Effects of Pancreatin on the Growth of Reovirus

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ABSTRACT

WALLIS, CRAIG (Baylor University College of Medicine, Houston, Tex.), JOSEPH L. MELNICK, AND FRED RAPP. Effects of pancreatin on the growth of reovirus. J. Bacteriol. 92:155-160. 1966.—The influence of pancreatin and other proteolytic enzymes on the growth, plaque formation, and antigenicity of reovirus was studied. Single-cycle yields of virus in the presence of enzyme were not increased, but multiple-cycle yields of virus were greatly enhanced. Immunofluorescence studies demonstrated that the transmission of reovirus from cell to cell is more rapid in the presence of the enzyme. These findings led to the development of a rapid plaque assay system for reovirus, a virus which has previously been difficult to assay by the plaque method. In the recommended procedure, pancreatin is incorporated into the agar overlay. Monkeys immunized with enzyme-treated reovirus yielded higher antibody titers than animals receiving the same amount of untreated virus.

The low plaquing efficiency of reoviruses has been attributed to inhibitors in the agar overlay (9). However, since the proteolytic enzymes have been shown to enhance the infectivity of reovirus (2, 6), a protease covering on the virus may be the responsible factor in suppressing cell-to-cell transmission under agar. This protein may also explain the long period required to grow reovirus in cells bathed in fluid medium. Previous electron microscope studies (5, 7) have actually revealed the virus to be embedded in a matrix as it is formed in the course of its replication cycle. The present study is concerned with the effect of proteolytic enzymes on the growth, plaque formation, and antigenicity of reovirus, aspects which have not been previously investigated.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys from immature rhesus monkeys were trypsinized, grown in M-H medium, and maintained in M-E medium, as described in detail elsewhere (4).

Viruses. Type 1 reoviruses, strains 716 and Lang, were grown in MK cultures maintained with M-E medium.

Virus assays. The viruses were assayed by plaque-counting methods, with results expressed as plaque-forming units (PFU). Overlay medium consisted of Earle’s salt solution, 0.4% NaHCO₃, 0.1% skim milk, 1:60,000 neutral red, and 1.5% agar (Difco). The preparation of the reagents for use in the overlay medium has been described (8).

Pancreatin. Buffered pancreatin tablets were each dissolved in 50 ml of distilled water, as described by the manufacturer (Oxoid, Ltd., distributed by Colab Laboratories, Inc., Chicago Heights, Ill.), and filtered through a 0.22-μm Millipore filter. This solution was frozen as undiluted stock until used, at which time it was diluted as indicated in the text below. This enzyme preparation gave reproducible results from lot to lot, whereas purified forms of pancreatin from other manufacturers tested at different levels did not give reproducible results. Other enzymes used in this study were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and were either two or three crystalline (except for lipase, which was reagent grade).

Immunofluorescence technique. Cells were grown on round 15-mm cover slips in petri dishes incubated in an atmosphere of 5% CO₂ at 37°C. When monolayers had formed, the cover slips were drained of growth medium, transferred to dry petri dishes, and inoculated with 0.1 ml of virus per cover slip. The virus was allowed to adsorb for 1 hr at 37°C, and then the cover slips were flooded with M-E medium, some with and some without pancreatin diluted 150-fold. Cells harvested for immunofluorescent staining were washed three times with warm tris(hydroxy-methyl)aminomethane (Tris) buffer (pH 7.4), air-dried, and fixed for 10 min in acetone. Viral antigen was detected by reacting the cells first with reovirus antisera in monkeys, and then with horse globulin that had been prepared against normal monkey serum globulin and labeled with fluorescein isothiocyanate. Detailed fractionation and labeling methods have been described (3).

