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Morphologic Characteristics and Nucleic Acid Type of Transmissible Gastroenteritis Virus of Pigs[1]

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With 11 Figures

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Introduction

The present system for the classification of viruses is based on their chemical and physical properties (25, 30). Information available on the transmissible gastroenteritis (TGE) virus of pigs with regard to these characteristics is fragmentary especially with respect to morphology.

With the exception of one report (47) ultrafiltration studies (8, 9, 43, 27) as well as direct measuring of virus particles (42, 35) have indicated that TGE virus is of medium size and measures between 80 and 100 mμ in diameter.

Ristic et al. (42) reported that particles purified from the intestines of pigs infected with TGE virus had an envelope and cubical symmetry which resembled the members of the herpesvirus group. *Okaniwa* et al. (35) observed in electron micrographs of thin sections of infected cell cultures particles which had an electron dense nucleoid and a limiting membrane with an average diameter of 95 mμ. These particles were found in the cytoplasm and its related membranous system (35).

The sensitivity of this virus to ether (17, 8, 42, 39, 5, 43, 27, 46) indicates the presence of essential lipids.

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Experiments with DNA inhibitors suggested that the nucleic acid of TGE virus is of the RNA type (5, 43).

The latter finding is not compatible with the characteristics of a herpesvirus. The fact that no evidence has been presented so far which would indicate that TGE virus replicates in the cell nucleus also does not support its classification as a member of the herpesvirus group. A re-examination of the morphologic characteristics and nucleic acid type of TGE virus appeared therefore to be indicated.

This paper describes the electron microscopic observations of thin sections of fetal pig thyroid cells harvested at various intervals after infection with TGE virus and on partially purified material from infected cultures prepared for electron microscopy by the negative contrast technique. Information is also presented on the nature of the nucleic acid.

Materials and Methods

1. Viruses

TGE Virus. The Purdue strain, passaged 144 times in fetal pig kidney (FPK) cell cultures was used as inoculum in the experiments reported here. The isolation and purification of this virus strain has been described (46).

Vesicular Stomatitis (VS) Virus. The New Jersey serotype of the virus was used. This virus was isolated in 1961 by Dr. *M. L. Kuns* from cattle during an outbreak of VS in Jones County, Georgia, and has been passaged 6 times in mouse brains and once in FPK cell culture.

Pseudorabies (PR) Virus. The Aujeszky strain of pseudorabies virus was originally obtained from the American Type Culture Collection and has since been passaged 5 times in mouse brains and once in FPK cell culture.

2. Cell Cultures

FPK cells were grown in 32 oz. prescription bottles. Fetal (FPT) and adult (APT) pig thyroid cells were grown in plastic dishes as described previously (46).

3. Concentration and Partial Purification of TGE Virus

From Infected FPK Cell Cultures. Four liters of cell culture fluid were harvested 42 hours after infection with $10^{6.8}$ plaque forming units (p.f.u.) per monolayer and clarified by low speed centrifugation (1000 *g* at 4° C) for 1 hour. The virus was concentrated by partition in an aqueous dextran[1]-methylcellulose (D-M) and dextran[1]-polyethylene glycol (D-PEG) system as described for the concentration of adeno and influenza viruses (1). A precipitate was removed from the bottom phase of the D-M system by low speed centrifugation for 1 hour and discarded. The turbid interphase of the D-PEG system was added to an equal volume of Eagle's basal medium without $NaHCO_3$. This mixture, the pH of which was 6.5, was then homogenized for 2 minutes with a Sorvall Omni-Mixer[2] at speed setting 10 while

[1] Dextran 2000, Pharmacia Uppsala, Sweden, was substituted for the D 68 dextran fraction.

[2] Ivan Sorvall, Inc., Norwalk, Connecticut.

submersed in ice water. The sediment obtained after low speed centrifugation for 3 hours was suspended in 3 ml of Hanks' balanced salt solution (HBSS) and sonicated (46). Then 2.5 ml of this preparation were placed on top of a linear density gradient (10—70% [w/v] sucrose in 0.85% saline) in a 1×3 in. nitrocellulose tube. The tube was filled to capacity with 4 ml of distilled water. The gradient was centrifuged in the SW 25.1 rotor of the Spinco model L ultracentrifuge at 63,000 *g* for 8 hours. Fractions of 1.0 ml each were collected from the top with an ISCO[1] model D density gradient fractionator and titrated. Fractions 18 and 19 (the 4 fractions containing the distilled water not counted) were pooled, dialyzed at 4°C against 1000 volumes of phosphate buffered saline (PBS), pH 7.4, and concentrated by pervaporation. Densities of the fractions were determined by weighing 50 λ volumes in a micropipette.

From Infected FPT Cell Cultures. FPT cell cultures in 60×15 mm plastic dishes were inoculated with 10^6 p.f.u. of virus and after 2 hours af adsorption 2 ml of maintenance medium (46) were added. Twenty-seven hours after infection 36 ml of cell culture fluid were harvested and clarified by low speed centrifugation for 1 hour. The supernatant fluid with a titer of $10^{7.6}$ p.f.u./ml was then centrifuged at 80,000 *g* for 1 hour in the No. 40 rotor of the Spinco ultracentrifuge. The resulting pellets were suspended in 12 ml of PBS and subjected to another cycle of low and high speed centrifugation. The final pellet was suspended in 0.5 ml of PBS or of 1% ammonium acetate.

4. Ether Treatment

Equal volumes of the virus concentrate obtained from FPT cell cultures and of absolute ethyl ether (Mallinckrodt) were mixed and shaken in 1 dram vials at room temperature for periods of 15 minutes to 3 hours. Subsequently the ether was allowed to evaporate.

5. Growth Curve in FPT Cell Cultures

FPT cell monolayers in 35×10 mm dishes were washed with HBSS and exposed to an input multiplicity of $10^{-1.8}$ p.f.u. of TGE virus. After 2 hours of adsorption at 37°C the monolayers were washed twice with warm (37°C) HBSS and 2 ml of maintenance medium (46) were added. Incubation was continued at 37°C. For the assay of total virus, cells and medium of one culture dish were subjected to sonication (46) followed by low speed centrifugation for 1 hour. For the assay of spontaneously released virus the medium from a second culture dish was centrifuged without prior sonication. Titers of the supernatant fluids were determined by plaque assay in APT cells (46).

