Inactivation of Foot-and-Mouth Disease Virus Vaccine Strains by Activation of Virus-associated Endonuclease

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SUMMARY
A new inactivation process for foot-and-mouth disease virus (FMDV) has been developed. This process is based on the activation of the FMDV endonuclease by incubation of unfractionated viral suspension or purified virions at 37 °C in the presence of high concentrations of monovalent cations such as K⁺, Cs⁺ or NH₄⁺ at pH 8.5. This procedure completely inactivated several FMDV vaccine strains yielding preparations having similar amounts of 140S particles to untreated controls. The inactivation followed first-order kinetics and the rate of inactivation was faster than that achieved with other agents, e.g. binary ethyleneimine. Testing in suckling mice or tissue culture revealed no residual infectivity after inactivation. Virus particles purified from inactivated preparations showed (i) the same sedimentation coefficient as non-inactivated preparations, (ii) electrophoretic patterns of their viral capsid proteins identical to those derived from non-inactivated preparations, and (iii) extensive degradation of the 35S viral RNA. This method is safer than inactivation with aziridines because only innocuous chemicals are used in the process.

INTRODUCTION
Foot-and-mouth disease virus (FMDV) has an endoribonuclease activity within purified virions (Denoya et al., 1978b). Evidence supporting the internal location of this enzymic activity was obtained by incubating the purified virions in NET buffer (100 mM-NaCl, 1 mM-EDTA, 50 mM-Tris-HCl, pH 7.4) under conditions that promoted degradation of the genomic RNA without alteration of the capsid structure; this degradation is not caused by external RNases (Denoya et al., 1978b). Under these conditions, the enzymic activity is very low and long incubation periods are required in order to detect an appreciable degradation of the genomic RNA (Denoya et al., 1978b). The RNA degradation apparently correlates with a loss of infectivity since, as shown by Brown & Wild (1966), purified virions lose one log₁₀ unit of infectivity after incubation for 8 h at 37 °C in 0.04 M-phosphate buffer pH 7.6. This rate of inactivation is unsuitable for a vaccine production process. Therefore, we decided to look for conditions capable of activating the enzyme inside undisrupted particles.

In this report, we describe the necessary conditions of incubation during which complete loss of infectivity is obtained without alteration of the physical and chemical properties of the protein components of the viral particles. This process has been proved to be appropriate for the inactivation of several FMDV vaccine strains.

METHODS

32P-labelled viral suspensions. Monolayers of baby hamster kidney cells (BHK-21 clone 13) were infected at 10 p.f.u./cell with FMDV type O, strain Caseros, as described by Denoya et al. (1978b). After adsorption, the monolayers were washed twice and maintained in phosphate-free Eagle's medium for 15 min, before the addition of 5 μg/ml actinomycin D. After 30 min, 200 mCi/ml[³²P]orthophosphoric acid (carrier-free, New England Nuclear)
were added. At 5 h post-infection the cells and the medium were frozen and then thawed to disrupt the cells. Nuclei and cellular debris were removed by centrifugation (3000 g, 5 min) and the supernatant used as \(^{32}\)P-labelled virus suspension.

**Virus purification and RNA analysis.** \(^{32}\)P-labelled virus suspensions were incubated at different pH and ionic conditions, as indicated in Results. After incubation, 2 ml of the viral suspensions were brought to 0.2% \(N\)-lauroyl-sarcosine (Sarkosyl), 20 mM-EDTA, pH 7.4, and layered on top of a 35 ml 10 to 30% (w/v) sucrose gradient made in NET buffer and centrifuged at 27000 r.p.m. for 3-5 h in an SW27 rotor (Beckman ultracentrifuge) at 4°C. One ml aliquots were collected from the bottom of the tube using a peristaltic pump. Fractions containing the 140S virus peak were pooled, made 0.5% in SDS and the RNA extracted twice with phenol–chloroform (Perry et al., 1972), ethanol-precipitated and analysed as described in Results.

**Innocuity tests.** Tests for residual infectivity were made in suckling mice and in BHK cells, as described in ‘CPFA Manual de Procedimientos para el Control de Vacuna Antiaftosa’ (1980) from the Foot-and-Mouth Disease Panamerican Center, Rio de Janeiro, Brazil. Alternatively, tissue culture innocuity tests were carried out using BHK monolayers (Anderson et al., 1970).

