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Propagation of the Virus of Porcine Epidemic Diarrhea in Cell Culture

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Porcine epidemic diarrhea virus (PEDV) was adapted to serial propagation in Vero cell cultures by adding trypsin to the medium. PEDV-infected cells showed a distinct cytoplasmic fluorescence when examined by a fluorescent-antibody-staining technique. Cytopathic effects, such as vacuolation, formation of syncytia, and fusion of cells, were detected even at passage 1 of the PEDV in Vero cells. Once adapted, the virus induced numerous syncytia containing over 100 nuclei. From virus passage 5 on, all cells forming the monolayer were fused and totally destroyed within 24 h after inoculation. Cell culture-grown PEDV had typical coronavirus morphology when viewed by electron microscopy. Attempts to propagate PEDV in several primary and secondary fetal porcine cell cultures in the presence or absence of trypsin were unsuccessful.

Members of the family *Coronaviridae* may cause severe diarrhea in humans and animals, especially in newborn individuals (33). In swine, the viruses of transmissible gastroenteritis (7, 14, 23, 37) and porcine epidemic diarrhea (PEDV) (1, 4, 11, 15, 18, 20, 21, 32, 38, 39), serologically not related to each other (22, 27, 35), are known as causative agents in digestive tract infections. Clinically, the two viral diseases can hardly be differentiated (4–6, 32, 38, 40).

Serial propagation of transmissible gastroenteritis virus is feasible in several cell culture systems, e.g., primary porcine kidney cells, thyreoidea cells, and CPK cells (16, 36, 37). Previous attempts to propagate PEDV in different cell-tissue or organ culture systems were unsuccessful, however (4, 11, 20, 21, 38). Propagation of PEDV in cell cultures is essential to circumvent the production of PEDV in colostrum-deprived piglets and to provide large quantities of virus for detailed characterization.

On the one hand, fetal calf serum (FCS) is known to inhibit adsorption of coronaviruses onto cell membrane receptors (10). On the other hand, the presence of trypsin in cell culture media is necessary for propagation of several viruses that cause enteric infections. For example, bovine coronavirus growth is facilitated by trypsin in the cell culture medium (3, 13, 26, 31). Similarly, this enzyme allows the in vitro propagation of astroviruses (17) and rotaviruses (41). In addition, the isolation of transmissible gastroenteritis virus is favored by trypsin as an ingredient of cell culture media (16); however, the type of cell culture, as well as the presence of trypsin, may be important (25).

This paper reports the first successful serial adaptation of PEDV to Vero cell cultures.

MATERIALS AND METHODS

Media. Growth medium was Eagle minimal essential medium (Amimed, Basel, Switzerland) buffered with 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and supplemented with 10% heat-inactivated FCS (North American Biologicals, Miami, Fla.) and antibiotics (100 IU of penicillin and 100 μg of streptomycin per ml). Maintenance medium consisted either of Eagle minimal essential medium with 2% FCS (SM) when experiments were started or, later, of Eagle minimal essential medium supplemented with 0.3% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and 0.02% yeast extract (E. Merck AG, Darmstadt, Federal Republic of Germany) and containing 10 μg of trypsin (1:250; Difco) per ml (TM). Since trypsin is thermolabile, 80% of TM was changed daily.

Cells. Different primary and secondary fetal pig cell cultures (intestine, lung, kidney, liver, spleen, brain, and skin) and several permanent cell lines which are susceptible to different coronaviruses (2, 31, 34) were used. They were Vero (African green monkey kidney cells), PD5 (porcine thyreoidea cells), PK15 (porcine kidney cells), and HRT18 (human rectal tumor cells [30]). Primary fetal pig cells were prepared by standard methods (19, 37). Briefly, organs of fetuses from slaughtered pregnant sows were removed under aseptic conditions, cut into small fragments, and dispersed by trypsin. Cell suspensions in growth medium were seeded in 150-cm^2 cell culture flasks (Corning Glass Works, Corning, N.Y.), and after 24 h of incubation, growth medium was changed to remove cell debris and nonviable cells.

