. (Baylor University College of Medicine, Houston, Texas) AND LUCY RASMUSSEN. Morphology of cytomegalovirus (salivary gland virus). J. Bacteriol. **85:**1319–1325. 1963.—The size and fine structure of cytomegalovirus was compared with that of herpes virus. Cytomegalovirus stains as a DNA virus and is almost identical in size to herpes virus. Both viruses have enveloped forms and display protein membrane subunits which are almost identical in number, size, and arrangement. Cytomegalovirus closely resembles herpes virus produced early in its growth cycle (9 hr) in that most of the particles are coreless and somewhat ragged in appearance. The particle-infectivity ratio with cytomegalovirus is extremely high, approximating 10^7 to 10^8. Although the average yield per cell was approximately 10,000 particles, only about one infectious unit per 1,000 cells was obtained. The biological explanation for this inefficiency of infectious virus synthesis is presently unknown, but the physical explanation appears to be that the nucleic acid cores are not complete. Not one particle was found, among over 1,000 examined, which stained as if it had a complete core.

The human cytomegalovirus, often called salivary gland virus, causes a generalized infection of newborn infants. The virus has been isolated and propagated in tissue cultures in several laboratories (Smith, 1959: Weller et al., 1957; Rowe et al., 1956). The virus appears to be specific for human fibroblast cultures and requires adaptation to tissue culture by serial passage. Several days are usually required after inoculation before substantial numbers of cells show cytopathic effects. Biological titrations indicate that this agent has a low degree of infectivity for the tissue-culture systems employed. Smith (1959) observed (80 to 120 mμ) virus-like particles in thin sections of infected tissue-culture cells, but the fine structure of cytomegalovirus is still unknown. Recent advances in virus particle staining technique (Smith and Melnick, 1962*a*) have made it possible to determine the actual number of virus particles and the fine structure of the virus in the same crude virus preparation. This study utilizes that technique to compare the structure of cytomegalovirus with herpes simplex virus. Some preliminary quantitative data are also presented.

MATERIALS AND METHODS

Two different strains were used for examination. The first was isolated from an 8-week-old male with a clinical diagnosis of cytomegalic inclusion disease. Positive isolations in this case were made from the urine sediment and from a liver biopsy. The second strain was isolated from a post-mortem kidney. All isolations were made in cultures of human fibroblast tissues derived from embryonic lung cells. The incubation temperature was 37 C. The virus employed for electron microscopy had been passed serially four to six times in tissue cultures. The agent was harvested several days postinoculation, when 25 to 50% of the cultures showed cytopathic changes. The agent was neutralized by convalescent sera from the child, but not by antiherpes serum. (The cytomegalovirus strains described in this report are two of several isolated as part of a collaborative study on latent viruses in children which is being carried out between M. Benyesh-Melnick's group in the Department of Virology and Epidemiology and Henry Rosenberg and Donald Fernbach of the Department of Pediatrics, Baylor University College of Medicine, Houston, Texas.)

Infected tissue-culture cell suspensions were treated for 10 sec in a 9-kc Raytheon sonic oscillator (model S-102A) or frozen-thawed one time to disrupt the infected cells, enzyme-treated for 10 to 20 min at 37 C to partially digest cell debris, diluted appropriately in saline, and sedi-

mented in the ultracentrifuge upon agar blocks (Smith and Benyesh-Melnick, 1961). They were pseudoreplicated in either 0.5% potassium phosphotungstate (pH 7.0) or 0.25% unneutralized uranyl acetate (Smith and Melnick, 1962*a*). Electron micrographs were taken in an RCA-EMU 3-F microscope. Particle counts and calculations were done in the manner described by Smith and Benyesh-Melnick (1961), except that virus staining was substituted for metal shadowing (Smith and Melnick, 1962*a*). The herpes virus used for morphological comparison with cytomegalovirus was obtained directly from a herpetic lesion (Smith and Melnick, 1962*b*) and from human lung-cell tissue culture passages of three recently isolated herpes strains.

