Correlation of Surface and Internal Ultrastructural Changes in Cells Infected with Foot-and-Mouth Disease Virus

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ABSTRACT

The surfaces of primary and continuous line cell cultures displayed the same sequence of morphological changes during the course of infection with foot-and-mouth disease virus. These changes could be classified into four broad stages: I) cells were flattened, closely attached to one another and microvilli appeared, II) cells rounded, microvilli began to disappear and the cells started to separate from one another by cytoplasmic strands, III) cells were discrete, rounded structures and IV) cells were rounded and had numerous attached buds, some of which contained virus. The internal changes included the appearance of increasing amounts of smooth membranous vacuoles lined with the viral induced RNA polymerase and the presence of buds, some with viral particles inside. While the different cell cultures showed similar internal and external changes as a result of infection, they responded to infection at different rates and contained subpopulations of resistant cells.

Key words: Foot-and-mouth disease, morphology of viral-infected cells, viral-induced RNA polymerase.

RÉSUMÉ

La surface de cultures de lignées cellulaires primaires ou continues manifesta la même séquence de changements morphologiques, à la suite d'une infection par le virus de la fièvre aphteuse. Cette séquence correspondait à peu près aux quatre stades suivants: 1— les cellules étaient aplaties, étroitement attachées les unes aux autres et des microvillosités apparurent éventuellement; 2— les cellules s'arrondirent et leurs microvillosités commencèrent à disparaître, tandis que les cellules commencèrent à se séparer les unes des autres par des filaments cytoplasmiques; 3— les cellules correspondaient à des structures discrètes et arrondies; 4— les cellules étaient arrondies et elles arboraient plusieurs bourgeons périphériques, dont certains contenaient du virus.

Les changements internes incluaient l'apparition d'un nombre croissant de vacuoles membraneuses et lisses, tapissées par l'ARN polymérase suscitée par le virus, et la présence de bourgeons, dont certains contenaient des particules virales.

Même si les différentes cultures cellulaires affichèrent des changements internes et externes similaires, à la suite de l'infection, elles y réagirent avec plus ou moins d'intensité et elles contenaient des sous-populations de cellules résistantes.

Mots clés: fièvre aphteuse, morphologie des cellules infectées par le virus, ARN polymérase suscitée par le virus.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) infects cattle, swine, sheep, goats and other domestic and wild cloven-footed animals. The disease is characterized by vesicular lesions on the mouth and feet. The snout, rumen, myocardium and mammary glands may also be affected.

Foot-and-mouth disease virus produces cytopathogenic changes in cell cultures prepared from various animal tissues (1, 2, 3). Light microscopy revealed intracytoplasmic processes and acidophilic masses (4, 5) and electron microscopy has shown cytoplasmic crystals of FMDV (6) and evidence for a prelytic release of FMDV in cytoplasmic blebs (7). Ultrastructural studies on another member of the picornavirus family have shown extensive proliferation of smooth membranous vacuoles in poliovirus-infected cells (8, 9).

In the study reported here, we examined surface and internal ultrastructural changes in FMDV-infected cell cultures using transmission and scanning electron microscopy. We established stages of infection that will be of use in the examination of animal tissues infected in the field. Four stages of infection were common to each of several different cell types infected with different viral types and will be described. The localization of

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the viral-induced RNA polymerase will be related to the stages of infection.

MATERIALS AND METHODS

CELL CULTURES

First and second passages of cultures of two different primary cells, bovine kidney (BK) and bovine calf thyroid (CTh) and cultures of two different pig kidney cell lines, Mengeling-Vaughn (MVPK) and PK-15, were grown in 25 cm^2 plastic flasks and infected when they contained about 5×10^6 cells. Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 5 to 10% bovine serum was used to grow all cell cultures except CTh cell cultures for which the MEM was mixed with one-half part of Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate (10, 11, 12).