Virus strains. Two strains of PEDV were used for the propagation experiments. One isolate, designated V215/78, was obtained from K. H. Witte, Arnsberg, Federal Republic of Germany. Newborn colostrum-deprived, specific-pathogen-free piglets were infected with this material. After 28 h, at the beginning of diarrhea, the inoculated piglets were sacrificed. The scraped-off mucosa and the content of the small intestine were pooled, diluted 1:5 in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate buffer [pH 7.4]), and homogenized by ultrasonication. The resulting suspension containing PEDV was clarified by centrifugation at 10,000 × *g* for 5 min to prevent bacterial or fungal contamination of the cell cultures.

The other isolate was obtained from M. B. Pensaert, Ghent, Belgium, and was used unpassaged for cell culture inoculation experiments.

To avoid cytotoxic reactions, the clarified virus suspensions were finally diluted 1:5 in SM or TM.

Inoculation of cell cultures. Before inoculation, the growth medium of confluent cell cultures grown either in 25-cm^2 flasks or on glass cover slips in 24-well tissue culture plates (Multiwell Falcon 3047; Becton Dickinson Labware, Lincoln Park, N.J.) was removed, and the monolayers were washed twice with SM or TM. Then the cells were inoculated with 0.5 ml per flask or 0.2 ml per well of the clarified and diluted

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virus suspension. After adsorption in the dark for 2 h at room temperature, maintenance medium was added (5 ml per flask, 0.8 ml per well) without removing the viral inoculum, and the cultures were incubated at 37°C. Control cultures were mock inoculated with the same volume of maintenance medium instead of viral inoculum and further treated in the same manner as virus-inoculated cultures.

Monitoring virus growth. Virus replication and production of virus-specific proteins were monitored by three different methods.

(i) CPE. Inoculated cell cultures were checked microscopically for cytopathic effects (CPE) daily. If the cell layer did not show CPE after 7 days of incubation, cells and supernatant fluids were frozen and thawed three times to release intracellular virus into the medium. The fluid was clarified by low-speed centrifugation (1,000 × *g* for 10 min) and inoculated onto freshly prepared monolayers. Three blind passages were done if no CPE were detected.

(ii) Immunofluorescence test. Rounded and detached cells were harvested at 48-h intervals, pelleted by low-speed centrifugation (200 × *g* for 10 min), washed once by being suspended in PBS, and dried on slides. After fixation with 100% ethanol for 20 min at 4°C, the cells were incubated for 1 h with an anti-PEDV-fluorescein isothiocyanate (FITC) conjugate, diluted 1:10 in PBS (kindly provided by M. B. Pensaert), and further processed for direct immunofluorescence tests. Cell monolayers grown on cover slips were washed once with PBS before fixation and were thereafter treated in the same way. If cells exhibiting a specific fluorescence could be seen, a second sample of the same cell preparation was incubated with another anti-PEDV-FITC conjugate (provided by K. H. Witte) to confirm the identity of the PEDV protein. The two conjugates, tested previously in gut sections of the small intestines of experimentally PEDV-infected piglets, led to bright cytoplasmic fluorescence of epithelial cells.

(iii) Electron microscopy. Clarified supernatant fluids from frozen and thawed cell cultures (see above) were placed on Parlodion-coated grids in 50-μl aliquots. After adsorption for 5 min, the grids were negatively stained with 2% phosphotungstic acid (pH 6.6) for 2 min and subsequently viewed with a Philips EM 201 electron microscope (Philips, Eindhoven, The Netherlands).

Staining of cell cultures. After inoculation, cover slip cultures were washed once with PBS. They were then fixed with 4% formaldehyde for 1 h and stained for 5 min with 0.5% (wt/vol) crystal violet. After being washed, the preparations were air dried and mounted in synthetic Canada balsam (Eukitt; O. Kindler, Freiburg im Breisgau, Federal Republic of Germany).

RESULTS

Search for a cell culture system to propagate PEDV. By using maintenance medium containing 2% FCS (SM), neither virus replication (CPE, electron microscopy) nor viral protein synthesis (direct immunofluorescence) could be detected after inoculation with either the German or Belgian PEDV in any of the fetal pig cell cultures or in PD5, PK15, and HRT18 cells. In Vero cell cultures, however, rounded and detached cells floating in the supernatant medium were observed 48 h after inoculation with both virus strains. These changes were not observed in the control cultures. When examined by direct immunofluorescence, about 5% of the detached cells showed cytoplasmic fluorescence. In inoculated cover slip cultures of Vero cells, a small number of single cells had brilliant granular cytoplasmic fluorescence, especially in the perinuclear region. A few fluorescing granules also were detectable in the nucleus (Fig. 1). After 6 days of incubation, the fluorescing cells had degenerated and detached from the monolayer, in which no further infected cells could be made out by immunofluorescence. No fluorescing cells were detectable in mock-inoculated cultures.