RESULTS

Figure 1 shows selected fields of cytomegalovirus particles negatively stained with potassium phosphotungstate. It can be seen that each particle is penetrated by the stain, which indicates a partial or complete absence of nucleic acid in the core area (Brenner and Horne, 1959; Wildy, Russell, and Horne, 1960; Smith and Melnick, 1962*a*). Capsomerelike substructure is evident around the less densely stained periphery of the particles. The number and spacing of the subunits seen around the periphery of the herpes and cytomegalovirus particles are approximately the same (22 to 23 capsomeres), which suggests a close similarity in their total number and arrangement in the two viruses. One particle in Fig. 1 is less densely stained and more of the capsomeres can be seen. The average distance between capsomeres, measured from center to center, was found to be about 125 A. The diameter of the hole in the capsomere was about 40 mμ, the same as that of herpes. The subunits closely resemble the capsomeres seen in the predominantly coreless herpes virus particles produced early in the growth cycle (Smith, *in preparation*), and prepared in the same way (Fig. 2). The average diameters of the phosphotungstate-penetrated cytomegalovirus and herpes virus particles were almost identical, measuring about 113 mμ. Many of the cytomegalovirus particles were somewhat ragged in appearance and displayed various degrees of fragmentation. Some of the particles contained an inner ringlike structure (Fig. 1, black

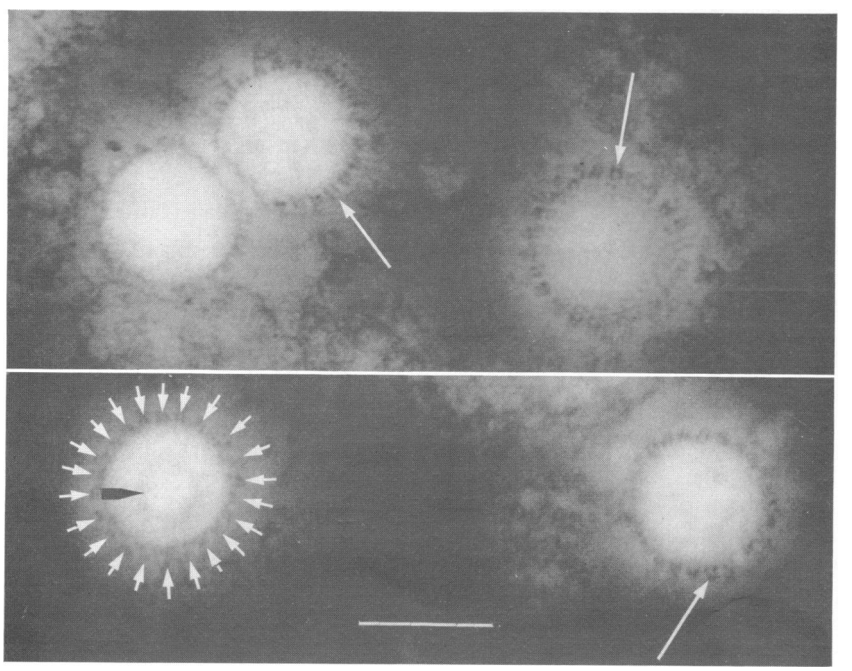

FIG. 1. *Phosphotungstate negative stain of cytomegalovirus showing core penetration of all particles. White arrows indicate capsomerelike substructure around less densely stained periphery of particles; black arrow indicates inner ringlike structue. Micron marker is 100 mμ in all figures.*

