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Nonviral Microbodies with Viral Antigenicity Produced in Cytomegalovirus-Infected Cells

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Masses of homogeneous electron-dense material accumulate in the cytoplasmic inclusions of cultured fibroblasts which have been infected with "wild" and "adapted" strains of human cytomegalovirus. The substance appears to be produced by microtubular membranes and the Golgi apparatus; ultrastructural histochemistry suggests that it is not lysosomal in nature nor is it comprised of lipids or polysaccharides. The dense material "buds" into cytoplasmic tubules forming circumscribed bodies having an investing membrane similar to the viral envelope. After transport to the extracellular milieu in cytoplasmic tubules and vesicles, virions and dense bodies can be demonstrated by immune electron microscopy. The homogeneous dense body appears to be a unique product of the cytomegalovirus-infected cell which possesses a limiting membrane having antigenic determinants common with the viral envelope.

Irregular masses of homogeneous dense material have been noted repeatedly in electron micrographs of the cytoplasm in cells infected with human cytomegalovirus (CMV) (2, 10, 11, 12, 14, 15). The nature and biological significance of this nonviral electron opaque substance has received surprisingly little attention despite its consistent presence in cells exhibiting cytopathic alterations. We report here ultrastructural studies concerned with the synthesis and composition of the material and its disposition in the cell. The material appears to be produced in the cytoplasm where it is invested by membranes to form circumscribed homogeneous dense bodies which possess viral antigens. These structures then are transported to the extracellular milieu in cytoplasmic tubules and vesicles.

MATERIALS AND METHODS

The AD-169, Davis, and Esp strains of human CMV were supplied by W. P. Rowe and T. E. Weller. These viruses had been passaged repeatedly in human fibroblast cell cultures and produced relatively large amounts of extracellular infectious virus. A "wild" strain (1974) was present in the pulmonary tissue of a renal allotransplant recipient with cytomegalic inclusion disease (patient D. F.) (5). Although the titer of this lung was $\geq 10^6$ tissue culture infectious dosages, 50% effective ($TCID_{50}$/g), little extracellular infectious virus was produced by cell cultures inoculated with the human material. Many of the ultrastructural features of the replicative sequences of these strains of CMV in human diploid fibroblast cultures (WI-38) have been described in detail elsewhere (8).

Electron microscope studies were carried out on WI-38 cells which had been inoculated with $10^{4.5}$ $TCID_{50}$ of each of the virus strains. Transmission microscopy was done on thin sections of cells which had been grown as monolayers in plastic dishes. At intervals after inoculation, cultures were fixed in situ with 2% glutaraldehyde. In most studies, the cell monolayers were postfixed in buffered 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in epoxy resins (8). After polymerization, the plastic dish was peeled from the Epon block and foci of cytopathic effect in the monolayer were identified by light microscopy. These areas were cut from the larger block and glued to plastic blanks for thin section preparation. This technique permitted the selection of localized sites of cytopathology in a monolayer which exhibited predominantly unaltered fibroblasts.

Uranyl acetate and lead citrate were used routinely for the staining of thin sections. A periodic acid-methamine silver stain to detect alpha glycols in polysaccharides was carried out by the method of Jones (7). Acid phosphatase reactivity was determined by the technique of Erickson and Trump (6).

Negative staining was carried out on supernatant culture medium and homogenized preparations of infected WI-38 cells which exhibited extensive cytopathic changes (infectivity titers $\geq 10^5$ $TCID_{50}$). Virus particles were concentrated by centrifuging the preparations for 30 min at 20,000 $\times$ g. The deposits then were stained with 3% phosphotungstic acid at pH 7.0 and layered onto grids which had been coated with Formvar and carbon. For immune electron microscopy, human sera with a CMV CF titer of 1:64 (patient D. P.) (4, 5) was diluted 1 to 20 in 0.85% NaCl and heat inactivated (56 C for 30 min). Equal

quantities of the virus preparation and dilute serum were incubated together for 60 min at 37 C and then held at 4 C for approximately 18 hr (1). The mixture was centrifuged for 30 min at 20,000 × *g*, and the pellet was negatively stained in the manner described above.

Philips 300 electron microscopes were used in all studies.

RESULTS

Cytopathic changes developed in fibroblast cell cultures 48 to 96 hr after inoculation of the four virus strains. By 120 hr distinctive nuclear and cytoplasmic inclusions were evident. In a previous publication we described in detail the ultrastructural features of the several morphological types of cytoplasmic inclusions in cells infected with CMV (8). In this report attention will be directed specifically to the homogeneous electron-dense material which accumulates in these inclusions in association with CMV virions.

The largest and most distinct inclusion in the cytoplasm of infected cells (Fig. 1a and b) was comprised of smooth endoplasmic reticulum and vesicles of differing sizes. Varying numbers of viral particles and circumscribed dense bodies, as well as the masses of homogeneous electron-dense material referred to above, also were present. The latter substance appeared to form in the cytoplasmic matrix in proximity to microtubular membranes and the Golgi apparatus (Fig. 2). It assumed an array of configurational forms, often being surrounded by, or intimately associated with viral capsids and nucleocapsids (Fig. 3). Although the material was not invested by a limiting membrane, it frequently impinged upon tubules, many of which contained virions within their lumina (Figs. 3 and 4).

Nucleocapsids acquired an enveloping membrane by "budding" into tubules in the cytoplasmic inclusion (Fig. 5). Masses of the homogeneous dense material also budded into these tubules in a fashion similar to the viral particles (Figs. 6a and b). Thus, they acquired a limiting membrane which was demonstrable using the periodic acid-methenamine silver strain (Fig. 4). Within the tubules, these structures, termed homogeneous dense bodies, exhibited an ovoid or spherical configuration. They varied in size but usually were two or three times the diameter of the enveloped virions. With time the ultrastructural features of the cytoplasmic inclusions seemed to change inasmuch as homogeneous dense bodies and virions in tubules were the prominent components (Fig. 7).