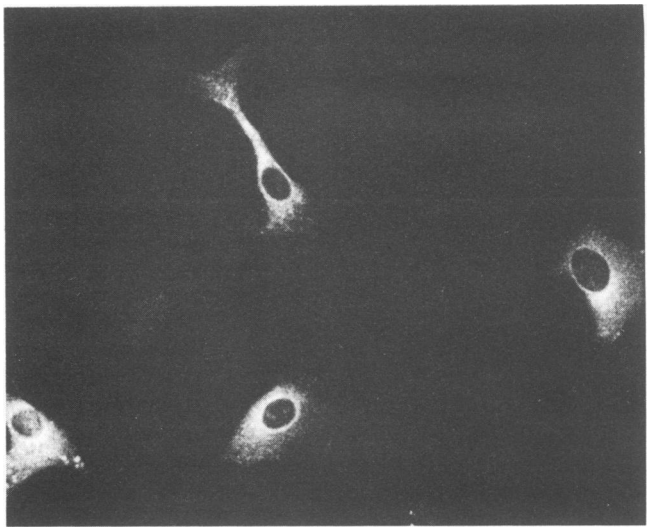

FIG. 1. Immunofluorescence of Vero cells inoculated with PEDV of gut origin and incubated for 3 days with SM. Single cells exhibiting a distinct cytoplasmic fluorescence after staining with anti-PEDV-FITC conjugate. Magnification, ×240.

Optimizing conditions for PEDV replication. With TM, all the primary and secondary fetal pig cell types tested, as well as PD5, PK15, and HRT18 cell lines, were nonpermissive for PEDV replication. In Vero cells, however, syncytia involving 5 to 10 nuclei could be detected microscopically after an incubation time of 3 to 5 days (Fig. 2). The formed syncytia contracted and detached from the intact surrounding cells 48 h later. The resulting gaps in the monolayer were slowly refilled with new uninfected cells.

Adaptation of PEDV to Vero cells. The daily-changed TM was pooled with the supernatant of the cells which had been frozen and thawed 7 days after inoculation. This mixture was transferred to new Vero cell cultures. Now syncytia had already formed 24 h after inoculation (Table 1), and before detaching from the flask the polycarions contained up to 50 nuclei. In the remaining monolayer, CPE progressed slowly and reached 40% after 5 days of incubation. On a subsequent two passages of PEDV, the number of syncytia containing over 100 nuclei (Fig. 3) increased; and on passage 4 of the virus, CPE of the monolayer reached 100% in less than 4 days of incubation. After further adaptation of PEDV to Vero cells, syncytia grew faster and all cells were fused and detached from the flask after 24 h of incubation.

Formation of PEDV protein-containing syncytia. In the course of adaptation, the morphology of emerging syncytia changed progressively during the virus replication cycle. In the beginning, some cells fused, losing their individual demarcation, and numerous distinctly swollen nuclei accumulated in the center of the rounded polycarions (Fig. 4). After 6 h, nuclei migrated to the periphery of syncytia and were aligned like strings of beads. At this point, large vacuoles were formed and the cytoplasma began to burst in the center of syncytia (Fig. 5), with nuclei becoming pycnot-