VIRUS INFECTION AND IMMUNOLABELING PROCEDURES

Foot-and-mouth disease virus serological subtypes A_{12} and O_1 Brugge were produced in baby hamster kidney cells grown in 2 L Baxter bottles in a Tris-buffered modified Eagle salt solution (13). Virus concentrates were prepared from infected cells by two cycles of precipitation with 6% polyethylene glycol. Cells for experimental use were grown in small plastic flasks and inoculated at a multiplicity of about 50 plaque forming units per cell with virus contained in 0.1-0.2 mL. After a 30 minute adsorption period, 5 mL of growth medium was added to each culture and incubation was continued at 37°C. At intervals from one to 12 hours postinfection, cells were washed with phosphate buffered saline, pH 7.5, and fixed in 2% glutaraldehyde for subsequent processing for electron microscopy. For those experiments which detected the FMDV-induced polymerase antigen, cells were first exposed to a solution of 1% saponin in 0.05% glutaraldehyde for 30 minutes. The cells were then treated with antipolymerase antibody and protein A conjugated peroxidase (14).

PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY

The glutaraldehyde fixed cells were postfixed with 2% osmium tetroxide for one hour. For transmission electron microscopy, cells were removed by scraping and dehydrated in graded ethanol solutions. The cells were then embedded in Epon 812 and thin sections were examined in a Philips 201 electron microscope. For scanning electron microscopy, the fixed cells were dehydrated in graded ethanol solutions *in situ* and the plastic flask was broken into small pieces for critical point drying in carbon dioxide. The dried cells were sputter-coated with gold and visualized in an Amray 1000A instrument at a tilt angle of 30° and 20 KV.