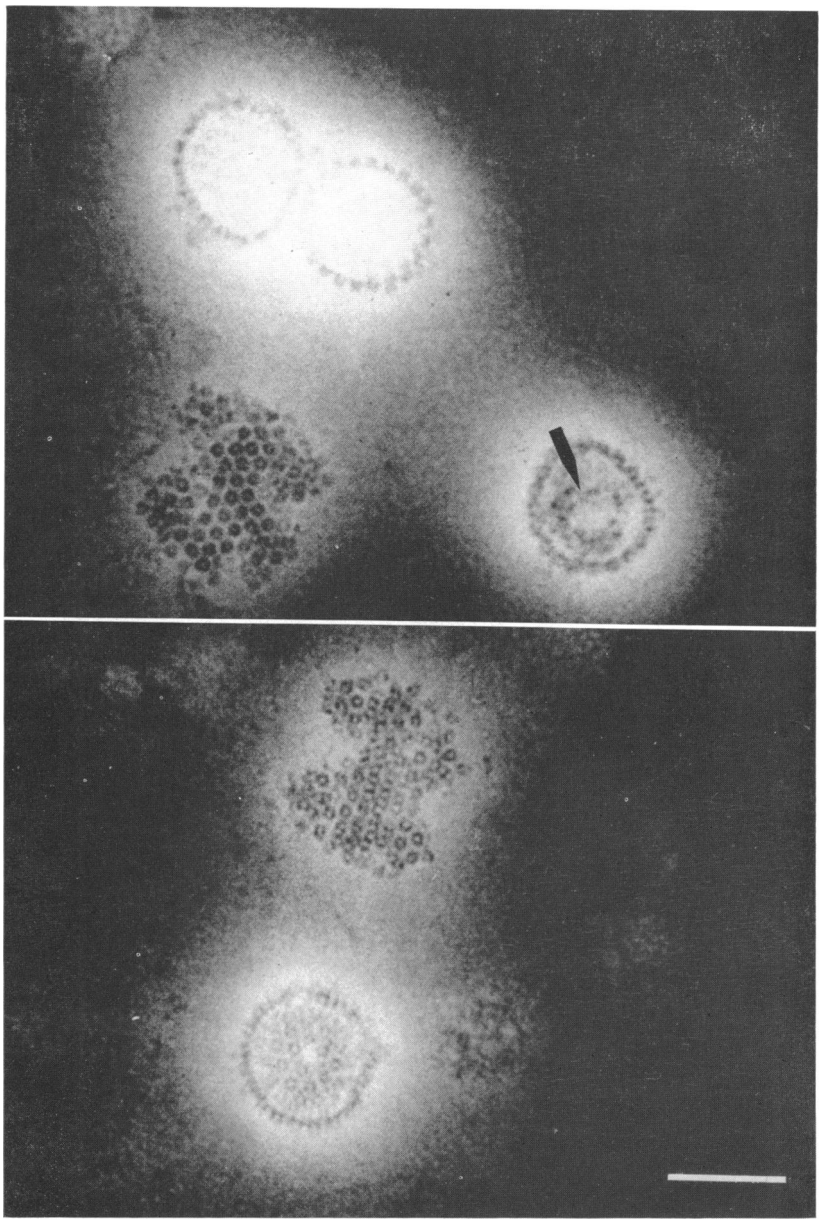

FIG. 2. *Phosphotungstate negative stain of herpes simplex virus typical of that obtained 9 hr postinfection, showing core penetration of most particles. Black arrow indicates ringlike structure.*

arrow). Similar ringlike structures are seen in herpes virus particles (Fig. 2, black arrows).

Uranyl acetate stains the cores of several deoxyribonucleic acid (DNA) animal viruses quite intensely with very mild treatment (10 to 20 sec in 0.2% aqueous solution; Smith and Melnick, 1962*a*, *b*). Figure 3 shows a field of cytomegalovirus particles stained in this way. Many particles show partial staining in the core area, but an examination of several hundred particles revealed none in which the core area appeared completely stained. Figure 4 shows a field typical of 9-hr postinoculation herpes virus particles similarly treated for comparison. The structural similarity

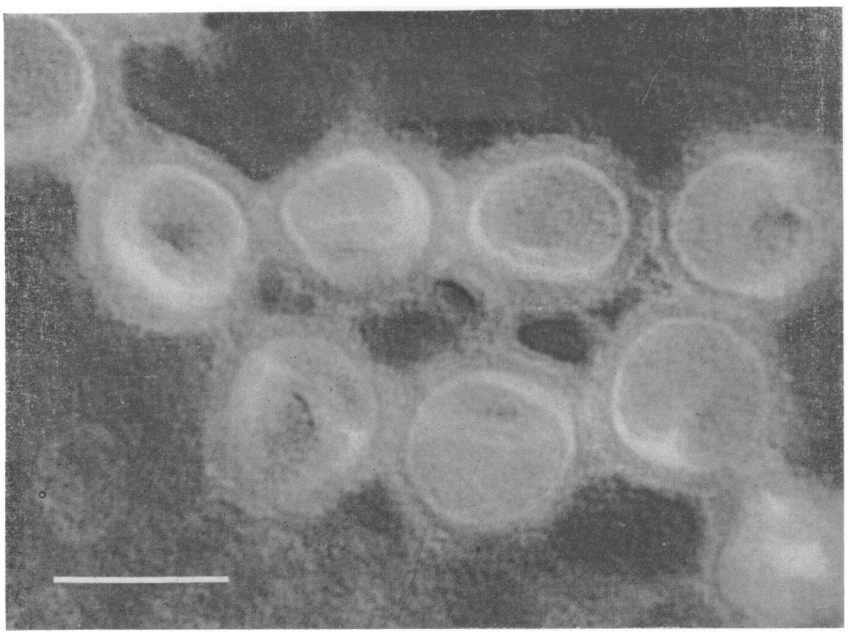

FIG. 3. *Uranyl acetate stain of cytomegalovirus showing incomplete staining of cores.*