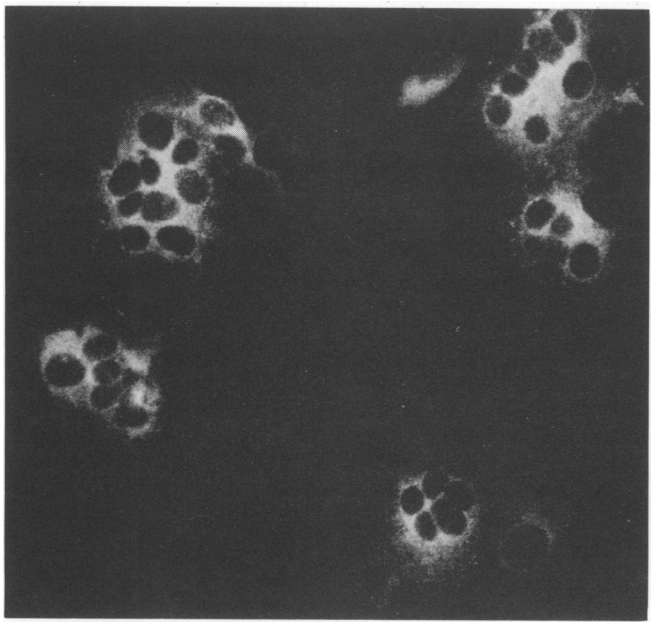

FIG. 2. Immunofluorescence of Vero cells inoculated with PEDV of gut origin and incubated for 3 days with TM (containing trypsin). Bright cytoplasmic fluorescence of syncytia involving different numbers of nuclei. Magnification, ×240.

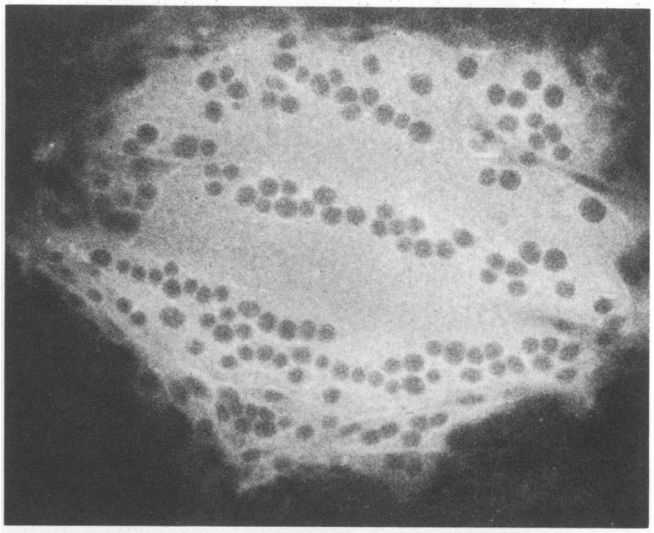

FIG. 3. Immunofluorescence of Vero cells inoculated with cell culture-adapted PEDV. Syncytium containing over 100 nuclei (15 h after inoculation). Magnification, ×120.

ic. A few hours later, all the syncytia detached from the flask like "flying carpets." As soon as the syncytia had reached a certain size, cells at the periphery of the polycarions remained unaffected and syncytial cells detached, leaving a punched-out, plaquelike hole in the monolayer (Fig. 6). Secondary syncytia were obviously induced by free virus particles in the supernatant, since they did not originate from existing polycarions.

Identification of PEDV protein by immunofluorescence. Cell cultures grown on cover slips were inoculated with PEDV of gut origin in TM. After 24 h of incubation, single cells and very few polycarions (up to 10 nuclei) exhibited bright fine-granular cytoplasmic fluorescence (Fig. 2). After three virus passages, fluorescing syncytia were already detected after an incubation time of 12 h and, in a later phase, contained more than 100 nuclei (Fig. 3). No fluorescing cells or syncytia were observed in uninfected cultures. All immunofluorescence studies led to the same results with both anti-PEDV-FITC-conjugates. Therefore, Vero cells showing specific fluorescence obviously contained PEDV proteins.

Electron microscopy. Clarified supernatant fluids of infected cell cultures, as well as of diarrheic feces of PEDV-infected piglets, always displayed numerous round-to-pleomorphic particles with diameters from 90 to 160 nm when examined by electron microscopy. Widely spaced peplomeres with lengths of 12 to 15 nm formed a crown around the virions (Fig. 7), showing a typical coronavirus morphology. No such particles were ever detected in mock-inoculated control cultures.

Trypsin dependence of in vitro replication of PEDV. If SM (containing FCS) was used for inoculation of Vero cell cultures with cell culture-adapted PEDV, single cells supported virus replication as revealed by immunofluorescence, but no syncytia were formed. After one passage, virus antigen-containing cells could no longer be detected in the inoculated monolayers. Therefore, the presence of trypsin in the maintenance medium was necessary to ensure PEDV replication even after 25 passages in Vero cells.