A cup-shaped depression invested by seemingly loose, membrane fragments frequently was evident at a pole of the homogeneous dense bodies (Fig. 8 and 9). It would appear that the depression was formed as the dense material protruded into the tubular lumen, and the membrane fragments were derived from the wall of the tubule. Alternatively, virions occasionally abutted on a pole of the budding dense body in a fashion which suggested that the depression was a "cast" of the viral particle (Fig. 9).

The electron-dense material in the cytoplasmic matrix and tubules of glutaraldehyde-fixed cells failed to exhibit acid phosphatase enzymatic activity in repeated studies (Fig. 10) and was not removed by lipid solvents (Fig. 11).

Numerous vesicles usually were evident in the cytoplasm of infected cells 120 hr after virus inoculation. These structures, which appeared to be sites of dilation of the cytoplasmic tubules, contained enveloped virions and membrane-bound homogeneous dense bodies as well as debris (Fig. 12). Thin sections of cultured cells at this time revealed extracellular virions and abundant numbers of homogeneous dense bodies (Fig. 13).

The sediments prepared from the cell-free medium and homogenates of cultures infected with the AD-169 and Davis strains revealed virions and nucleocapsids when examined by negative staining. These structures were scattered randomly throughout the specimen, and only occasionally could two be found in the same field (Fig. 14). Homogeneous dense bodies were not identified with certainty because of the presence of amorphous fragments of cell debris.

When immune serum was mixed with the same preparations, the sediments exhibited aggregates of enveloped virions and variable numbers of large opaque bodies having a granular external membrane similar to the viral envelope (Fig. 15). These latter structures are believed to be identical

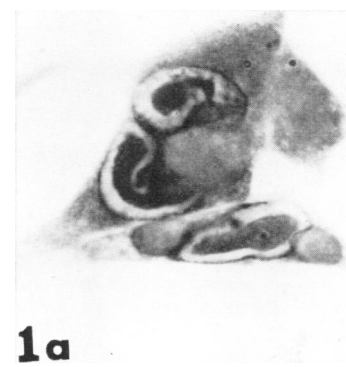


FIG. 1a. *CMV (1974 strain)-infected cells with dense nuclear inclusions and pale-staining cytoplasmic inclusions 148 hr after inoculation. Hematoxlin and eosin stains.* ×*1,400.*