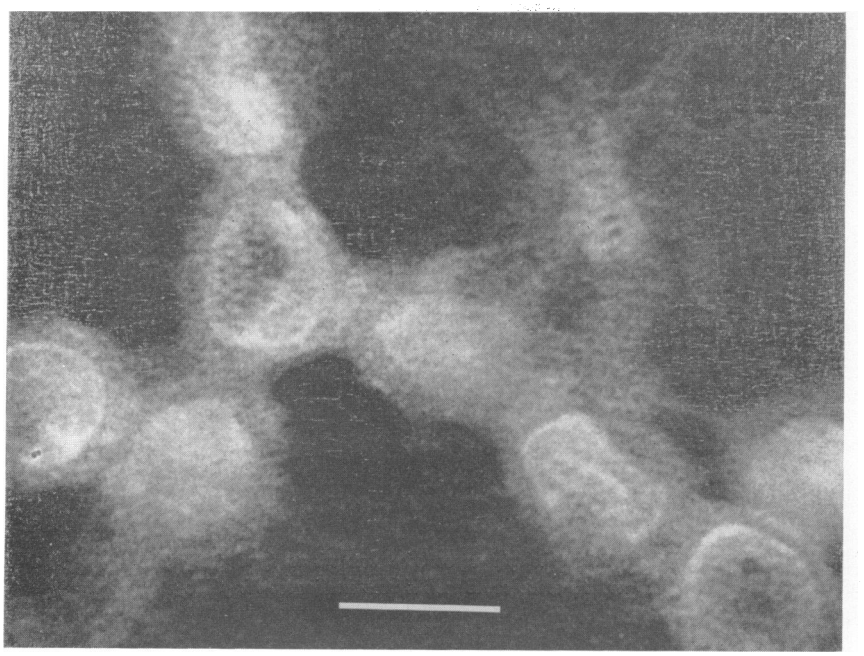

FIG. 4. *Uranyl acetate stain of 9-hr herpes simplex virus showing incomplete staining of cores.*

between the two viruses is apparent. A large fraction of herpes virus taken at the peak of the growth cycle (20 hr) or obtained directly from vesicular lesions usually stains intensely in the core area when treated with uranyl acetate (Smith, *in preparation*) as seen in Fig. 5. This is in sharp contrast to the predominantly unstained often ragged-looking particles seen 9-hr post-

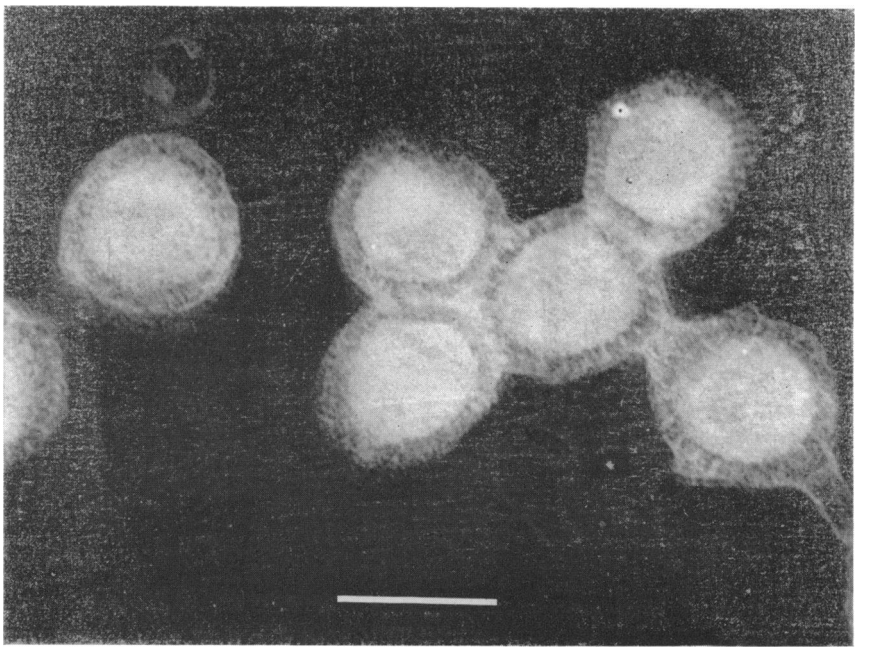

FIG. 5. *Uranyl acetate stain of herpes simplex virus showing complete staining of cores.*

inoculation (Fig. 4). The average diameters of cytomegalovirus and herpes virus after uranyl acetate treatment were almost identical, averaging 108 mμ. The average diameter of the partially stained cores of both herpes virus and cytomegalovirus was 77 mμ. The cytomegalovirus particles shown in Fig. 1 and 3 are hardly distinguishable morphologically from similarly stained early cycle herpes virus (Fig. 2 and 4).