DISCUSSION

This is the first report of an in vitro adaptation of PEDV to serial propagation in Vero cell cultures. In contrast to transmissible gastroenteritis virus, PEDV requires trypsin in the medium for in vitro replication. However, PEDV originating from feces of infected piglets is able to induce at least the primary steps of replication in Vero cells in the presence or absence of trypsin, as revealed by immunofluorescence. Such an effect may be the result of residual intestinal content in the viral inoculum, as described for a porcine calicivirus-like virus (8). In contrast, in vitro passage of PEDV without

TABLE 1. Adaptation of PEDV to Vero cells during subsequent passages

Maintenance medium[a]	No. of PEDV passage in Vero cells	Detection of fluorescing cells[b]	Appearance of syncytia[b]	No. of nuclei in syncytia[c]	Incubation time after inoculation	Degree of CPE at end of incubation period (%)
1	Primary inoculation	+	−	—	7 days	0
1	1	−	−	—	7 days	0
2	Primary inoculation	+	+	10	5 days	0
2	1	+	+	50	5 days	40
2	3	+	+	>100	4 days	100
2	24	+	+	>100	20 h	100

[a] 1, Containing FCS but no trypsin; 2, containing trypsin but no FCS.
[b] +, Detected; −, not detected.
[c] —, No syncytia.

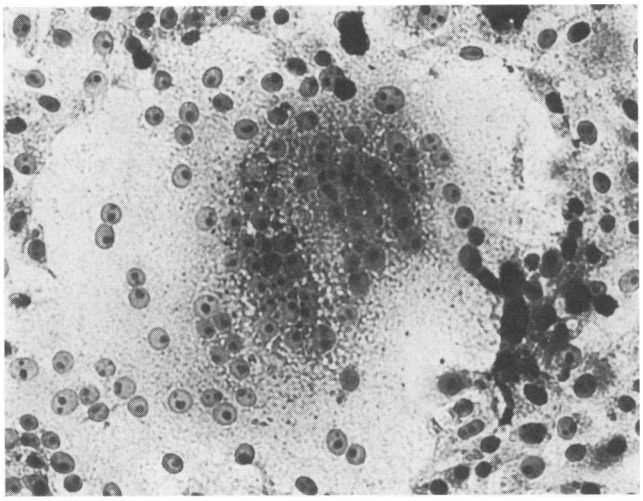

FIG. 4. Early state of cell culture-adapted PEDV-induced cell fusion, 9 h after inoculation. Numerous nuclei are accumulated in the center of the growing syncytium. Crystal violet staining; magnification, ×240.

trypsin was not possible and virions released in the cell culture supernatant had to be activated by trypsin to render them infectious for cells cultured in vitro. Possibly, such an activation is based on the same mechanism(s) responsible for the adsorption and the cell-fusing activity of other coronaviruses (9, 28, 29). It is known that for a successful adsorption and penetration, the E2 spike glycoprotein of certain coronaviruses has to be cleaved into two subunits (29).

The CPE caused by PEDV in Vero cells are quite similar to the cell alterations induced by other coronaviruses in Vero and other cell types (fusion of cell membrane and syncytium formation [12, 24, 29]).

Surprisingly, PEDV could not be propagated in primary or secondary fetal porcine cell cultures under any conditions.