6. Determination of Nucleic Acid Type

Serial tenfold dilutions of TGE, VS, and PR viruses were prepared in HBSS with lactalbumin hydrolysate (0.5%) — yeast extract (0.1%) and a plaque test performed as described for TGE virus (46) with the following modifications: Mycostatin was omitted from the agar medium. Groups of 3 culture dishes per virus dilution were overlayed either with regular agar medium or agar medium containing 0.2 μM of 5-iododeoxyuridine[2] (IUDR) or 0.2 μM of IUDR plus 1.0 μM of thymidine[2] per ml, respectively. Plaques were counted 48 hours after inoculation.

[1] Instrumentation Specialities Company, Lincoln, Nebraska.

[2] California Corporation for Biochemical Research, Los Angeles, California.

7. Negative Staining of Partially Purified TGE Virus

One drop of virus suspension in PBS was placed on carbonized Formvar-coated grids. The specimens were blotted dry and washed with 1% ammonium acetate to remove residual salts. One drop of 2% phosphotungstic acid adjusted to pH 7.0 with normal potassium hydroxide (potassium phosphotungstate — PTK) was then placed on the grids and the excess fluid was blotted dry. Virus suspensions in 1% ammonium acetate were mixed with equal parts of PTK and then mounted onto the grids. Some specimens were fixed with formaldehyde or treated with ethyl ether prior to staining with PTK. The grids were examined with a Hitachi HU-11B type electron microscope at an instrumental magnification of 40,000 or 60,000 using double condenser illumination.

8. Thin-sectioning Techniques for Cultured Cells

FPT cell cultures, infected as described for the growth curve experiment were harvested 4, 6, 10, 14, 21 and 33 hours after inoculation. The cultured cells were detached from the plastic dish with a "rubber policeman" and

Table 1. Concentration of TGE Virus by Partition in Aqueous Dextran-Methylcellulose and Dextran-Polyethylene Glycol

Preparation	Volume (ml)	Infectivity ($\log_{10}$ p.f.u./ml)	Infectivity recovered (% of original total infectivity)	Concentration factor
1. FPK cell culture fluid, clarified	4000	6.9	100	1
2. D-M system, bottom phase	440	7.6	50	5
3. D-PEG system, interphase	15	8.2	8	20
4. Interphase of 3, homogenized; sediment after low speed centrifugation	7.5	9.4	63	316

centrifuged at a low speed for 5 minutes to form a pellet. The cell pellets were fixed in buffered 1% osmium tetroxide solution, dehydrated with graded alcohols, and embedded in a plastic mixture according to *Mollenhauer* (31). Thin sections were cut with glass knives using a Porter-Blum ultramicrotome and double stained with uranyl acetate (45) and lead citrate (41). Uninoculated cultures were similarly harvested and processed, and used as controls for possible nonspecific cellular changes.

Results

Concentration and Partial Purification

The virus was effectively concentrated from FPK cell cultures by partition in the D-M and D-PEG systems as demonstrated by the data presented in Table 1. A concentration of at least 2.5 $\log_{10}$ was obtained and 63% of the infectious virus present in the original preparation was

recovered. The lower infectivity yields in steps 2 and 3 of the procedure (Table 1) are probably caused by aggregation of the virus particles. The sedimentation of the infectious virus by low speed centrifugation of the interphase obtained in step 3 was unexpected and probably was due to adsorption of the virus to an impurity. Separation from this impurity was, however, possible by sonication followed by equilibrium

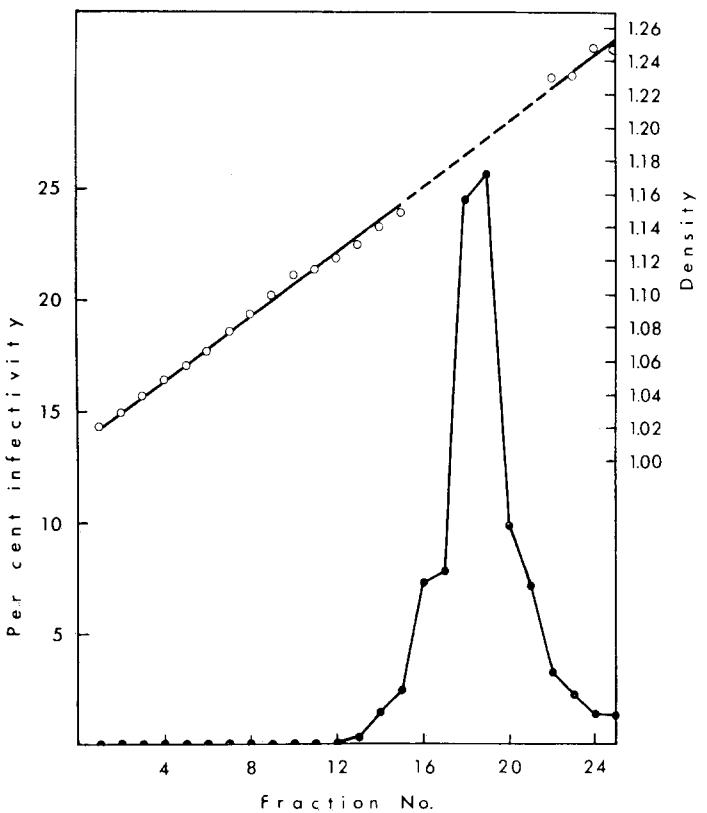

Fig. 1. Sedimentation of TGE virus by equilibrium zonal centrifugation in a sucrose gradient. ● infectivity; ○ density.

zonal centrifugation (7). Three heavy bands, not associated with infectivity, formed in the upper third of the liquid column. The peak infectivity was recovered at a level just below a broad slightly opalescent zone in the bottom third of the column at a density of 1.1925 (Fig 1).

Differential centrifugation of the virus propagated in FPT cells resulted in a 10 fold concentration (titer of final suspension: $10^{8.6}$ p.f.u./ml).