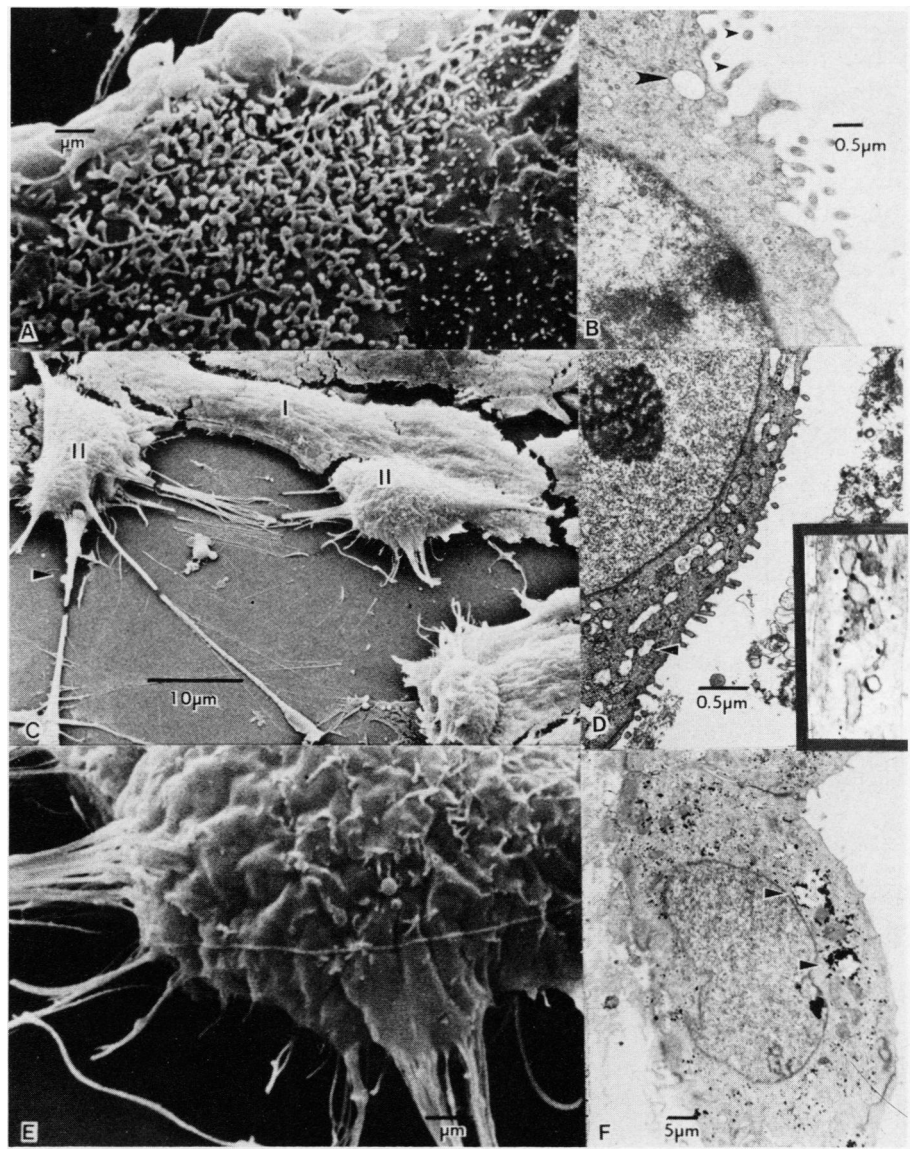

Fig. 1. Scanning (SEM) and transmission (TEM) electron micrographs of cells in the early stages of infection. A is an SEM of MVPK cells in stage I, showing many microvilli, X36,000. B is a TEM of BK cells in stage I, showing microvilli (*small arrows*) and the start of smooth membranous vacuole (SMV) formation (*large arrow*), X14,000. C is an SEM of PK-15 cells in stages I and II with the cells in stage II showing rounding and cytoplasmic strands, X10,000. D is a TEM of PK-15 cells in stage II, showing SMV lined with immunoperoxidase labeled viral induced RNA polymerase, X20,000. Insert shows higher magnification of immune labeled SMV. E is an enlargement of the stage II PK-15 cell seen in C, showing the disappearance of microvilli, X42,000. F is a TEM of CTh cells in stage II showing the SMV associated with the immunoperoxidase labeled RNA polymerase and with microvilli practically disappeared, X3,000.

RESULTS

The surfaces and internal structures of infected cells displayed a continuum of morphological changes during the course of infection. These changes could be classified into four broad stages which appeared in the same sequence in all infected cells, although not all stages were always present for a particular cell type in every series of experiments.

Uninfected cells and cells at the start of infection grew in flattened layers closely attached to each other (figure not shown). Early in infection, the surfaces of cells in stage I had microvilli of varying sizes extending from them (Fig 1A). Internally (Fig 1B), the cells showed newly formed smooth membranous vacuoles (SMV), previously reported to be the site of viral RNA synthesis in infected cells (15) and many microvilli projections. As infection progressed to stage II, the microvilli began to disappear, the cells started rounding and separating from one another and cytoplasmic strands were seen (Figs. 1C, E). The cytoplasmic strands from one cell were often unattached to other cells and an occasional microbud was seen (Fig 1C, arrow). Internally (Figs 1D, F), numbers of SMV increased in which immunoperoxidase labeling with antisera established the presence of the viral induced RNA polymerase. The microvilli gradually disappeared. Stage III cells were rounded and quite smooth (Figs 2A, C), Internally (Fig 2A), the rounded cell had many larger SMV, apparently resulting from the coalescing of smaller vacuoles. Stage IV cells were rounded with numerous buds, some of which contained virus (Figs 2B, C).

The four cell types, while showing the same sequence of morphological changes, responded differently to infection with respect to time, as shown in Table I. At one hour postinfection, more than 50% of primary BK cells were in stage II of infection and that status remained for the next two hours. At four hours postinfection, more than 50% of the BK cells had changed to stage III and at six hours, the majority of the BK cells were in stage IV. It is important to point out that at the different postinfection times there were cells in

TABLE I. Kinetic Study of Surface Structural Changes in FMDV-Infected Cell Cultures[a,b]

Cell	Hours Postinfection						
	1	2	3	4	5	6	7
Bovine Kidney (BK)	II	II	II	III	III	IV	
Pig Kidney (MVPK)	I	I	II	III	III		IV
Pig Kidney (PK-15)	I	II	II	III	IV		