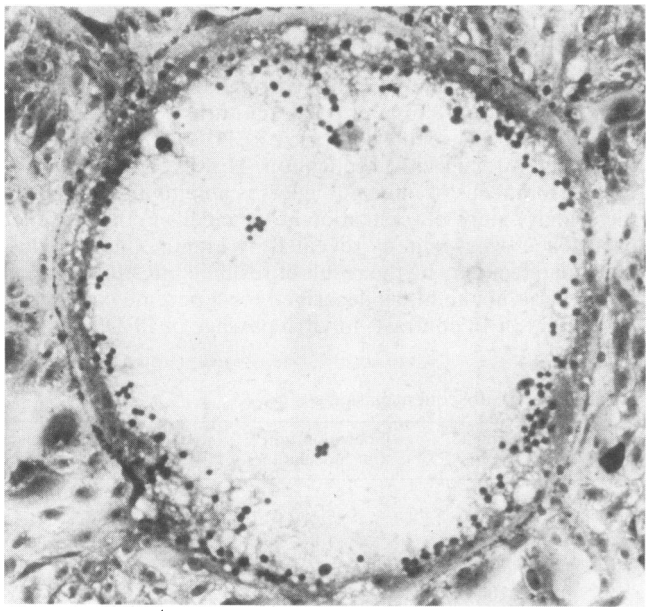

FIG. 5. Late state of cell culture-adapted PEDV-induced polycarion, 15 h after inoculation. Pycnotic nuclei are accumulated at the periphery; the center begins to burst. Crystal violet staining; magnification, ×120.

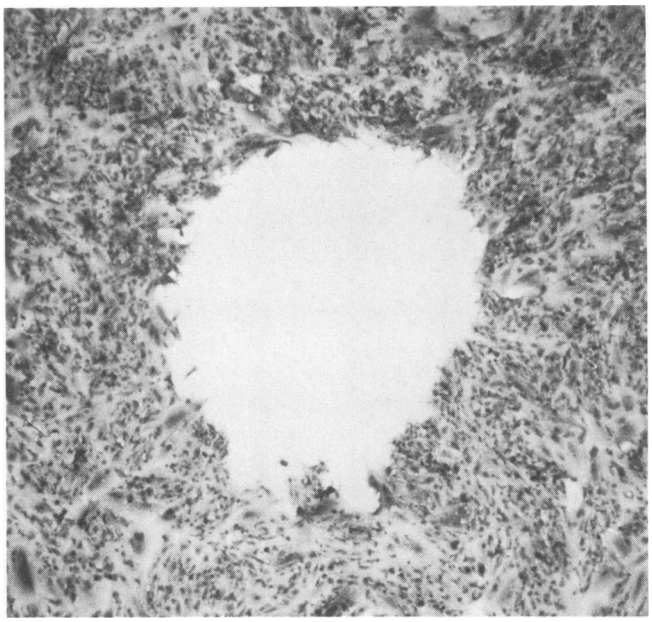

FIG. 6. Characteristic plaquelike hole in the cell monolayer after detachment of a PEDV-induced syncytium 19 h after inoculation. Surrounding cells are unaffected. Crystal violet staining; magnification, ×48.

Additionally, unlike Vero cells, primary and secondary cells were damaged (rounded and detached) by the action of trypsin (data not shown). Presumably, the damaged cells were not able to support virus replication.

The method described to propagate PEDV in cell culture should provide larger quantities of virus for the development of virus assays and serological tests. Further, it should make possible a better characterization of PEDV. Studies to develop a plaque test for virus titration and an enzyme-linked immunosorbent assay to detect antibodies to PEDV and viral antigens in feces are under way.

With the cell culture system described here, animals in medical experiments can be replaced.

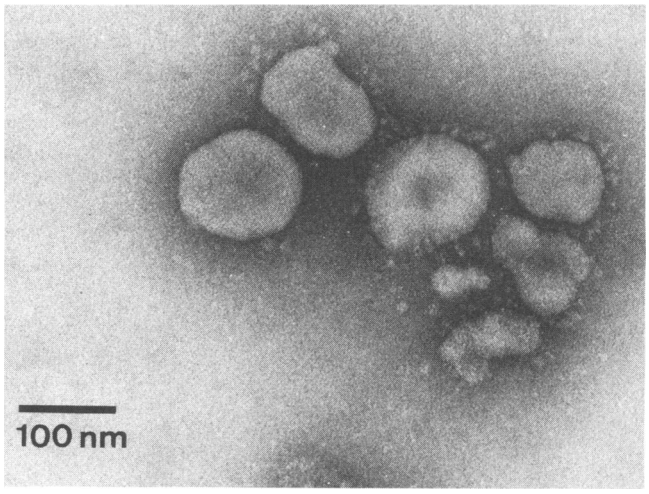

FIG. 7. Electron micrograph of typical coronavirus particles obtained from supernatant of cell culture-adapted PEDV-inoculated Vero cells (passage 26 of PEDV in Vero cells). Note the characteristic widely spaced, club-shaped surface projections. Phosphotungstic acid negative staining; magnification, ×128,000.

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