Numerous larger bodies were seen in cytomegalovirus preparations which varied considerably in size, measuring between 180 and 300 mμ. Some of these were seen to contain one or more virus particles. Particles within these envelopes were usually difficult to see because of the density of the envelopes. These structures closely resemble the phosphotungstate-stained envelope forms described by Wildy et al. (1961) for herpes virus.

The particle concentrations in sonic-treated or frozen-thawed disrupted cytomegalovirus-infected cell suspensions were determined. The conditions used for cell rupture and virus release were the mildest possible so as to minimize physical damage to the virus. No morphological or quantitative differences between sonic-treated and freeze-thaw released virus particles were seen. Particle counts were not precise, because many particles were obviously fragmented or so incomplete as to be difficult to recognize. Only clearly defined particles possessing the structure described above were counted, so the concentration estimates are less than the true values. The particle concentration in crude tissue culture materials approximated 10^{10}/ml, and the infectivity titers in different samples ranged between 10^2 and 10^4/ml. These concentrations were observed in suspensions containing the debris of approximately 10^6 cells/ml. Therefore, the yield per cell was about 10,000 particles, but only 0.001 infectious unit. This means that only 1 in 1,000 cells made a mature infectious particle. Some of the difficulties in regard to passage and precise biological characterization of this virus become apparent.

Discussion

The staining reaction similarities and physical resemblance of cytomegalovirus to herpes virus are such as to indicate that they should be grouped in the same classification. Andrewes et al. (1961) already suggested that cytomegalovirus be classified with herpes in the Nita group, because it was thought to be a DNA virus, is 100 to 200 mμ in diameter, contains essential lipids, multiplies

within the nucleus, and does not seem to undergo a maturation phase at the cell membrane. The morphological findings here give additional support to classifying cytomegalovirus with the herpes group. Although no cytomegalovirus particles were found which appeared to possess structurally complete cores, the staining reactions with phosphotungstate and uranyl acetate strongly suggest that its nucleic acid is DNA. Since every particle we examined was partially or completely penetrated by phosphotungstate, it was difficult to observe the entire capsomere arrangement. We conclude from micrographs, however, that cytomegalovirus possesses 162 capsomeres in an arrangement identical or similar to that of herpes virus.

The infectivity titers obtainable with cytomegalovirus suspensions described in this study have been between 10^2 to 10^4 ID_{50}/ml. The ratio of particles to infectious units is therefore on the order of 10^6 to 10^8, an unusually large figure. This high ratio is almost certainly due in part to the incompleteness of the nucleic acid core, which is apparent in the electron micrographs.

It is significant that not one particle was found which possessed a completely filled core. Infected cells produce extremely large numbers of protein coats with incomplete cores. Thus, the efficiency with which particles containing a full complement of nucleic acid are produced is very poor. The resemblance of cytomegalovirus to the first viral structures observed in the herpes virus growth cycle suggests that cytomegalovirus production is imperfect in one of the earlier phases. This may be due to a failure to produce essential components in proper relative amounts or to a failure to assemble these components after they are formed.

The particle-infectivity ratio of rabbit papilloma virus is also 10^7 to 10^8 (Bryan and Beard, 1940). The reason for this extremely high figure is not clearly understood, although it seems likely that host sensitivity may be a factor. A single crude preparation of rabbit papilloma virus which we examined contained over 10^{11} particles/ml, although the infectivity was only about 10^3/ml. (This preparation was kindly assayed and supplied to J. L. Melnick by Charles A. Evans, Department of Microbiology, University of Washington College of Medicine, Seattle.) This confirms the estimates made by Bryan and Beard (1940) on the basis of particle weight. Both phosphotungstate (negative) and uranyl (positive) staining showed that the cores of most of these papilloma particles were quite complete. It is interesting that the human cytomegalovirus and the rabbit papilloma virus both have extremely high particle-infectivity ratios, but probably for different reasons.

Studies are now under way to find conditions which will permit more efficient growth of cytomegalovirus. An attempt will be made to correlate any increases in biological activity with changes in the physical appearance of the particles.

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