Growth of Foot-and-Mouth Disease Virus in the Different Layers of Bovine Omasum in Suspended Cultures

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When suspended cultures of bovine omasum were cultured without agitation, the epithelium soon degenerated and foot-and-mouth disease virus multiplied mainly in the corium cells. Five days of preincubation were needed to reach a population of corium cells that could yield virus at a titer of 10^{8.75} to 10^{8.95} mean tissue culture infective doses per ml. The virus was freely released from the cells into the medium only when the degenerated epithelium was removed from the subepithelial tissue prior to virus inoculation. In agitated cultures, the viability of the epithelium was retained, the virus multiplied in all the layers of the epithelium and was freely released into the medium, and a virus titer of 10^{8.85} mean tissue culture infective doses per ml was obtained without preincubation. The omasal laminae could be separated along the line of apposition of the two mucous membranes of the organ. The virus yield from these thin separated membranes was 0.5 to 1.0 log higher than that obtained from nonseparated laminae.

In our earlier paper (4), the cultivation of foot-and-mouth disease virus (FMDV) type A in suspended cultures of bovine reticulum and omasum was reported. A later communication (5) dealt with the cultivation of FMDV types O, A, and C in these cultures and with the dynamics of virus production and release. These tissues, which are histologically similar to the tongue epithelium, were discussed as to their potentialities for FMDV vaccine production in reference to the Frenkel method (4).

The present paper describes the histological changes of omasal tissue occurring under different culturing conditions and their relation to the dynamics of FMDV growth and release. Cultures of omasal tissue as a whole and of the different layers were compared as to their capacity to support growth of FMDV.

MATERIALS AND METHODS

Virus. FMDV type A_{12} (Near East variant 1964) at the fifth passage level in baby hamster kidney cell line BHK-21 was used.

Media. (i) The medium for the omasal cultures consisted of Earle balanced salt solution containing 1% glucose, 0.5% lactalbumin hydrolysate, and 0.01% yeast extract. No serum was added. Penicillin, streptomycin, kanamycin, neomycin, and mycostatin were added at a concentration of 1,000 IU, 1,000 µg, 25 µg, 20 µg, and 100 IU per ml, respectively. The pH was 7.15 to 7.20. (ii) The medium for transporting and washing the tissue was phosphate-buffered saline without Ca^{2+} and Mg^{2+} (1). (iii) For growth of BHK-21 cells, modified Eagle medium (3) was used with 10% calf serum. Maintenance medium for virus titration contained 2% inactivated horse serum.

Quaternary ammonium bromide. This is an aqueous solution containing 10% didecyl dimethyl ammonium bromide and 30% ethyl alcohol. This compound was used as an antifoam and disinfecting agent, at a final concentration of 50 µg/ml in phosphate-buffered saline. Quaternary ammonium bromide is a joint product of Bromine Compounds Ltd. and Abic Ltd.

Preparation of the tissue, virus cultivation and virus assay. The omasal tissue was collected immediately after slaughter and transported in cold phosphate-buffered saline at 1:3 (wt/vol). Twenty grams of tissue cut into pieces of 7 to 10 cm in length and 5 to 7 cm in width were washed with 100 ml of phosphate-buffered saline in 1-liter Erlenmeyer flasks that were shaken for 10 min at 37 C. This was repeated three times, and then 100 ml of medium was added to the washed tissue in each flask.

Viral inoculum of 2 ml per flask (titer of 10^{8.95}/ml) was added either immediately or after a period of incubation (preincubation). In the latter case, the medium was changed prior to virus inoculation.

The cultures were incubated at 37 C either without agitation or agitated by a homemade shaker moving horizontally back and forth with an amplitude of 2 cm. Two speeds were employed, 152 and 456 movements (one direction) per min.

Each sample for virus assay consisted of a pool from two cultures. All samples were stored at -70 C until titration.

Extraction of tissue-associated virus (TAV) by freezing and thawing was done as previously described (5).
For each of the conditions tested for influence on virus growth, a series of three experiments was carried out with three different omasses. As in each series of experiments different maximal virus titers were obtained, the experiment in which the maximal virus titer was intermediate was selected to represent the entire series.

Titration of virus was performed in BHK-21 cells in tubes, and the mean tissue culture infective dose was calculated by the method of Kärber (2) and expressed in log base 10 per milliliter.

**Histological examination.** Morphological aspects of cells were examined in conventional paraffin sections stained with hematoxylin and eosin.

**RESULTS**

The behavior of uninoculated omasal tissue under various conditions of culture. After 20 to 24 h of incubation of nonagitated cultures (stationary cultures), the epithelium generally detached from the subepithelial tissue (Fig. 1) and could then be peeled off. In some cultures, 48 to 72 h of incubation were needed before complete removal of the epithelium could be achieved. These differences were found to be related to the freshness of the tissue.