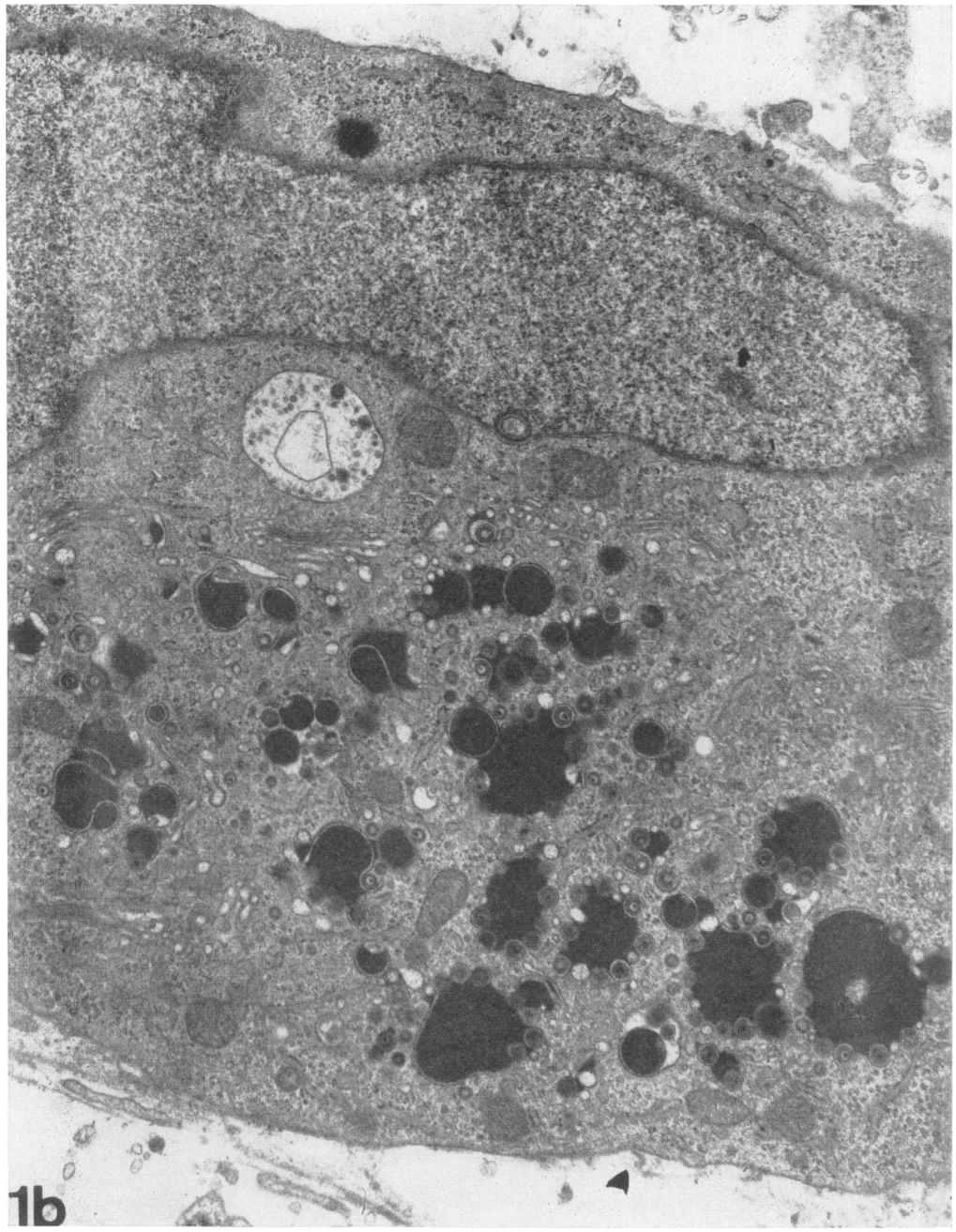
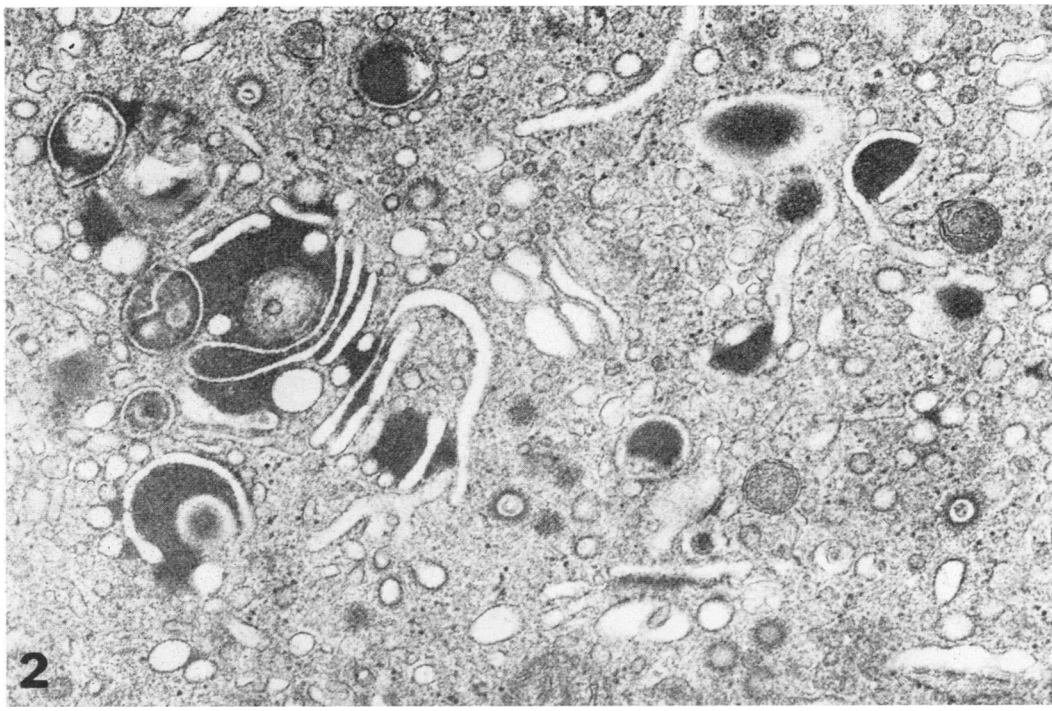


FIG. 1b. *Homogeneous masses of electron-dense material, viral particles, and microtubules comprise the cytoplasmic inclusion. Golgi apparatus is situated at the periphery.* ×*10,300.*

to the homogeneous dense bodies because of (i) their consistent presence in the cell-free medium of infected cultures but not of uninfected cultures and (ii) their relative size relationship to intact virions.

Aggregates made up exclusively of nucleocapsids also were demonstrable by immune electron microscopy (Fig. 16). These complexes always were segregated from the aggregates comprised of enveloped virions and dense bodies.