**Virus protein analysis.** Unlabelled suspensions of \(O\) Caseros virus were divided into two 10 ml aliquots. One was brought to 0.1 M-Tris-HCl, 0.5 M-NH₄Cl, pH 8.5 and incubated at 37°C for 24 h; the other aliquot was kept at approximately 0°C for the same period. Virus was purified from both samples and fractions corresponding to the 140S peak were pooled. Approximately 30 to 40 \(\mu\)g of purified virions were precipitated with 4 vol. acetone at -20°C for at least 12 h. Virus was collected by centrifugation (100000 g for 30 min) and the pellets were re-suspended in 20 pL electrophoresis buffer. Samples were then analysed on 12.5% SDS–polyacrylamide gels as described by Laemmli (1970). After electrophoresis the gels were fixed, stained with Coomassie Brilliant Blue and destained.

**RESULTS**

**Effect of pH on enzyme activity**

\(^{32}\)P-labelled virus suspensions, obtained as described in Methods, were brought to different pH values with NaOH and incubated for 4 h at 37°C; the virus was then purified and the RNA from the virus peak was extracted twice with hot phenol–chloroform and analysed on sucrose gradients as described in Methods. An increase of activity was obtained by increasing the pH by just 1 unit (Fig. 1). The sedimentation coefficient of 35S naked viral RNA was affected upon incubation at the highest pH used. Although some 35S RNA remained upon incubation at pH 8.5 for 4 h there was RNA cleavage under these conditions (Fig. 1d); pH values lower than 7.5 were not tested because of the known lability of FMDV below pH 7.0. On the other hand, pH values higher than 8.5 promoted some alkaline degradation of naked RNA controls (results not shown). When Tris–HCl (100 mM final concentration) was used instead of NaOH, a similar activation of the enzyme was obtained. However, in this case, the RNA degradation was more extensive than that obtained when NaOH was used, indicating a possible effect of the Tris buffer on the enzyme activity.

**Effect of ionic concentration on RNA degradation in situ**

The effect of different cations and ionic concentrations on the enzymic activity was studied. Fig. 2 shows the effect of increasing the \(\text{NH}_4\text{Cl}\) concentration on viral RNA degradation, and indicates that RNA degradation increased proportionally to the ammonium ion concentration, reaching a maximum at 500 mM-\(\text{NH}_4\text{Cl}\). A similar effect was observed at pH 7.5 and 8.0 (results not shown). By using \(\text{K}^+\) or \(\text{Cs}^+\) instead of \(\text{NH}_4^+\) identical results were obtained; \(\text{NaCl}\) did not promote activation (not shown).

In contrast to what is observed with disrupted particles, where the enzyme is strongly activated by bivalent cations such as \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) (M. A. Lebendiker et al., unpublished results), these ions had no effect on intact particles (data not shown). The reason for this difference between monovalent and bivalent cations is not known.

Similar results to those presented in Fig. 2 were obtained by incubating highly purified virions (free of any external nuclease contamination) in 100 mM-\(\text{NH}_4\text{Cl}\), 500 mM-Tris–HCl, pH 8.5, thus excluding the possibility of RNA degradation by external nucleases. The combined effect of higher pH and higher ionic strength on the nuclease activity produced a marked enhancement of the RNA degradation when compared with that obtained with our previous conditions of
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Fig. 1. Effect of pH on virus RNA degradation in situ. $^{32}$P-labelled virus suspensions in Eagle’s medium were brought to the desired pH by addition of 0.1 M NaOH and incubated for 4 h as follows: (a) pH 7.5, 0 °C; (b) pH 7.5, 37 °C; (c) pH 8.0, 37 °C; (d) pH 8.5, 37 °C. After incubation the virions were purified, the RNA extracted twice with phenol–chloroform and analysed in 17 ml 10 to 30% (w/v) sucrose gradients made in 1% NET-SDS buffer pH 7.4 (Beckman SW27.1, 17 h, 22000 r.p.m. at 20 °C). ○, Sedimentation gradient.

incubation (Denoya et al., 1978a, b). Under the present conditions, the virus RNA was extensively degraded after 4 h incubation (Fig. 2e) and the extent of degradation was greater than that obtained after 50 h incubation in NET buffer (Denoya et al., 1978b). The degradation profile shown in Fig. 2(e) seemed to be maximal, since no further degradation was observed after longer incubation periods (Fig. 2f).