In stationary cultures incubated for 24 h, the epithelium could be removed more easily and completely when the culture flasks were completely filled with medium (1 liter) rather than with the standard of 100 ml of medium. A similar effect resulted when the pH of the medium (standard volume) was maintained above 7.3 throughout the first 24 h of incubation by adding a 7.5% solution of NaHCO₃.

On the other hand, prolonged adherence of the epithelial tissue (up to 72 h of incubation) was observed when the air-medium interface was enlarged by using culture flasks of the standard volume (1 liter) but with a bottom 1.5 times larger in diameter than the standard flasks. A similar effect of prolonged adherence of the epithelium resulted when the tissue was treated prior to culturing with 0.25% trypsin for 30 min at 37°C without stirring.

When the cultures were incubated under agitation (shaking), the entire epithelium remained firmly adherent to the subepithelial tissue for at least 48 h with slow agitation and for at least 96 h with fast agitation.

An early effect of agitation could be observed histologically after 2.5 h of incubation, namely, the dilaceration of the keratinous layers from the epithelium (Fig. 2).

The behavior of the tissue in stationary and agitated cultures after 2.5 and 24 h of incubation is shown in Fig. 1–3.

When fast agitation was employed, foam formation was observed in vessels containing the culture but not in vessels containing medium without tissue. Foam formation could be prevented when quaternary ammonium bromide was incorporated into the phosphate-buffered saline used for the first washing cycle at a final concentration of 50 µg/ml. This treatment resulted in a nearly 100% reduction of bacterial and fungal contamination present in the washing fluid of untreated controls.

**Stationary cultures.** Growth of FMDV in

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**Fig. 1.** Tissue from stationary culture after 24 h of incubation. The epithelium has become detached from the subepithelial tissue. The keratinous layers are dilacerated and in some places detached from the epithelium. ×63.
FIG. 2. (a) Tissue from stationary culture after 2.5 h of incubation. The keratinous layers are still intact. ×63. (b) Tissue from agitated culture after 2.5 h of incubation. Note the dilaceration of the keratinous layers. ×63.
the different tissue layers and its distribution in tissue and medium. After 24 h of stationary culturing, cultures to be tested for virus growth were divided into three groups. One group consisted of whole omasal tissue, whereas the other two groups were obtained by splitting mother cultures into separate cultures, i.e., epithelium and subepithelial tissue. Virus inoculation was done at intervals of 24 h during preincubation, commencing at the time of splitting the cultures and continuing up to a total of 120 h (Fig. 4).

During preincubation, proliferation of corium cells was histologically observed both in cultures of whole and subepithelial tissue. Virus growth was demonstrated in cultures of subepithelial tissue and of whole tissue. No virus growth was detected in cultures of detached epithelium.

In cultures of whole tissue, significant amounts of TAV (0.75 to 1.25 log) could be extracted by freezing and thawing. This could be obtained after 48 h of preincubation. In cultures of subepithelial tissue, no significant amounts of TAV could be extracted.

A comparison of the maximal virus titers (virus in medium or TAV) obtained in each kind of culture at each stage of preincubation shows that the ability of whole tissue and subepithelial tissue to produce virus was mate-

![Fig. 3. Tissue from agitated culture after 24 h of incubation. The epithelium is still adherent to the subepithelial tissue. The keratinous layers have become entirely detached. The uppermost dark layer is the stratum lucidum of the epithelium. ×63.](image-url)

![Fig. 4. Growth of FMDV in stationary cultures. The diagram shows the effect of preincubation on virus growth in cultures of whole tissue and subepithelial tissue and virus distribution in medium (MV) and in tissue (TAV). Sampling for virus assay was done at 20 h after inoculation.](image-url)
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...ually the same, the difference between them being in the distribution of the virus in the tissue and medium.

Agitated cultures. Growth of FMDV and its distribution in tissue and medium. Figure 5 shows the growth of FMDV in omasal cultures of whole tissue incubated without agitation as well as under slow and fast agitation.

Two characteristics of virus growth were profoundly affected by agitation, i.e., the maximal virus titer obtained and the time in hours after inoculation at which this peak occurred. This observation was further supported by employing agitation of different intensity. Thus, without preincubation, the maximal virus titers obtained were $10^{3.20}$ (at 32 h after inoculation) in stationary cultures, $10^{5.20}$ (at 28 h after inoculation) in slow agitated cultures, and $10^{8.70}$ (at 20 h after inoculation) in rapidly agitated cultures. Comparison of the results obtained in stationary and slowly agitated cultures shows that the effect of agitation in favoring higher virus yield was more pronounced and reflected throughout the growth curve when preincubation of 24 h was employed.