[a]The Roman numerals represent one of four morphological stages seen during infection which are described in the text. Briefly, in Stage I, cells are flat with appearance of microvilli, in Stage II, cells are rounding and have cytoplasmic stranding, in Stage III, cells are rounded and in Stage IV, cells are rounded with buds. The hour postinfection at which a state is listed represents the time when approximately 50% of the cells infected with FMDV, type A_{12} or O_1 Brugge, at a multiplicity of infection of 50, are in the stage

[b]Calf thyroid cells are not listed in the table because a majority of the cells never were in any one stage at a given time

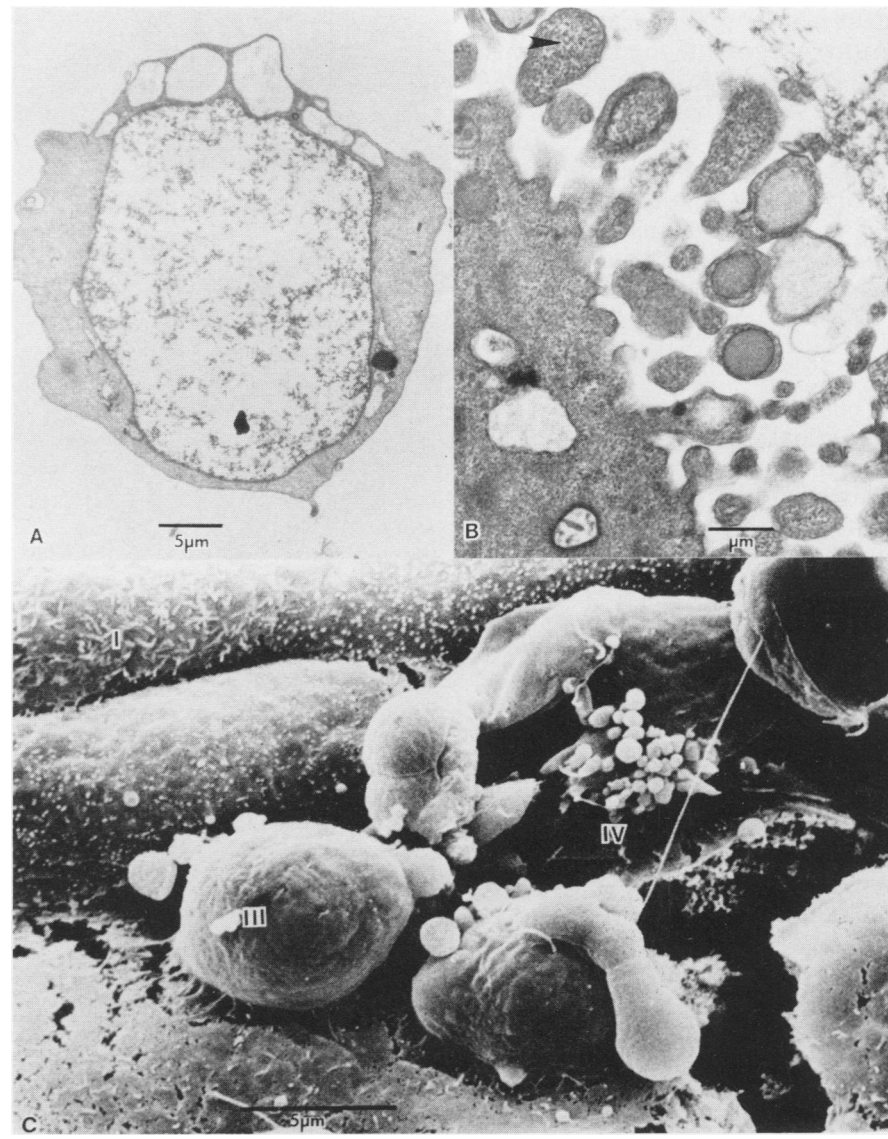

Fig. 2. Transmission (TEM) and scanning (SEM) electron micrographs of cells in the late stages of infection. A is a TEM of MVPK cells in stage III, showing a rounded cell with larger smooth membranous vacuoles, X7,200. B is a TEM of BK cells in stage IV showing numerous buds, some with viral particles inside (*arrow*), X35,000. C is an SEM of BK cells in stages I, III and IV. Microvilli, rounding and budding are seen as previously described, X22,000.

earlier and later stages in lesser proportions than 50%.