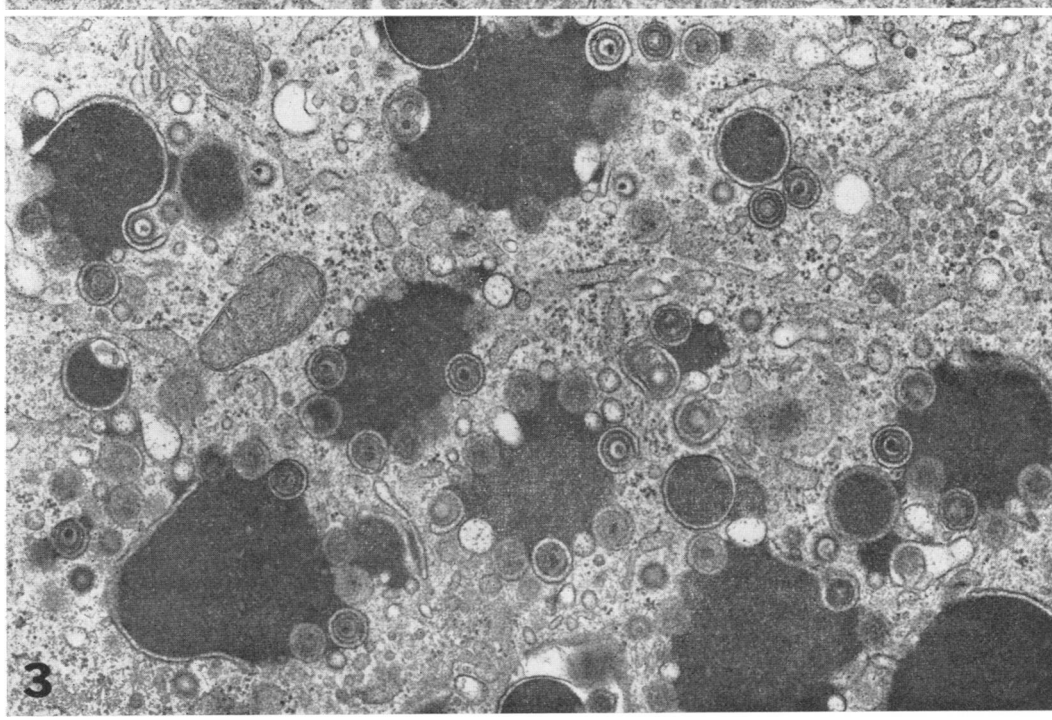
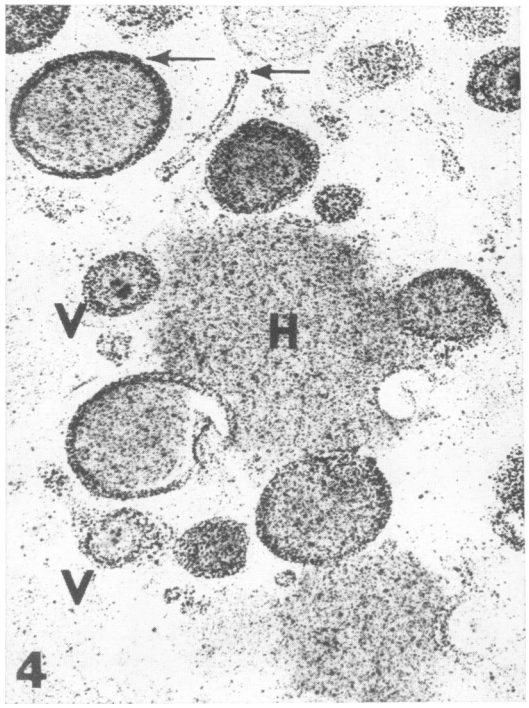
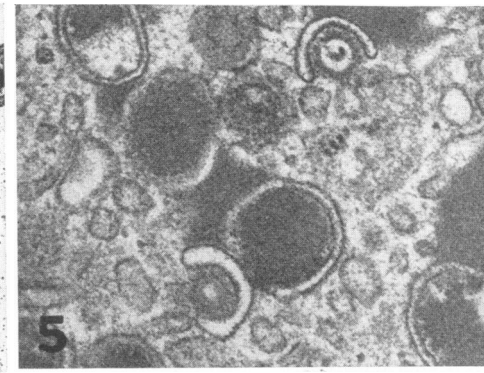
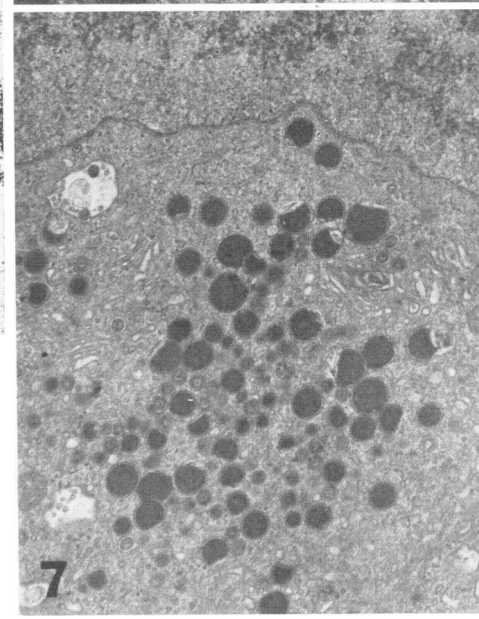


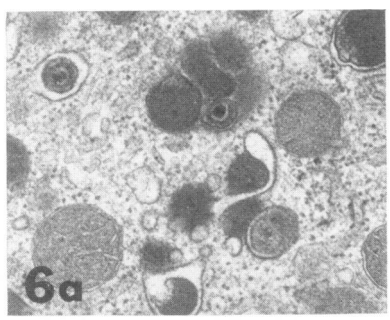
FIG. 2. *Golgi region in CMV (1974 strain)-infected cell 96 hr after inoculation. The homogeneous dense material has accumulated adjacent to membranes.* ×*33,000.*

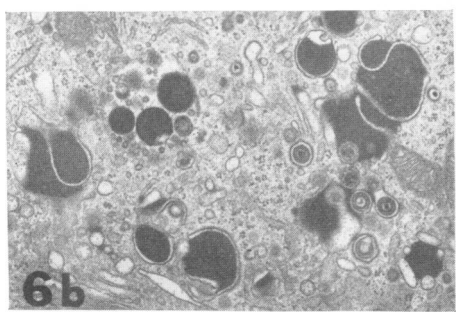
FIG. 3. *Accumulations of dense material in the cytoplasmic matrix of CMV (1974 strain)-infected cell 148 hr after inoculation. Note its intimate association with enveloped virions in tubules and the nucleocapsids free in the cytoplasm.* ×*24,200.*











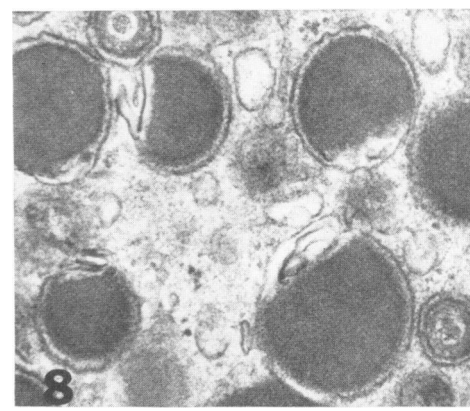


FIG. 4. *Dense material (H) in the cytoplasmic matrix of this CMV (AD-169 strain)-infected cell failed to stain with methenamine silver after periodic acid digestion. Note the heavy silver deposits in the walls of tubules (arrow). Virions (V). ×37,500.*