Figure 6 shows the prolonged duration of virus production in slowly agitated cultures of whole tissue. No significant amounts of TAV

![Figure 5](image)

**Fig. 5.** Growth of FMDV in stationary and agitated cultures. The growth curves of virus present in the medium in rapidly agitated (Fa. ag.), slowly agitated (Sl. ag.) and stationary (Sta) cultures are compared. The figures on the growth curves (0, 24) indicate the time of preincubation in hours.

![Figure 6](image)

**Fig. 6.** Growth of FMDV in slowly agitated cultures preincubated for 24 h. A comparison of virus content in medium (MV) and in tissue (TAV) is shown.
could be extracted when samples were taken at 4-h intervals during the period from 20 to 44 h after inoculation. Thus, maximal virus titers obtained at the various times (whether virus in medium or TAV) did not differ by more than 0.25 log.

In these cultures, all the layers of the epithelium were engaged in virus synthesis as observed by the cytopathic effect in histological sections (Fig. 7).

**Separation of the omasal laminae and its effect on virus growth in agitated cultures.** Each omasal lamina consists of two mucous membranes, the deeper layer of connective tissue of both membranes being in apposition and loosely connected. The two membranes can be mechanically separated along the line of junction (Fig. 8). This separation, which usually proceeds easily and smoothly, yields two membranes, each being about one-half the thickness of the original lamina and consisting of all the elements of the omasal mucous membrane except for some degree of damage in the deeper layer caused by the separation.

In this series of trials, separation was the first step taken at the laboratory in preparing the tissue for culturing. Cultures of separated and nonseparated tissue of the same weight were tested for virus yield (virus in medium). In addition, changing the entire medium at inter-

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**Fig. 7.** (a) Tissue from agitated culture after 72 h of incubation. \(\times63\). (b) Same tissue 48 h after virus inoculation. Arrows point to the cytopathic effect in all the layers of the epithelium. \(\times63\).
vals of 4 h, as against retaining the original medium, was tested for its effect on virus yield in cultures of separated tissue.

Separated tissue was superior to non-separated tissue in its ability to support virus growth (Fig. 9). This was so during the entire period from 20 to 32 h after inoculation, the difference in virus titers ranging from 0.5 to 1.0 log.

In addition, in separated tissue, complete medium change affected only slightly virus titers tested at 4-h intervals.

**DISCUSSION**

The behavior of omasal tissue in culture could be physically and chemically affected to a great extent. The effect of each of the factors tested was reflected first in the state of the epithelial layer. Adherence of the epithelium to the subepithelial tissue was found to be a reliable indicator of cell viability. On the other hand, detachment of the epithelium from the subepithelial tissue was obviously due to a degenerative or a lethal process.

In agitated cultures, a virus titer of $10^{6.95}$/ml could be obtained without preincubation, whereas in stationary cultures 120 h of preincubation were needed to obtain similar virus titers (Fig. 4, 5). Agitation enhanced the viability of the epithelial cells, due probably to better aeration of the cultures. In addition, agitation enhanced also the dilaceration of the keratinous layers (Fig. 2), thus rendering the exposed epithelial cells more accessible to oxygen and nutrients as well as to the infecting virus. This might explain the difference in time of preincubation (0 and 24 h) needed to reach similar virus titers in cultures agitated at high and low speeds (Fig. 5). In this connection the effect of trypsin and quaternary ammonium bromide could be attributed to removing proteinaceous material adhering to the epithelium.

In stationary cultures, significant amounts of TAV (from 0.75 to 1.25 log) could be extracted from cultures of whole tissue but not from cultures of subepithelial tissue. Under these conditions, the cover of nonviable epithelium prevents the free release of virus multiplying in the subepithelial tissue. Rupturing of the epithelium by freezing and thawing removes the obstacle to virus release. Virus multiplying in exposed subepithelial tissue is freely released into the medium (Fig. 4). In agitated cultures, no significant amounts of TAV could be extracted. Under these conditions, the virus multiplied in the viable epithelium, and there was no hindrance to its free release into the medium during the period from 20 to 44 h after inoculation (Fig. 6). As shown by the cytopathic effect (Fig. 7b), all the layers of the epithelium were engaged in virus synthesis, which provides an explanation for the prolonged high-yield virus production (Fig. 6, 9). This factor is as practically important for vaccine production, since by several complete medium changes the total virus yield is considerably higher.

The effect of separation of the omasal laminae in promoting higher virus yield might be

![Fig. 8. Longitudinal section of omasal lamina showing the mucous membrane on either border. The arrows point to the line of separation of the two membranes composing the lamina. ×25.](http://aem.asm.org/)

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Fig. 9. Growth of FMDV in cultures of separated and nonseparated tissue, slowly agitated and preincubated for 24 h. Growth curves of virus present in the medium were obtained by sampling at 4-h intervals. The yield of virus in the medium at the same time intervals, but after complete medium change (MC), was tested in cultures of separated tissue only.

explained by a more efficient diffusion of oxygen and nutrients in a thinner tissue.

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