RESULTS

Effect of pancreatin in overlay medium on plaque formation. The two reovirus strains were selected.
because they were known to differ in their plaque-forming ability. Without special treatment, the Lang strain fails to produce plaques, whereas the 716 strain produces tiny plaques about 1 to 2 mm in diameter in about 5 days. In the present experiments, 0.1 ml of each strain in serial 10-fold dilutions was inoculated onto drained cultures (in 1-oz bottles containing 10^6 cells), and after an adsorption period of 2 hr at 37 °C, representative cultures were overlaid with medium containing the pancreatin stocks that had been diluted 30 to 120 times. The results are shown in Table 1.

By the 3rd day after inoculation, the 716 strain had not produced plaques under the usual overlay; however, the virus titer in cultures containing 1:30 pancreatin in the overlay was 10^4.5 PFU/ml, and the diameter of plaques ranged from 2 to 5 mm. By the 6th day, the virus in the control cultures had attained a titer of 10^6.5 with 1- to 2-mm plaques as compared with 10^7.7 under the pancreatin-containing overlay, in which the plaques averaged 6 to 8 mm. Figure 1 illustrates the differences in plaque size found between control and pancreatin overlays on the 3rd day.

The Lang strain gave similar results. However, no plaques were visible in the pancreatin-free cultures even on the 6th day, whereas the titer under pancreatin-containing overlays was 10^4.4 PFU/ml with an average plaque diameter of 2 to 3 mm.

Trypsin and chymotrypsin were also tested, but the toxicity of these enzymes for MK cells precluded their routine use. At enzyme concentrations where plaque enhancement occurred, readings could only be made up to the 3rd day, since cell integrity was lost thereafter.

**Effect of pancreatin on reovirus during growth cycles under fluid medium.** These experiments were designed to test the effect of the enzyme on the growth of the virus in single and multiple cycles of replication.

Strain 716 was inoculated at a multiplicity of 1 PFU per cell onto drained 1-oz cultures, containing 10^6 cells. After an adsorption period at 37 °C for 2 hr, the cultures were repeatedly washed to remove unadsorbed virus. Some of the cultures were then held in M-E medium at 37 °C. Duplicate cultures were maintained in the same medium containing pancreatin diluted 150 times, which is well beyond the toxic concentration of enzyme for the cells. Representative cultures were frozen at different intervals, beginning at 2 hr and ending at 16 hr after the addition of virus. Harvests were assayed by thawing cultures, centrifuging them lightly for a few minutes, and plating the clarified supernatant fluids under pancreatin overlays. In these experiments on the single growth cycle, no differences between the pancreatin-treated and enzyme-free cultures were detected. Newly formed virus was first detected after 10 hr in both enzyme-free and control pancreatin-treated cultures; both stocks were similar in titer (10^6 PFU/ml). At 12 hr, titers of 10^6.5 and 10^6.4 (control and pancreatin) were obtained, and at 16 hr 10^6.3 and 10^6.3, respectively.

Multiple-cycle experiments were performed in similar fashion to those above. The input of virus was 10 PFU per culture of 10^6 cells, and harvests were collected at 2 hr through 6 days. The results of a typical experiment are illustrated in Fig. 2. Cytopathic effects (CPE) were first evident in the pancreatin-containing cultures on the 2nd day, at which time 10% of the cells were involved. Infected pancreatin-free cultures showed similar degrees of CPE only on the 4th day.

Virus harvested 24 hr after inoculation contained 10^6.0 PFU/ml in the infected control cultures and 10^6.4 PFU/ml in cultures maintained.

![Table 1. Effect of pancreatin on plaque titers and diameters of type 1 reoviruses](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Overlay</th>
<th>Plaque titer (PFU/ml)</th>
<th>Plaque size (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Strain 716</td>
<td>Lang</td>
</tr>
<tr>
<td></td>
<td>3rd day</td>
<td>6th day</td>
</tr>
<tr>
<td>No added enzyme</td>
<td>&lt;2.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Pancreatin added</td>
<td>1:30</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>1:60</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>1:120</td>
<td>&lt;2.0</td>
</tr>
</tbody>
</table>

* Viruses were inoculated onto drained MK cultures at various dilutions and, after 2 hr of adsorption at 37 °C, cultures were overlaid with control medium (see Materials and Methods) or medium containing pancreatin at dilutions indicated. Dilutions of 1:15 caused slight toxic effects on cells, and higher concentrations destroyed them.