Growth Curve in FPT Cell Cultures

New infectious virus was first demonstrable between 3 and 4 hours after infection (Fig. 2). Spontaneously released virus appeared approximately 1 hour later and continued to be released at approximately the same rate that new virus was formed. One growth cycle was apparently completed within 6 hours. A cytopathic effect visible by light micro-

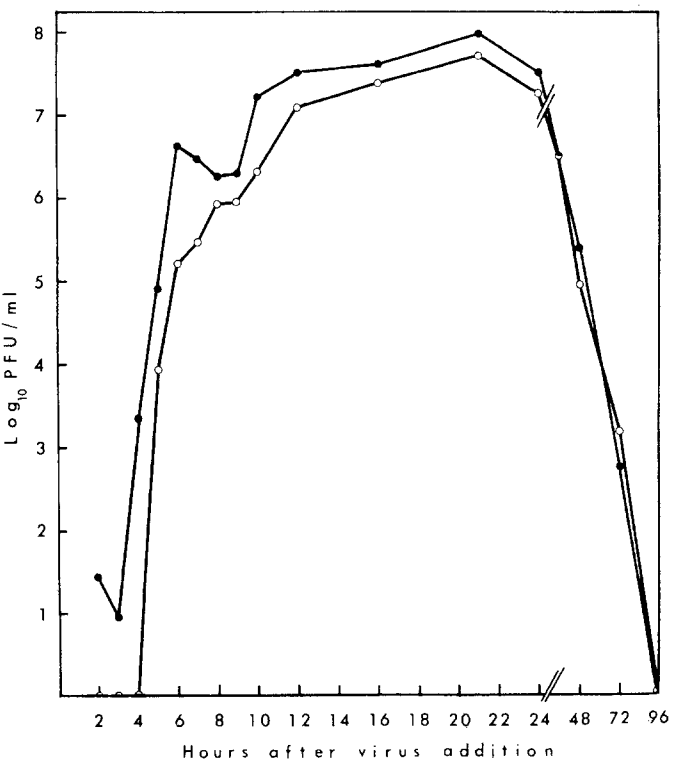

Fig. 2. Growth curve of TGE virus in FPT cell cultures. ● total virus; ○ spontaneously released virus.

scopy was first noted 7 hours after infection, and all cells were either destroyed or had signs of degeneration by 24 hours after infection.

Effect of DNA Inhibitor

The number (Table 2) and size of plaques were not reduced when TGE virus was propagated in the presence of IUDR, an inhibitor of DNA viruses (18). The same was true for VS virus, known to be a RNA virus, while plaque production by the DNA containing PR virus was

suppressed in the presence of the inhibitor. The inhibitory effect was completely reversed in the presence of a 5 fold concentration of thymidine. The results of this experiment suggest, as have previous experiments by other workers (5, 43) that the nucleic acid of TGE virus is RNA.

The Structure of TGE Virus as Observed by the Negative Staining Technique

Numerous particles thought to be virions were seen, either singly or in clusters, in preparations partially purified and concentrated from both kinds of infected cell cultures. They were variable in size and shape but were predominantly spherical in outline and with an over-all diameter of 75—120 mμ. Most of them were intact, and neither an outer membrane nor an internal structure could be demonstrated (Fig. 3). In some spontaneously disrupted particles, however, as a result of some penetration of phosphotungstate to the interior, an outer membrane approximately

Table 2. Titers[1] of TGE, VS, and PR Viruses in the Absence and Presence of IUDR

Virus	Concentration of nucleoside/ml		
		0.2 μM IUDR	0.2 μM IUDR 1.0 μM thymidine
TGE	6.5	6.7	6.7
VS	6.1	6.2	6.2
PR	6.3	<0	6.1

[1] $\log_{10}$ p.f.u./ml

10 mμ in thickness was observed, and a concentric arrangement of the interior was suggested. Prefixation with formaldehyde tended to preserve the spheroidal form of virus particles and allowed phosphotungstate penetration, revealing a well-defined outer membrane and some aspects of internal structure. Some particles had parallel or concentric filaments which were found to consist of a series of spherical units with a diameter of approximately 2.5 mμ (Fig. 4). Ether treatment resulted in partial or complete disruption of virus particles, and the particles tended to aggregate. In partially disrupted particles, the outer membranes were broken at one or more points, and the contents of some particles were released. The particles became more irregular in shape and larger in size, and the parallel or concentric arrangement of the beaded filaments was visible within them (Figs. 5 and 6). On treatment with ether for 3 hours, most particles were completely disintegrated, the internal structure was hardly recognizable, and only fragments of the outer membrane remained.

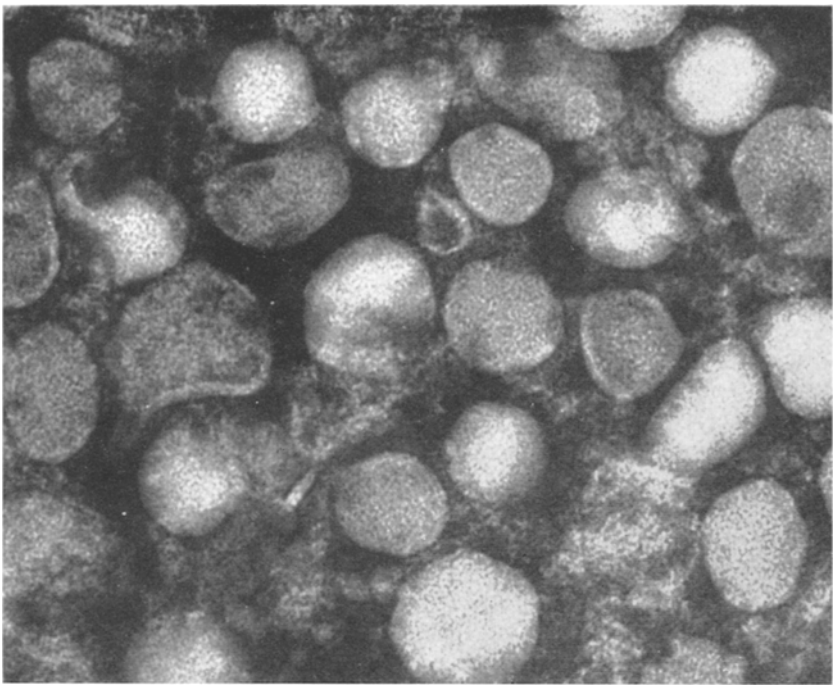

Fig. 3. Virus was purified by phase partition and density gradient centrifugation and stained with PTK. Some spontaneously disrupted particles are penetrated by PTK and show an outer membrane. ×200,000.