FIG. 5. *"Budding" of nucleocapsids into cytoplasmic tubules of this CMV (1974 strain)-infected cell. ×31,500.*

FIG. 6. *"Budding" of homogeneous dense material into cytoplasmic tubules of this CMV (1974 strain)-infected cells. ×27,000.*

FIG. 7. *Cytoplasmic inclusion in CMV (1974 strain)-infected cell 360 hr after inoculation. Homogeneous dense bodies and virions in tubules are prominent in contrast to the inclusion illustrated in Fig. 1b. ×8,400.*

FIG. 8. *Homogeneous dense bodies in cytoplasmic tubules of a CMV (1974 strain)-infected cell. Note the reflected membrane fragments at one pole. ×33,000.*

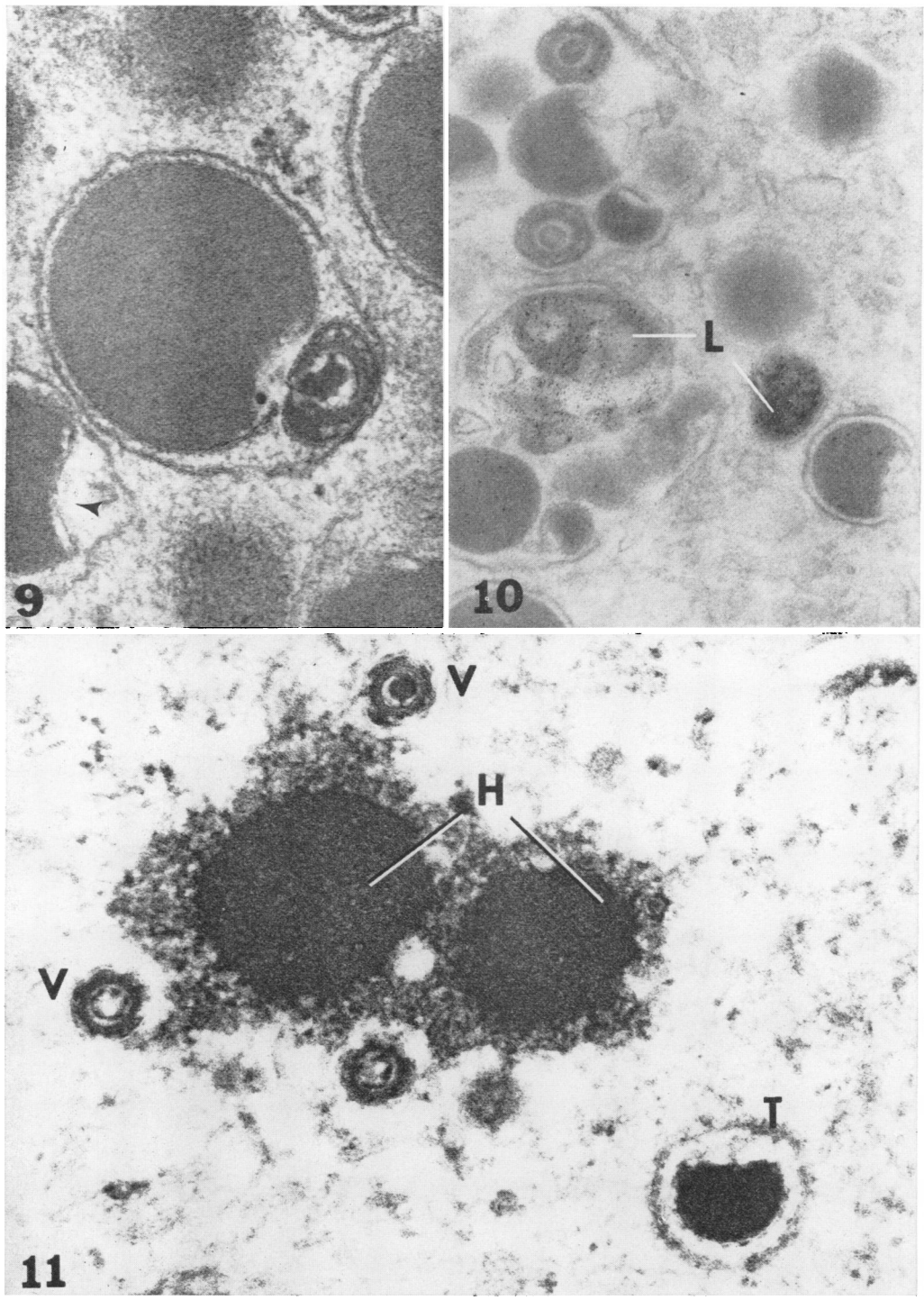
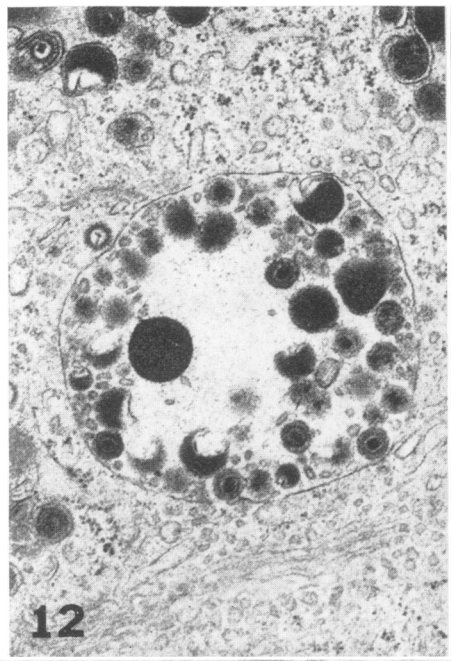
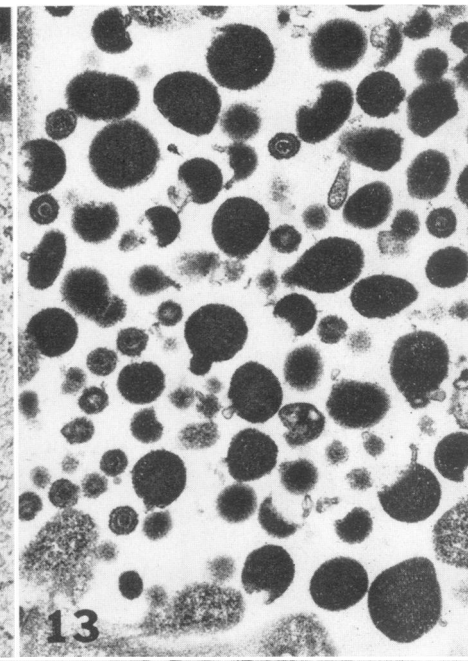