The sedimentation coefficient and the amount of 140S particles in the incubated samples was the same as in the non-incubated controls.

Analysis of the capsid proteins of the inactivated FMDV particles

The possibility of breakdown of virus protein during the process of inactivation was assessed. Fig. 3 shows that there were no detectable changes in the protein pattern of the incubated virions, indicating that this method does not cause extensive degradation of the virus proteins.
Fig. 2. Effect of ionic concentration on virus RNA degradation in situ. $^{32}$P-labelled virus suspensions in Eagle's medium were incubated for 1 h at 37 °C in 1 mM-Tris-HCl pH 8.5, with NH$_4$Cl at the following concentrations: (a) 100 mM; (b) 250 mM; (c) 500 mM; (d) 750 mM. (e, f) 100 mM-Tris-HCl pH 8.5 with 500 mM-NH$_4$Cl at 37 °C for 4 h (e) or 14 h (f).

Fig. 3. Control of integrity of the structural proteins of inactivated virions using PAGE analysis of O$_1$ Caseros FMDV structural proteins. (a) Control; (b) inactivated by activation of viral endonuclease with 100 mM-Tris-HCl, 500 mM-NH$_4$Cl, pH 8.5.

**Correlation between RNA degradation and virus infectivity**

We determined the loss of infectivity with virus samples at different times of incubation with NH$_4$Cl and binary ethyleneimine (BEI; extensively used as an inactivating agent in FMDV vaccine production). As shown in the example presented in Fig. 4, NH$_4$Cl inactivation of O$_1$ Campos FMDV followed first-order kinetics and the rate of inactivation was faster than that achieved with BEI. In the absence of NH$_4$Cl and at 37 °C, pH 8.5 (Na$_2$CO$_3$) the inactivation was slower and much slower when only the ionic strength was increased (0.5 mM-NaCl) (not shown), indicating the possibility of a combined specific effect of pH and the ionic environment on the endonuclease activity. Samples were taken 20 h after incubation at 37 °C in 500 mM-NH$_4$Cl, 100 mM-Tris-HCl, pH 8.5 for innocuity tests. No c.p.e. was observed; similar results were obtained when the innocuity was tested in suckling mice.

Similar results were obtained with a large number of representative FMDV vaccine strains.
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Fig. 4. Rate of FMDV inactivation under different conditions. O1 Campos FMDV grown in suspensions of BHK cells (1.5 × 10^6 cells/ml) at 37 °C for 24 h was harvested after clarification by centrifugation (1500 g for 3 min). The infective virus suspensions were divided into aliquots which were treated in one of the following ways: incubated at 4 °C for 48 h (○); pH adjusted to 8.5 with Na_2CO_3 and incubated for 48 h at 37 °C (●); as for the latter but a solution of bromoethyleneamine added to form BEI, the virus suspension then incubated at 37 °C, a second addition of the inactivating agent made after 24 h and the sample kept at 37 °C for a further 24 h (▲); pH adjusted to 8.5 by addition of 0.11 vol. 1 M-Trizma base, 5.5 M-NH_4Cl and incubated at 37 °C for 48 h (△). Samples were taken at suitable time intervals (0, 1, 2, 3, 4, 5, 6, 16, 22, 24 and 48 h) for infectivity assays.

Table 1. Half-life of infectivity of FMDV in inactivation with aziridines or by action of virion-associated endonuclease

<table>
<thead>
<tr>
<th></th>
<th>Mean t_1/2 (min)</th>
<th>s.d.</th>
<th>n</th>
<th>Range</th>
<th>No. of strains</th>
<th>12 log_{10} units of virus (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEI</td>
<td>39.0</td>
<td>11.0</td>
<td>34</td>
<td>23.7-69.2</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>BEI</td>
<td>26.1</td>
<td>8.1</td>
<td>8</td>
<td>16.0-39.9</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>BEI (in situ)</td>
<td>41.1</td>
<td>9.8</td>
<td>35</td>
<td>29.1-68.8</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Endonuclease</td>
<td>18.1</td>
<td>5.6</td>
<td>9</td>
<td>12.0-32.0</td>
<td>9*</td>
<td>13</td>
</tr>
</tbody>
</table>

* O1 BFS 1860, O1 Campos, O1 Thailand, A22 Mahmatli, C1 Indaial, C Noville, SAT 1 Bot 1/68, SAT 2 Bot 3/77, SAT 2 SAR 3/79.

inactivated as clarified virus suspensions in Eagle’s BHK medium with added 0.1 M-Tris-HCl, 0.5 M-NH_4Cl, pH 8.5 (Table 1).