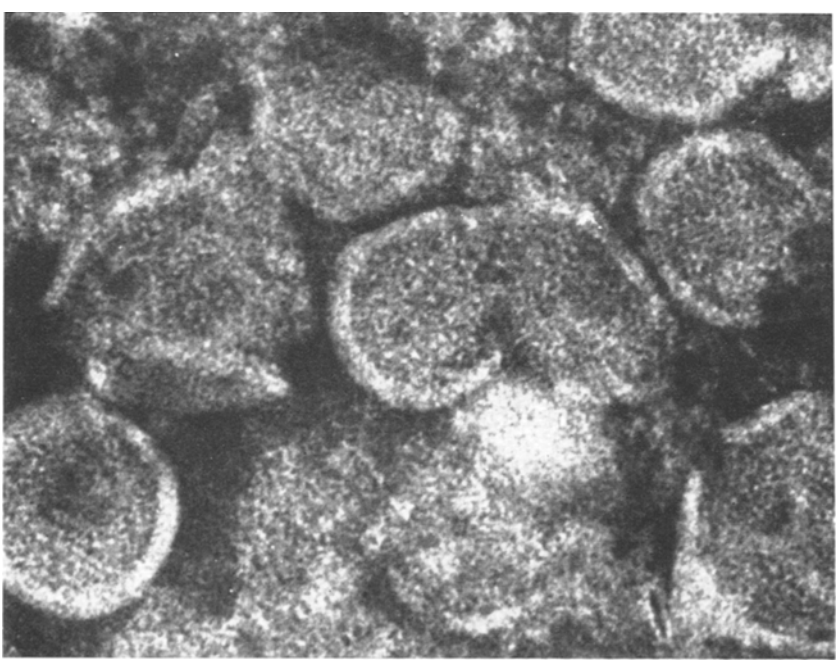

Fig. 4. Virus was purified by phase partition and density gradient centrifugation, fixed with formaldehyde, and stained with PTK. All particles are penetrated by PTK and show a distinct outer membrane; internal beaded filaments run parallel or concentrically. ×320,000.

The Morphology and Development of TGE Virus Observed in Thin Sections

Well defined particles, presumed to be those of TGE virus, were found in occasional cells prepared 4 hours after infection. The particles were observed individually within cisterns and tubuli of the granular and agranular endoplasmic reticulum, and in vacuoles bordered by double membranes. The origin of the vacuoles was obscure (Fig. 7).

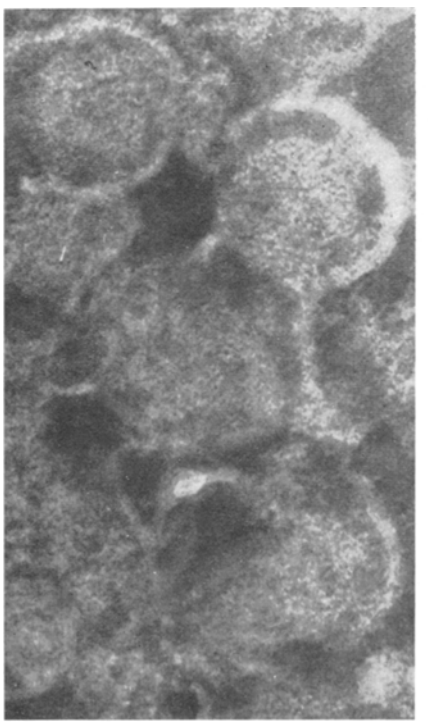

Fig. 5. Virus was purified by differential centrifugation, treated with ether for 45 minutes, and stained with PTK. Particles are partially disrupted and contents of some particles are released. ×200,000.

Fig. 6. Virus was purified by differential centrifugation, treated with ether for 60 minutes, and stained with PTK. A concentric arrangement of filaments can be seen. ×320,000.

Thereafter, the number of involved cells and virus particles per cell increased progressively with the lapse of time. In specimens harvested 6 hours or more after infection, the particles were found in both the cytoplasm and extracellular space (Figs. 9 and 10). They were circular or ellipsoidal in profile, varied in size, ranging from 50×63 to 76×87 mμ. Since the axes of lesser diameter corresponded to the direction of the knife marks on the sections, the apparent flattening of the particles might be interpreted as an artifact caused by knife compression.

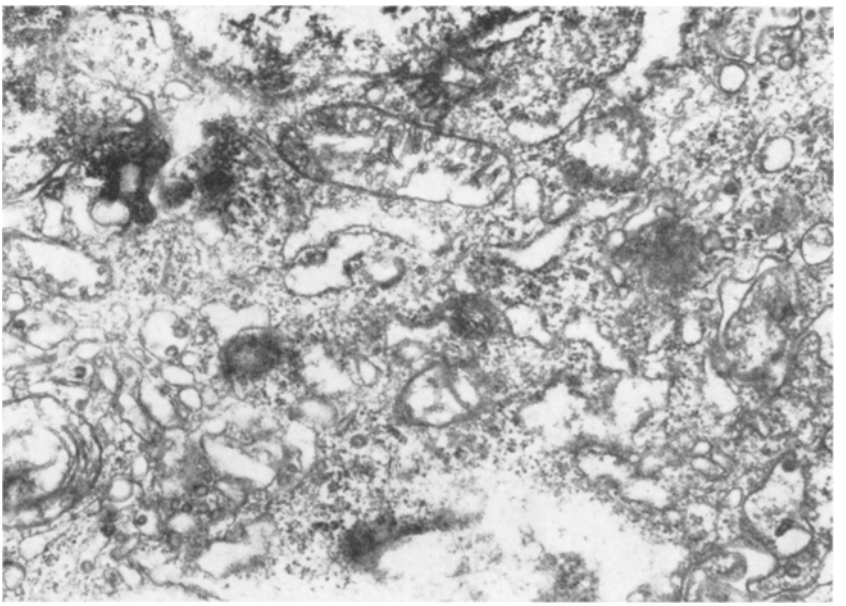

Fig. 7. Part of the cytoplasm of a cell 4 hours after inoculation. Several particles are seen within the cavities of endoplasmic reticulum and vacuoles. Mitochondria are swollen and their cristae are disintegrated. ×21,000.