FIG. 9. *A homogeneous dense body in a cytoplasmic tubule of a CMV (AD-169 strain)-infected cell. Note the double laminate membrane of the homogeneous dense body, viral envelope, and tubule. The virion is intimately associated with a pole of the body, a possible explanation for the cup-shaped defect (arrow).* ×51,000.

FIG. 10. *Acid phosphatase activity was demonstrable in lysosomes (L) but was not present in intratubular virions and homogeneous dense bodies of this CMV (AD-169 strain)-infected cell.* ×56,000.

FIG. 11. *The dense material in the cytoplasmic matrix (H) and the tubules (T) was not removed by lipid solvents after glutaraldehyde fixation. In contrast, the membranes of the tubules and the intratubular dense bodies and virions (V) are discontinuous and fragmented indicating an alteration in the lipid component.* ×56,000.





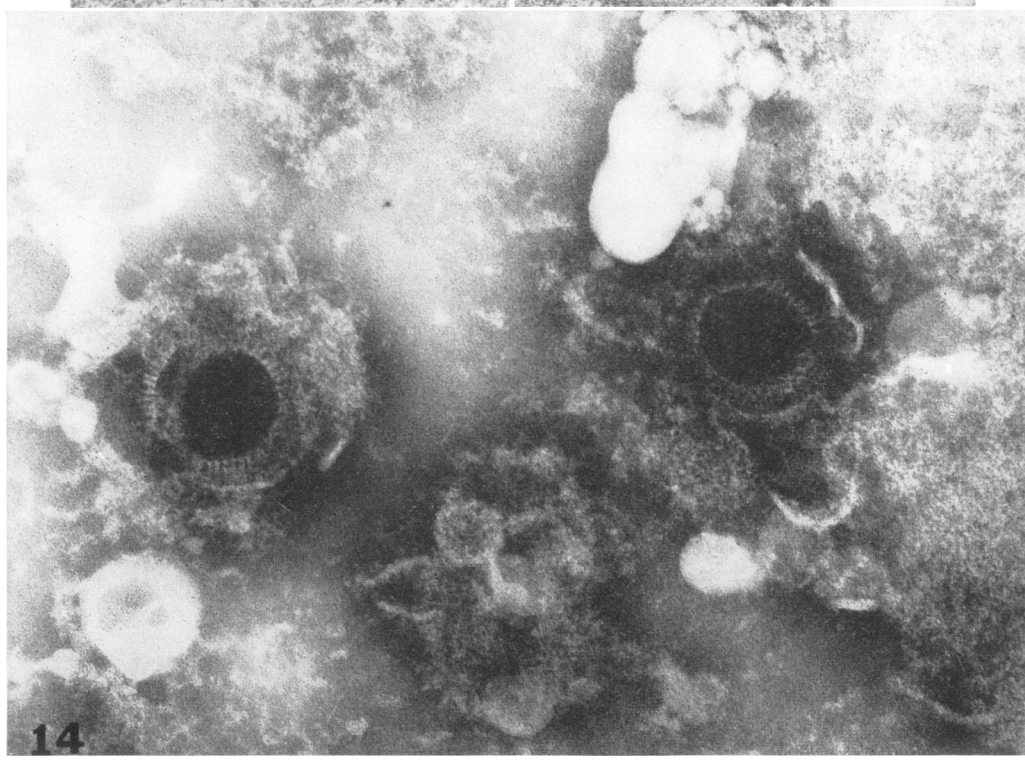


FIG. 12. *Cytoplasmic vesicle of CMV (1974 strain)-infected cell containing enveloped virions and homogeneous dense bodies.* ×*24,500.*

FIG. 13. *Extracellular particles in CMV (AD-169 strain)-infected culture 336 hr after inoculation.* ×*27,000.*

FIG. 14. *Direct negative staining of tissue culture supernatant from CMV-infected cells (AD-169 strain) revealed only occasional randomly scattered particles. This micrograph was unusual inasmuch as two of the particles appeared in the same field. As can be seen CMV displays typical herpesvirus morphology.* ×*200,000.*