* About 100 plaques were counted to determine average diameter.
with pancreatin. However the 2nd-day harvests were significantly different. Infected control cultures manifested titers of $10^{4.3}$, whereas the cultures containing pancreatin increased to $10^{5.0}$. The maximal titer of $10^{5.7}$ PFU/ml was reached in pancreatin cultures on the 4th day, as compared with only $10^{4.4}$ PFU/ml, which was not reached in the infected control cultures until the 6th day.

The effect of the enzyme on the production of viral antigen was also studied by immunofluorescence. Different dilutions of reovirus were adsorbed to monolayers on cover slips. After an adsorption period of 1 hr at 37°C, the cultures were rinsed repeatedly, and a series of cover slips were maintained in M-E medium. A duplicate series was made with medium containing 1:150 pancreatin. Cover slips were fixed at different times, reacted with antireovirus antiserum, and examined for fluorescence. The results of this experiment are shown in Table 2. In the pancreatin-containing cultures, the number of infected cells greatly exceeded the number of positive cells in the enzyme-free cultures. Thus, at an inoculum of $10^{-4}$, on day 2 only 1% of the cultures without added enzyme were positive, in contrast to 50% of the cells in the pancreatin-treated cultures. In the presence of pancreatin, cells showed such advanced cytopathic effects 2 days after inoculation of high doses of virus and 3 days after inoculation of even the low doses that immunofluorescence readings were not possible.

**Effect of pancreatin on adsorption of reovirus.** When reovirus is treated with trypsin or other proteolytic enzymes and then plated, the infectivity of the sample is increased over untreated samples (2, 6), suggesting that the enzyme acts by removing inhibitors from the virus surface. However, from the results of the multiple-cycle yields shown above, pancreatin might have acted by enhancing virus adsorption. This was tested experimentally.

Cultures were drained of medium and inoculated with 0.1 ml containing 5,000 PFU of reo-

![Figure 1](http://jb.asm.org/)

**Fig. 1.** Effect of pancreatin on plaque formation of reovirus (716 strain). Photographed 3rd day after inoculation. Left culture: inoculated with 20 to 40 PFU of reovirus; overlay made with pancreatin-free medium. Right culture: same inoculum, but pancreatin (1:50) included in overlay medium.

![Figure 2](http://jb.asm.org/)

**Fig. 2.** Effect of pancreatin on multiple-cycle yields of reovirus. The 716 strain was inoculated into drained cultures with an input of about 10 PFU per culture. Cultures were maintained with M-E medium, and M-E medium containing the enzyme (1:150).

**Table 2.** Fluorescence studies with reovirus (strain 716)

<table>
<thead>
<tr>
<th>Virus inoculum</th>
<th>Percentage of cells exhibiting fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cultures without added pancreatin</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>25</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>1</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0</td>
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</tbody>
</table>

* Unsatisfactory because of advanced cytopathic effects.
virus (716 strain), determined on the basis of previous titrations with the usual overlay. Duplicate drained cultures were inoculated with virus suspended in medium containing 1:10 pancreatin. After adsorption periods up to 40 min, 1 ml of Tris buffer was added to the cultures, and unadsorbed virus was recovered after rotary agitation of the diluent over the monolayer. The amount of virus recovered was the same, regardless of whether pancreatin had been present during adsorption.

Another experiment was performed with cells pretreated with pancreatin. After 2 hr of treatment at 37 C, the cell layer was repeatedly rinsed to remove residual pancreatin, and cultures were then inoculated with 10 to 20 PFU of virus contained in a 0.1-ml volume of M-E medium. Cultures pretreated with pancreatin manifested no enhancement of plaque counts.