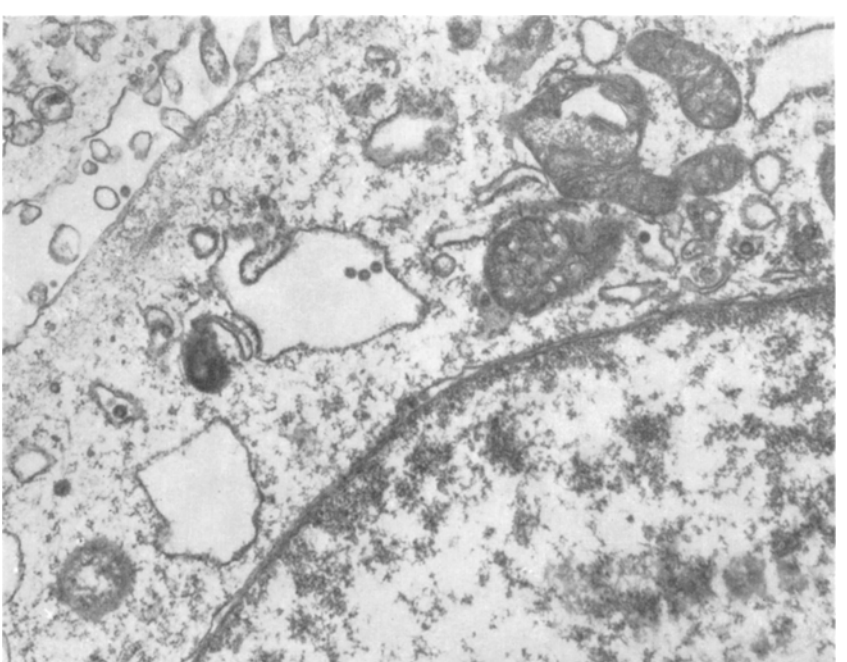

Fig. 8. A portion of a cell 10 hours after infection. Four particles are contained within the perinuclear cisterna of the nucleus which occupies the lower right corner of the picture. Several particles are also seen within cytoplasmic vacuoles. ×20,000.

The particle was composed of a dense central nucleoid, viroplasm and limiting membrane. The nucleoid was roughly circular in outline with a maximum diameter between 40—60 mμ. Most of them had a central region of diminished opacity, and some had the appearance of a doughnut, but others were uniformly dense (Figs. 10 and 11). The limiting membrane, approximately 8 mμ thick, had a unit membrane structure, consisting of two dark lines each about 3 mμ thick and separated by an intermediate electron-lucent line of about 2.5 mμ in thickness

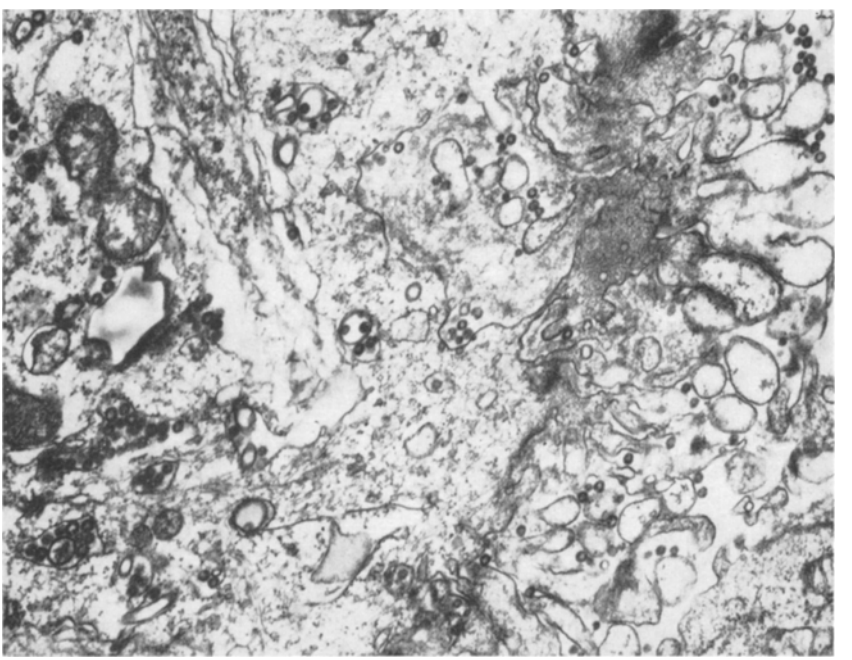

Fig. 9. A portion of a cell 33 hours after infection. The cell membrane with irregular-shaped microvilli is seen across the right portion of the micrograph and the cytoplasm containing numerous particles is left. Some cytoplasmic particles are in apposition with the vacuolar membranes. Many extracellular particles can be seen between the microvilli. ×20,000.

(Fig. 11). The viroplasm was a less electron-dense zone about 5 mμ in thickness, separating the nucleoid and the limiting membrane (Fig. 11). The majority of the particles were lodged, either singly or in a cluster, within the above-mentioned vacuoles or canaliculi and not infrequently in the perinuclear cisterna (Fig. 8). A few particles, especially those in cells at advanced stages of infection, were scattered in the cytoplasmic matrix, and their presence there may be due to disruption of the vacuoles. In the lumina of the vacuoles, some particles were observed to lie free, but some others were intimately associated with the inner surface of

the membrane bordering the vacuoles. The limiting membrane of the particles often appeared to be continuous with the vacuolar membrane (Fig. 10). Sometimes vacuoles were filled with the particles and amorphous material of moderate density.

Extracellular particles were identical in size and morphology with those seen in the cytoplasm. Some of them were free between microvilli and some were closely attached to the external surface of the cell membrane (Figs. 9 and 10).