The primary CTh cells are not listed in Table I because a majority of the cells never seemed to be infected. Less than 20% of the cells reacted to infection by passing through the various stages, but it is worthy of note that this small fraction of the CTh cells reached stage IV in about three hours, more quickly than any of the other cell types.

The MVPK cells reacted more slowly to infection than the other cell types. The majority of the cells did not reach stage IV until seven hours postinfection. Some MVPK cells were still in stage II at 12 hours postinfection. The other pig kidney cell line, PK-15, responded steadily to infection with most cells reaching stage IV by five hours.

The correlation of surface and internal cellular changes in infected cells is shown in Table II. The descriptions given refer to all the cell types.

DISCUSSION

The primary and continuous line cell cultures passed through similar internal and external changes in appearance as infection with FMDV progressed and these changes occurred at somewhat different rates for each culture. Primary CTh cells were the most sensitive to infection of the cells tested because they produced visually detectable viral progeny at the earliest time. However, less than 20% of the CTh cells were modified after infection whereas the majority of the other cell types passed through the four stages of infection. It is of interest that primary CTh cells have been reported to lose their susceptibility to infection with FMDV at the third cell passage (12). We assume that the small fraction of susceptible cells in the heterogeneous primary population does not survive repeated cell passage.

The apparently homogeneous cells of the MVPK continuous line were also variable in their response to infection as indicated by the presence of some stage II cells at 12 hours postinfection. A subpopulation of these cells seems to remain frozen in an early stage of infection for as yet unexplainable reasons. Culturing these individual cell subpopulations of greater and lesser sensitivity to viral infection would obviously be of value in experiments designed to understand their behavior, but as with the CTh cells, such passaging may not be feasible.

The kinetics of appearance of the immune-labeled viral-induced RNA polymerase in infected cells agrees with previous results for this enzyme activity shown in baby hamster kidney (BHK) cells (16). Enzyme activity in BHK cells peaked at about three and one-half hours and then decreased as infected cells became rounded and formed many virus-containing buds (Stage IV) at five hours postinfection. The concomitant loss of activity and immune response suggests that the viral-induced polymerase protein was probably being degraded in addition to its possible dissociation from the active *in vivo* RNA replication complex (17).

The studies reported here used cells infected at a high multiplicity of infection to insure synchronized infection. The results can be of value in analyzing nonsynchronized natural and induced infections of animals in which the tissues have been exposed to low multiplicities of virus. Previous examination of tissues from infected guinea pigs revealed a need for establishing known stages of cellular infection (14). Ultrastructural examination of such infected tissues would help establish the presence and stage of infection by comparison to known infected cells.

TABLE II. Correlation of Internal Changes with Surface Appearance of FMDV-Infected Cells[a]

Cell Surface	Internal Change
Stage I: Flattened, close contact with neighboring cells and appearance of microvilli	A few smooth membranous vacuoles (SMV), lined with viral RNA polymerase
Stage II: Rounding, with microvilli disappearing and with cytoplasmic strand attachments between cells	Increased amount of SMV with accompanying RNA polymerase
Stage III: Rounded, smooth and separated from neighboring cells	About 50% of cell cytoplasm is filled with SMV. Peak amount of RNA polymerase
Stage IV: Rounded, separate and with numerous buds, some of which contain virus	Many SMV; many buds, some with viral particles inside. Decreasing amount of RNA polyerase

[a]Bovine kidney, Mengeling-Vaughn pig kidney, PK-15 pig kidney and calf thyroid culture cells were infected with FMDV types A_{12} and O_1 Brugge as described in Materials and Methods

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