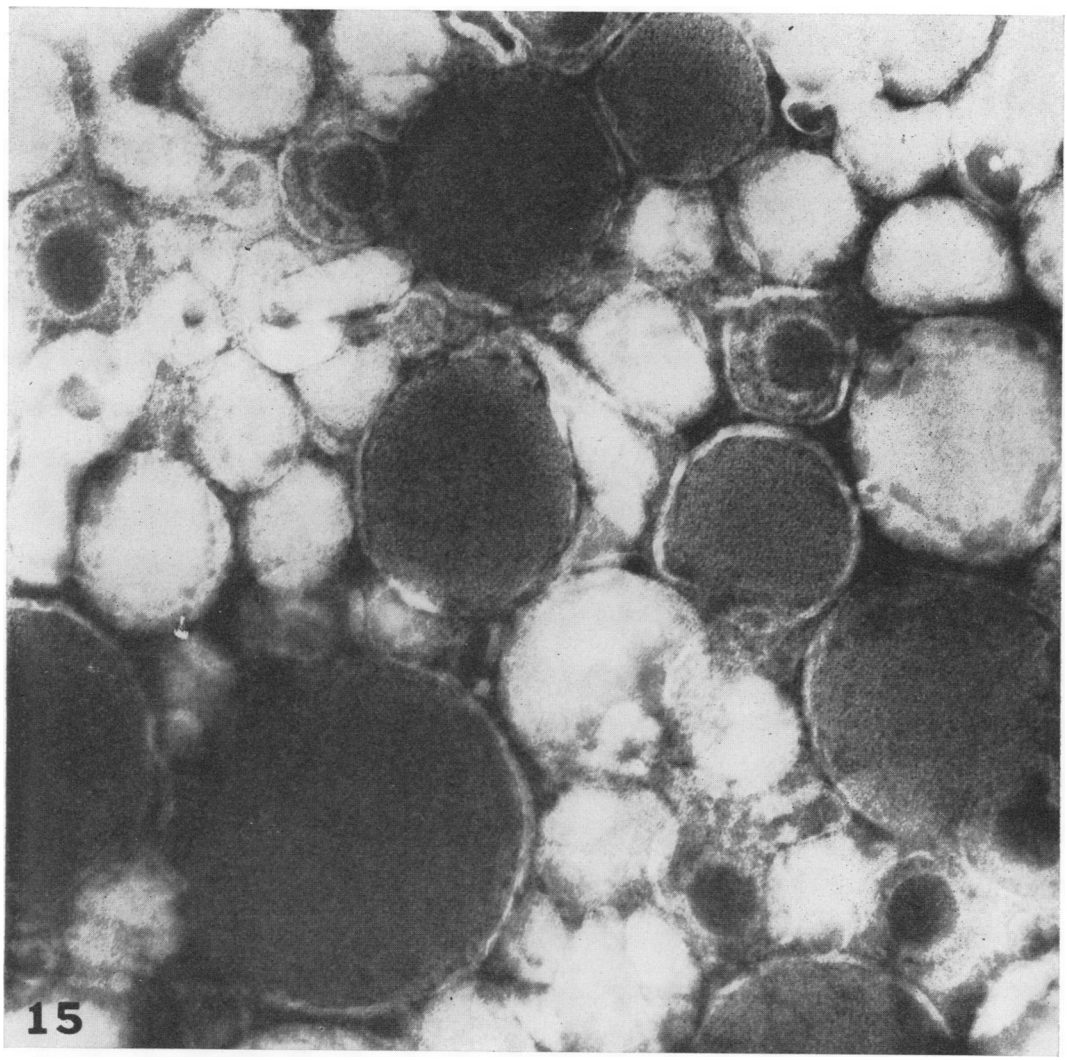


FIG. 15. *When specific antiserum was added to the type of preparation illustrated in Fig. 14, large rafts of virus particles and homogeneous dense bodies could be seen. The fact that these two types of structure were linked together by antibody showed that they shared at least one antigen.* ×*150,000.*

DISCUSSION

The homogeneous electron-dense material which accumulates in the cytoplasm of CMV-infected cells appears to be synthesized by both microtubular membranes and the Golgi apparatus. Within the cytoplasmic matrix it lacks an investing membrane as well as acid phosphatase activity and thus appears not to be lysosomal in nature. The material is not dissolved by lipid solvents and fails to react with the methenamine silver stain after periodic acid digestion. By exclusion it seems likely that the substance is comprised largely of proteins.

The presence of lysosomes in the cytoplasm of CMV-infected cells was reported by earlier workers (11, 14, 15) and documented in this study (Fig. 10). We wish to emphasize that the lysosomal structures which exhibited acid phosphatase activity in our material were morphologically distinct from the accumulations of homogeneous dense material which serve as the subject for this paper. Moreover, the latter substance lacked a limiting membrane when it was found free in the cytoplasmic matrix (Fig. 4). This distinction was not emphasized in the reports of previous investigators. To further assure ourselves that the homo-