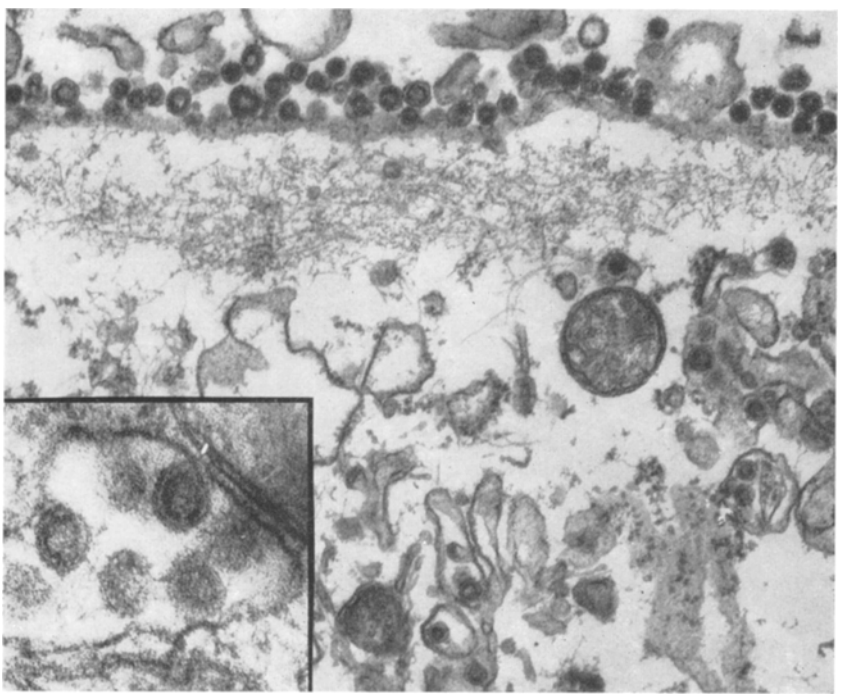

Fig. 10. Part of a cell 10 hours after inoculation. Numerous extracellular particles, some of which are intimately attached to the external surface of the cell membrane, are in the upper border of the picture. Several intracytoplasmic particles are also seen within vacuoles. The cytoplasmic organelles are disintegrated. At a higher magnification (see insert) the limiting membrane of a cytoplasmic particle appears to be continuous with the vacuolar membrane. ×45,000. Insert, ×100,000.

Various cytopathic changes were observed in the infected cells. Cytoplasmic alterations were characterized by vacuolization and condensation of the cytoplasm, swelling and disintegration of mitochondria, dilatation and fragmentation of endoplasmic reticulum, and rupture of the cell membrane. Within the nucleus there were disturbance of the chromatin pattern, displacement or loss of the nucleolus, and rupture of the nuclear envelope. In spite of such nuclear changes, no virus particles could be identified within the nucleus.

Discussion

The particles which we have observed and described are believed to be TGE virus. This belief is supported by the following:

1. the particles seen in partially purified material and in thin sections have been found only in preparations made from cell cultures infected with TGE virus;

2. the concentration of the particles seen in the purified material could be correlated with the infectivity titers. In thin sections, the number

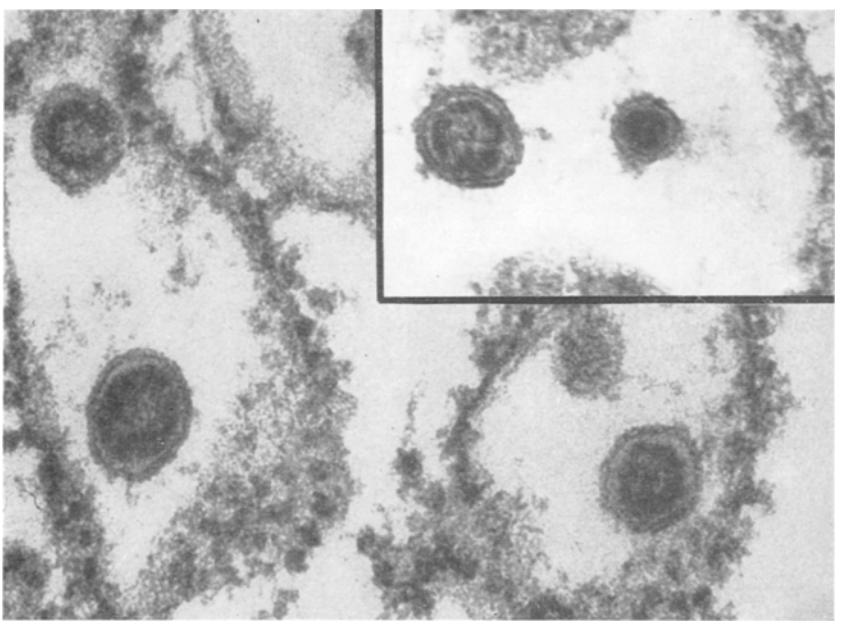

Fig. 11. A portion of the cytoplasm of a cell 10 hours after inoculation. Four particles within the dilated cisternae of granular reticulum are shown at a greater magnification. Insert shows two extracellular particles. The limiting membrane with a unit membrane structure, viroplasm and nucleoid are clearly visible. ×160,000.

of particles appeared to increase as the time of incubation increased. The increase in the number of particles could be correlated with the rise in infective titer in both the cellular and fluid phases of the cultures;

3. the particles demonstrated by both methods, negative staining and thin sectioning, were similar in shape and size. The size was in agreement with that reported by others (8, 9, 42, 43, 27, 35);

4. the morphology of the particles and possible mode of replication were similar to those of some other animal viruses.

Ristic et al. (42), based on the structural and biochemical properties of virus particles, indicated that TGE virus had similarities to both herpesviruses and myxoviruses. The present results obtained by examining negatively stained particles suggest that TGE virus bears some resemblance to the myxoviruses in that it is nearly in the same range of size as influenza virus and shows a considerable variation in size and shape (22). However, neither surface projections nor internal helical structure characteristic of myxoviruses could be recognized. Virus particles fixed with formaldehyde or disrupted with ether had a definite outer membrane and internal beaded filaments which ran parallel or concentrically. However, such treatments of TGE virus did not result in the visualization of helix and the formation of rosette-like structure. Our findings appear similar to those described for some oncogenic (15, 26, 6, 38, 23, 13), infectious bronchitis (4), and visna viruses (44). In these viruses, the symmetry of the internal structure has not been definitely established as being of the helical type.