**Penetration of virus.** Since there is no enhanced adsorption of virus which could be detected in the presence of enzyme, the possibility of more rapid penetration of the virus into cells was investigated. A typical experiment follows. Reovirus was diluted to contain 100 PFU/0.1 ml in (i) 1:10 pancreatin and (ii) pancreatin-free diluent. These suspensions were used to inoculate a large number of drained cultures. After adsorption periods ranging from 15 to 480 min, cultures were washed repeatedly to remove unadsorbed virus, and they were then treated with a concentration of reovirus antiserum capable of neutralizing 10,000 PFU, to neutralize the virus that had adsorbed but had not yet penetrated the cell. After 1 hr of incubation at 37 C, cultures were washed to remove free antibody. The cultures were then provided with maintenance medium, and were immediately frozen and thawed. The clarified supernatant fluids of these harvests were assayed to compare the amount of cell-associated virus beyond the reach of antibody but which had not yet been "eclipsed." The infected cultures with or without pancreatin yielded harvests that had insignificant differences in their virus content. Thus, the effects of pancreatin are apparently not due to enhanced cell penetration.

**Effects of pancreatin on harvests obtained at different stages in the growth cycle.** The following experiment was performed to determine when in the stage of virus growth the enzyme was effective. Reovirus grown in single-cycle yields (described above) in M-E medium was used as the source of virus. Different harvests (taken from 2 to 14 hr postinfection) were diluted 10-fold in (i) Hanks salt solution and (ii) 1:10 pancreatin in salt solution. Samples were held at 37 C for 1 hr and then assayed. The 10-hr harvest was the first harvest showing newly formed virus, and it was activated 30-fold by enzyme treatment. The 12-hr harvest was enhanced 25-fold by pancreatin treatment, and the 14-hr harvest, 40-fold. Thus, virus formed at different times during the single cycle of growth could be enhanced to approximately the same degree.

The following experiment was performed to determine whether the inhibitor was bound to reovirus during the intracellular replication phase or only after virus release.

Reovirus (716 strain) was inoculated onto drained cultures at an input of 1 PFU/cell. After 2 hr of adsorption at 37 C, the cells were washed repeatedly, and then M-E maintenance medium was added. After 24 hr, the fluid phase was harvested, and the cells were washed and harvested separately. Virus was released from the cells by freezing and thawing. The fluid and cell-associated samples were then diluted 10-fold in (i) 1:10 pancreatin or (ii) Hanks salt solution. Both samples were activated to the same degree (25- and 30-fold, respectively), which indicates that intracellular and extracellular virus are equally sensitive to the action of the enzyme.

In some experiments, a small input of 10 PFU per culture was used, and multiple-cycle harvests were collected after 4 days of incubation. In confirmation of the results of Spendlove and Schaffer (6), the extracellular virus in the late harvest could not be activated by pancreatin, whereas the late intracellular virus was readily activated 25-fold.

**Effect of enzymes on the antigenicity of reovirus.** In view of the enhanced infectivity described above, viral antigenicity was also tested in animals inoculated with enzyme-treated virus. The antigenicity of reovirus in 2 M MgCl2 was also tested, as this salt has also been shown to enhance infectivity (9). Reovirus (716 strain) was diluted with an equal volume of (i) Hanks salt solution, (ii) stock pancreatin, (iii) 0.1% chymotrypsin, (iv) 0.1% trypsin, (v) 0.1% papain, (vi) 0.1% lipase, and (vii) 4 M MgCl2. The samples in the enzymes were held at 37 C for 1 hr, and representative samples in MgCl2 were heated at 50 C for 15 min. All samples were then assayed for virus, and in addition 2-ml portions were injected intramuscularly into young rhesus monkeys. Preinoculation sera were taken from all the monkeys, and none was found to neutralize reovirus. After 10 days, all animals were bled and given a booster injection of the same inoculum used initially; a second bleeding was made 10 days thereafter. The results of the antibody tests are shown in Table 3.