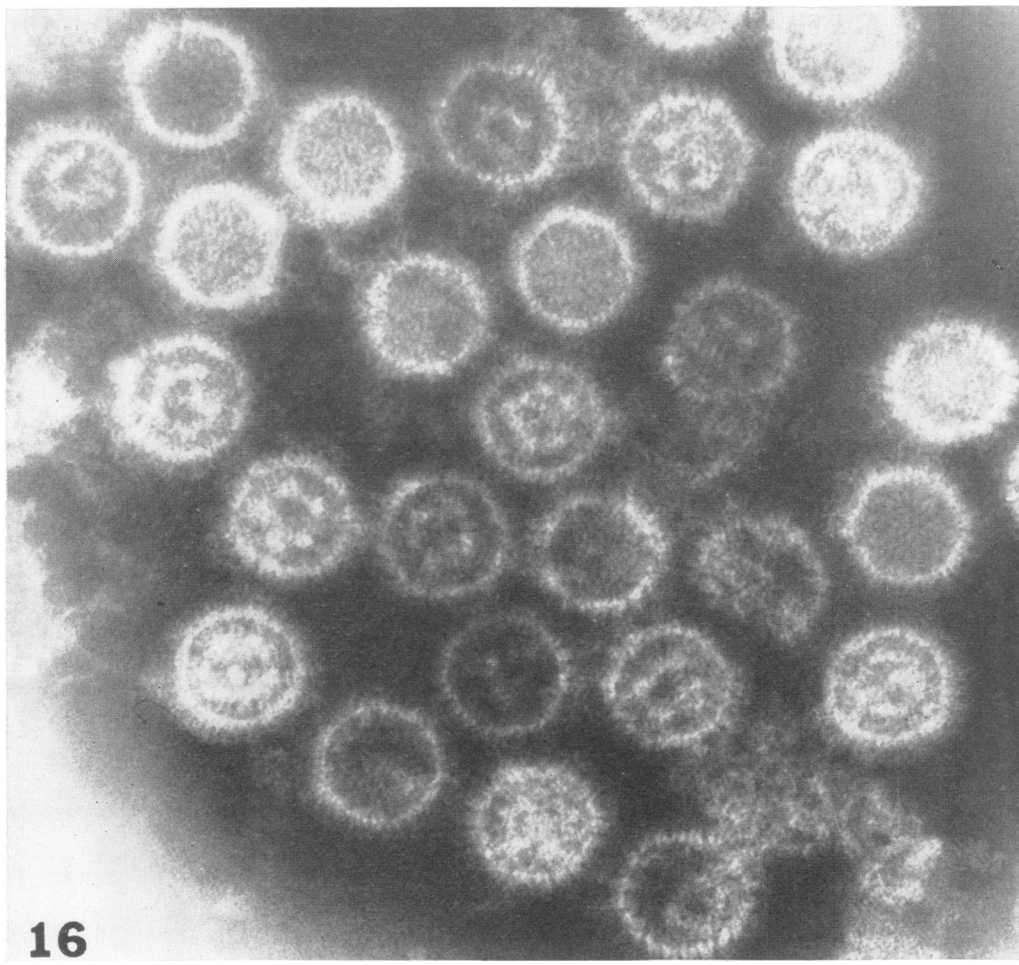


FIG. 16. *In addition to the aggregates of enveloped CMV particles and the homogeneous dense bodies, this preparation also revealed rafts of the capsids and nucleocapsids linked together with antibody. The fact that the two types of aggregates (i.e., Fig. 15 and Fig. 16) were never mixed is an indication of the specificity of the immune reaction as seen in the electron microscope.* ×*200,000.*

geneous dense material was not lysosomal, we treated infected cells with Triton X and by repeated cycles of freezing and thawing to increase membrane permeability before staining for enzyme activity. In addition, efforts were made to increase the sensitivity of the test by varying the concentrations of both the substrate and lead. Under most conditions cytoplasmic structures with acid phosphatase reactivity were present in the cells, but the homogeneous dense material failed to stain.

Periodic acid-Schiff staining material has been described in the cytoplasm of cells examined by light microscopic histochemistry (11, 14, 15). We are uncertain which organelles contain the reactive material demonstrated in these earlier studies. The periodic acid-methenamine silver stain used in our investigation and the work reported by Ruebner (14) specifically reacts with the alpha glycols in cytoplasmic membranes as well as the envelopes of the virions and homogeneous dense bodies. Since increased amounts of glycogen are not observed in infected cells by electron microscopy, it seems likely that the periodic acid-Schiff reactive material which has been demonstrated by light microscopy is located in the membranes which are found in great abundance in CMV cytoplasmic inclusions (8, 9).

As might be expected, the double laminate limiting membrane which invests the homogeneous dense bodies in the cytoplasmic tubules appears to be similar, if not identical to, the viral envelope.

This view is supported by our immune electron microscope observations which show aggregates of recognizable CMV virions linked to the dense bodies, an indication that the virions and homogeneous dense bodies share common antigenic determinants. One must assume from observations such as those illustrated in Fig. 13, that the dense bodies comprise a substantial component of the antigenic mass synthesized by infected cells. Thus, they may serve as a potent immunogen in the naturally infected host, even when little extracellular infectious virus is produced.

Although the stability of the dense bodies is not known, it is likely that their antigen components are detectable in complement fixation tests. Indeed, virus preparations for use in complement fixation and neutralization tests may be comprised of a disproportionately large number of dense bodies and relatively few infectious virus particles. This possibility could account for the discrepancies which frequently are observed when these two tests are employed in the evaluation of human sera for CMV antibodies (4).

Cytoplasmic accumulations of dense material, similar to the substance described in this report, are evident in published electron micrographs of cells infected with other strains of human CMV (2, 10–12), murine CMV (13), varicella-zoster virus (2, 3) and the herpes-type virus of frogs (16). It is suggested in several publications that the material is incorporated into the envelope of the virion as a coat surrounding the nucleocapsid, subjacent to the external limiting membrane. We have not found documented in the literature the production of the enveloped dense bodies described here by viruses other than human CMV.

It is difficult to envision a biological role for the homogeneous dense material based on the evidence accumulated thus far. Its intimate association with nucleocapsids in the cytoplasmic matrix supports the view that the material is associated with a structural component of the coat material which is acquired by the nucleocapsids in the cytoplasm. If so, it would appear to be produced in excess and could serve as a mediator concerned with the process of viral envelopment.

ACKNOWLEDGMENTS

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