From the data obtained by observing thin sections of infected cultured cells, TGE virus particles seem to differentiate at the inner surface of the membrane of endoplasmic reticulum, perinuclear cisterna and cytoplasmic vacuole of unknown origin. After maturation, it is presumed that virus particles detach from the membranes and then migrate by way of the cytoplasmic canalicular system to the surface of the cell, from where they are released. Similar observations of virus development have been made in cells infected with viruses of western equine encephalomyelitis (33), Venezuelan equine encephalitis (34), Japanese encephalitis (37), yellow fever (2, 29), and Rift Valley fever (28), and in various types of neoplastic cells (3, 24, 32, 12, 40, 10, 19, 16, 20, 21, 36, 11, 14). There is a possibility that virus particles may also mature at the surface of cells, because of their intimate attachment to the external cell surface. It should be emphasized, however, that extracellular particles are identical in size and morphology with intracytoplasmic particles. A budding process, as described in various types of neoplastic cells, could not be clearly demonstrated in stages of virus differentiation. This might be explained by assuming that the budding process of TGE virus occurs quickly.

Okaniwa et al. (35) suggested the presence of precursor particles in the cytoplasm of cells infected with TGE virus. We could not demonstrate such particles in the host cell.

Thus TGE virus bears, with regard to nucleic acid type, morphology, and possible mode of development, some similarities to some of the myxoviruses and some oncogenic viruses. However, further information is necessary to definitely classify this virus.

Summary

The morphology and development of transmissible gastroenteritis (TGE) virus of pigs have been studied with the electron microscope. By the negative contrast technique characteristic particles were found in preparations concentrated and partially purified either from infected fetal pig kidney cell cultures by phase partition in dextran-methylcellulose and dextran-polyethylene glycol followed by equilibrium zonal centrifugation or from infected fetal pig thyroid (FPT) cell cultures by differential centrifugation. Most of the particles had a spherical shape and were between 75 and 120 mμ in diameter. In some particles disrupted spontaneously, fixed with formaldehyde or split with ether, an outer membrane and internal beaded filaments were demonstrated.

In thin sections of cultured FPT cells infected with the virus, a small number of intracytoplasmic virus particles could be detected 4 hours after infection. The particles increased in number, both intra- and extracellularly, with the lapse of time, concomitant with the increase in amount of infective virus in cultures. The virus particle consisted of a dense nucleoid 40—60 mμ in diameter separated by a zone of lesser density from a limiting membrane 56—81 mμ in diameter. On the basis of morphological observations, it was suggested that the virus particle matures at the inner surface of the membrane which lines the cisternae of endoplasmic reticulum, perinuclear cisterna, and cytoplasmic vacuoles of unknown origin. Multiplication of TGE virus was not inhibited by 5-iododeoxyuridine suggesting that its nucleic acid is RNA. Similarity between TGE virus and some of the myxoviruses, as well as some oncogenic viruses was discussed.

References

1. *Albertsson, P. Å.:* Partition of cell particles and macromolecules. John Wiley & Sons/New York. p. 209—212 (1960).
2. *Bergold, G. H.,* and *J. Weibel:* Demonstration of yellow fever virus with the electron microscope. Virology **17,** 554—562 (1962).
3. *Bernhard, W.:* Electron microscopy of tumor cells and tumor viruses. Cancer Res. **18,** 491—509 (1958).
4. *Berry, D. M., J. G. Cruickshank, H. P. Chu,* and *R. J. H. Wells:* The structure of infectious bronchitis virus. Virology **23,** 403—407 (1964).
5. *Bohl, E. H.,* and *T. Kumagai:* The use of cell cultures for the study of TGE virus of swine. Proc. 69th annual meeting U.S. Livestock San. Ass. 343—350 (1965).
6. *Bonar, R. A., U. Heine, D. Beard,* and *J. W. Beard:* Virus of avian myeloblastosis (BAI strain A). XXIII. Morphology of virus and comparison with strain R (Erythroblastosis). J. nat. Cancer Inst. **30,** 949—997 (1963).
7. *Brakke, M. K.:* Density gradient centrifugation and its application to plant viruses. Advanc. Virus Res. **7,** 193—224 (1960).

8. *Cartwright, S. F., H. M. Harris, T. B. Blandford, I. Fincham,* and *M. Gitter:* Transmissible gastroenteritis of pigs. Vet. Rec. **76,** 1332—1333 (1964).
9. *Cartwright, S. F., H. M. Harris, T. B. Blandford, I. Fincham,* and *M. Gitter:* A cytopathic virus causing a transmissible gastroenteritis in swine. I. Isolation and properties. J. comp. Path. **75,** 387—396 (1965).
10. *Dales, S.,* and *A. F. Howatson:* Virus-like particles in association with L strain cells. Cancer Res. **21,** 193—197 (1961).
11. *Dalton, A. J., F. Haguenau,* and *J. B. Moloney:* Further electron microscopy study on the Moloney agent. J. nat. Cancer Inst. **33,** 255—275 (1964).
12. *De Harven, E.,* and *C. Friend:* Further electron microscope studies of a mouse leukemia induced by cell-free filtrates. J. biophys. biochem. Cytol. **7,** 747—752 (1960).
13. *De Thé, G.,* and *T. E. O'Connor:* Structure of a murine leukemia virus after disruption with tween-ether and comparison with two myxoviruses. Virology **28,** 713—728 (1966).
14. *Dirksen, E. R.,* and *R. Cailleau:* An electron microscopic study of the leukemia virus in AKR and hybrid mice inoculated with ascites passage or tissue-cultured leukemic cells. Cancer Res. **27** (1), 568—577 (1967).
15. *Dourmashkin, R. R.,* and *P. J. Simons:* The ultrastructure of Rous sarcoma virus. J. Ultrastruct. Res. **5,** 505—522 (1961).
16. *Haguenau, F.,* and *J. W. Beard:* The avian sarcoma leukosis complex: its biology and ultrastructure. In: *A. J. Dalton* and *F. Haguenau* (editors): Tumors induced by viruses. Academic Press. New York and London **1,** p. 1—60 (1962).
17. *Harada, K., T. Kumagai,* and *J. Sasahara:* Cytopathogenicity of transmissible gastroenteritis virus in pigs. Nat. Inst. anim. Hlth Quart. **3,** 166—167 (1963).
18. *Herrmann, E. C., Jr.:* Plaque inhibition test for detection of specific inhibitors of DNA containing viruses. Proc. Soc. exp. Biol. (N.Y.) **107,** 142—145 (1961).
19. *Heine, U., G. S. Beaudreau, C. Becker, D. Beard,* and *J. W. Beard:* Virus of avian erythroblastosis. VII. Ultrastructure of erythroblasts from the chicken and from tissue culture. J. nat. Cancer Inst. **26,** 359—388 (1961).
20. *Heine, U., G. de Thé, H. Ishiguro,* and *J. W. Beard:* Morphologic aspects of Rous sarcoma virus elaboration. J. nat. Cancer Inst. **29,** 211—223 (1962).
21. *Heine, U., G. de Thé, H. Ishiguro, J. R. Sommer, D. Beard,* and *J. W. Beard:* Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. II. Nephroblastoma (Wilms' tumor): Ultrastructure. J. nat. Cancer Inst. **29,** 41—105 (1962).
22. *Hoyle, L., R. W. Horne,* and *A. P. Waterson:* The structure and composition of the myxoviruses. II. Components released from the influenza virus particles by ether. Virology **13,** 448—459 (1961).
23. *Jordan, L. E., G. Plummer,* and *H. D. Mayor:* The fine structure of foamy virus. Virology **25,** 156—159 (1965).
24. *Lasfargues, E. Y., D. H. Moore, M. R. Murray, C. D. Haagensen,* and *E. C. Pollard:* Production of the milk agent in culture of mouse mammary carcinoma. J. biophys. biochem. Cytol. **5,** 93—96 (1959).