Assay of antigens used for injection of the mon-
keys showed an activation multiplicity of 30 times for reovirus treated with pancreatin, 6 times if treated with trypsin, 30 times with chymotrypsin, 3 times with lipase, 10 times with papain, and 10 times for the sample heated in MgCl₂. The antibody response in monkeys was consistently higher if the animals received treated samples that showed enhanced infectivity. The three monkeys injected with untreated virus having a titer of 10⁶ PFU/ml yielded neutralizing titers of <1:10 to 1:40 at the first postinjection bleeding and 1:80 to 1:160 at the second bleeding. The monkey inoculated with pancreatin-treated virus (30-fold activation) had titers of 1:80 and 1:320 (first bleeding) and 1:320 and 1:640 (second bleeding); trypsin-treated virus (sixfold activation) elicited titers of 1:160 and 1:320; chymotrypsin (30 times activation), 1:80 and 1:640; papain (10 times activation), 1:80 and 1:320; lipase (three times activation), <1:10 and 1:80. The sample heated in MgCl₂ (10 times activation) induced a serum titer of 1:80 at the first bleeding. Prior to the second bleeding, this monkey died, but not due to viral infection. The monkey immunized with virus treated with unheated MgCl₂ (which did not increase viral infectivity) had a low antibody response similar to the animals receiving untreated virus.

**Discussion**

The addition of pancreatin to the agar overlay results in the development of large reovirus plaques in rhesus kidney cells within 3 days after inoculation. In the presence of enzyme, the cell-to-cell transmission of the virus is greatly facilitated, and during multiple cycles of viral replication the yield of infectious virus is higher than in cultures maintained in the absence of enzyme. Enhancement of reovirus infectivity after exposure to either MgCl₂ (9) or to proteolytic enzyme (2, 6) has been noted previously. Spendlove and Schaffer (6) suggested that the enzymatic enhancement of reovirus infectivity was due to proteolytic action upon a virus-associated substrate. The present study indicates that pancreatin does not appear to enhance the adsorption of reovirus to the monkey cells, but that cell-to-cell transmission is enhanced in the presence of the enzyme, as shown by increased plaque size and the more rapid spread of viral antigens in the presence of pancreatin. The pancreatin may be activating noninfectious virus. One possible mechanism of activation might be the removal of the virus surface of inhibitors that interfere with penetration or uncoating of the virus. Experiments carried out in this study did not indicate that pancreatin enhanced reovirus penetration into cells more rapidly than untreated virus, nor was there any evidence of an earlier eclipse phase with pancreatin than in its absence.

The possibility exists that the enzyme is destroying interferon. However, Spendlove and Schaffer (6) showed that pelleted virus (presumably free from interferon) could still be activated by enzymes. Furthermore, in the present study we found that virus stocks diluted 10,000-fold, well beyond the range of interferon activity, were still activated by treatment with pancreatin.
Reovirus treated with enzymes was a more potent antigen in monkeys than untreated virus. The increased antibody response may be a reflection of the higher infectivity of the treated virus.

Experiments on virus harvested after multiple cycles of growth showed that intracellular virus was activated by pancreatin to the same degree as the extracellular virus obtained from a single cycle of growth. However, the extracellular virus obtained after multiple (but not single) cycles of growth showed little or no activation by the enzyme. This confirms the finding of Spendlove and Schaffer (6), who had shown that late fluid harvests were not amenable to activation with enzymes. However, by replacing the culture fluids with fresh nutrient just before release of virus into the medium, they obtained enzyme-sensitive virus. It seems to us that extracellular virus may be already enhanced by the proteolytic enzymes produced by the cells in the culture, and thus the effects of exogenously added enzymes would not be detectable. Production of proteolytic enzymes by MK cultures has been described by Baron and Barnett (1).

Although many of the observations reported in this study cannot be explained at the present time, the fact that treatment of reovirus with pancreatin is followed by the enhancement of reovirus infectivity and the formation of large and reproducible plaques within 3 days after the inoculation of monkey cells should prove useful for those working with these agents.

Acknowledgments

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Literature Cited