25. *Lwoff, A., R. Horne,* and *P. Tournier:* A system of viruses. Cold Spr. Harb. Symp. quant. Biol. **23,** 51—55 (1962).
26. *Lyons, M. J.,* and *D. H. Moore:* Purification of the mouse mammary tumor virus. Nature (Lond.) **194,** 1141—1142 (1962).
27. *McClurkin, A. W.,* and *J. O. Norman:* Studies on transmissible gastroenteritis of swine. II. Selected characteristics of a cytopathogenic virus common to five isolates from transmissible gastroenteritis. Canad. J. comp. Med. **30,** 190—198 (1966).
28. *McGavran, M. H.,* and *B. C. Easterday:* Rift Valley fever virus hepatitis: Light and electron microscopic studies in the mouse. Amer. J. Path. **42,** 587—607 (1963).
29. *McGavran, M. H.,* and *J. D. White:* Electronmicroscopic and immunofluorescent observations on monkey liver and tissue culture cells infected with the Asibi strain of yellow fever virus. Amer. J. Path. **45,** 501—517 (1964).
30. *Melnick, J.,* and *R. M. McCombs:* Classification and nomenclature of animal viruses, 1966. Progr. med. Virol. **8,** 400—409 (1966).
31. *Mollenhauer, H. H.:* Plastic embedding mixtures for use in electron microscopy. Stain Technol. **39,** 111—114 (1964).
32. *Moore, D. H., E. Y. Lasfargues, M. R. Murray, C. D. Haagensen,* and *E. C. Pollard:* Correlation of physical and biological properties of mouse mammary tumor agent. J. biophys. biochem. Cytol. **5,** 85—92 (1959).
33. *Morgan, C., C. Howe,* and *H. M. Rose:* Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. J. exp. Med. **113,** 219—234 (1961).
34. *Mussgay, M.,* and *J. Weibel:* Electron microscopic and biological studies on the growth of Venezuelan equine encephalitis virus in KB cells. Virology **16,** 52—62 (1962).
35. *Okaniwa, A., M. Maeda, K. Harada,* and *T. Kaji:* Electron microscopy of swine transmissible gastroenteritis (TGE) virus in tissue culture cells. Nat. Inst. anim. Hlth Quart. **6,** 119—120 (1966).
36. *Okano, H., A. Kunii,* and *J. Furth:* An electron microscopic study of leukemia induced in rats with Gross virus. Cancer Res. **23,** 1169—1175 (1963).
37. *Ota, Z.:* Electron microscope study of the development of Japanese B encephalitis virus in porcine kidney stable (PS) cells. Virology **25,** 372—378 (1965).
38. *Parsons, D. F.:* Structure of the Gross leukemia virus. J. nat. Cancer Inst. **30,** 569—583 (1963).
39. *Pehl, K. H.,* and *Ch. Ludwig:* Die Tenazität des Virus der infektiösen Gastroenteritis der Schweine, Typ Doyle-Hutchings. Arch. exp. Vet.-Med. **19,** 165—170 (1965).
40. *Pitelka, D. R., K. B. Deome,* and *H. A. Bern:* Virus-like particles in precancerous hyperplastic mammary tissues of C3H and C3Hf mice. J. nat. Cancer Inst. **25,** 753—777 (1960).
41. *Reynolds, E. S.:* The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17,** 208—212 (1963).
42. *Ristic, M., S. Sibinovic,* and *J. O. Alberts:* Electron microscopy and ether sensitivity of transmissible gastroenteritis virus of swine. Amer. J. vet. Res. **26,** 609—616 (1965).
43. *Sheffy, B. E.:* Characterization of transmissible gastroenteritis virus. Proc. 69th annual meeting U.S. Livestock San. Ass. 351—360 (1965).

44. *Thormar, H.*, and *J. G. Cruickshank:* The structure of visna virus studied by the negative staining technique. Virology **25,** 145—148 (1965).
45. *Watson, M. L.:* Staining of tissue sections for electron microscopy with heavy metals. J. biophys. biochem. Cytol. **4,** 475—478 (1958).
46. *Witte, K. H.*, and *B. C. Easterday:* Isolation and propagation of the virus of transmissible gastroenteritis of pigs in various pig cell cultures. Arch. ges. Virusforsch. **20** (3), 327—350 (1967).
47. *Young, G. A., R. W. Hinz*, and *N. R. Underdahl:* Some characteristics of transmissible gastroenteritis (TGE) in disease-free antibody-devoid pigs. Amer. J. vet. Res. **16,** 529—535 (1955).

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