**DISCUSSION**

FMDV is inactivated either with formaldehyde, aziridines [acetyleneimine (AEI) or BEI] or 1,2-epoxypropanal for the preparation of vaccines. The process of virus inactivation with formaldehyde causes alterations of the fine capsid structure which affects the immunogenicity (Wild & Brown, 1968). Moreover, it has been recognized that formalin-inactivated vaccines might contain infective virus (Moosbrugger, 1948; Schneider, 1955) and some outbreaks have been attributed to the use of formaldehyde-treated vaccines (King et al., 1981).

Aziridines are better virus inactivators than formaldehyde, since the virus infectivity is destroyed through an inactivation reaction that follows first-order kinetics (Graves &
Arlinghaus, 1968). Although it has been stated by the same authors that aziridines destroy viral infectivity without any noticeable change in the capsid proteins, the high reactivity of these compounds makes it difficult to discount completely the aminoethylation of sulphydryl, amino and carboxyl groups of viral proteins (Bachrach & Vande Woude, 1968; Bachrach et al., 1975). Moreover, as recently reported, AEI inactivation may reduce the thermal stability of 140S particles (Doel & Baccarini, 1981).

For all these reasons, a new inactivation method based upon the activation of a virus-associated endonuclease was developed by our group; this is a biological process which does not affect the viral capsid proteins. We described previously an endonuclease associated with highly purified virions and found that this enzyme degraded the genomic RNA when virions were maintained at 37 °C. These findings encouraged us to search for conditions capable of activating the enzyme inside intact viral particles. The present communication shows that the enzymic activity was enhanced by increasing the pH of the incubation medium (Fig. 1) and that this activity could be further enhanced when the process was carried out at higher ionic strength (Fig. 2). High ionic strength by itself did not mediate activation of the endonuclease since high concentrations of NaCl had no effect on the enzymic activity. However, we found that K⁺ or Cs⁺ ions could replace NH₄⁺ with similar efficiency and by combining both effects (i.e. pH 8.5 plus higher ionic strength) the RNA could be completely degraded to small pieces (6S to 8S) in 4 h. In vitro conditions that activate the enzyme when disrupted virus preparations are used as a source of enzyme have also been defined (M. A. Lebendiker, unpublished results).

Under similar conditions of incubation, Ward (1978) reported RNA degradation in poliovirus. However, it was not clearly demonstrated that the observed degradation of the RNA was mediated by an enzyme and it would be of interest to investigate whether poliovirus has an endonuclease similar to the one we have characterized in FMDV (Denoya et al., 1978a, b). Also, Esonov & Nikitin (1967) described a method of inactivation of FMDV based on incubation at 37 °C, pH 9.0 for 36 h. Their method yielded immunogenic preparations, but it may have involved alkaline degradation of the genomic RNA.

The breakdown of the genomic RNA was correlated with a loss of viral infectivity (Fig. 4); the rate of inactivation followed first-order kinetics and was faster than that achieved with a currently used aziridine (BEI). Moreover, this process completely inactivated several representative FMDV vaccine strains (Table 1), yielding preparations of similar antigenic content to untreated controls. In some cases the inactivated virus antigens produced by this technique have been shown to be stable for a period of at least 6 months at 4 °C.

When analysed on SDS-polyacrylamide gels, no differences were found between the protein patterns of purified inactivated virions and those of standard untreated virions. Innocuity tests in suckling mice and plaque assays showed that there was no residual infectivity left after 20 h of inactivation.

Recently, new approaches to the production of FMDV vaccines have been developed, namely production of synthetic VP1 by genetic engineering (Boothroyd et al., 1981; Kleid et al., 1981; Kupper et al., 1981) or synthetic peptides (Bittle et al., 1982). However, as stated by Bittle et al. (1982), VP1 from Escherichia coli appears to be no more effective than that produced from virus particles and, clearly, there is much to be learnt about the structural features of the antigenic determinants necessary for effective immunization before VP1 can be considered as a realistic alternative for vaccine production. On the other hand, although the synthetic peptide vaccines may represent an improvement upon the standard vaccines, the economic feasibility of chemical synthesis appears to be difficult to judge at present. Therefore, it is likely that vaccines produced by conventional tissue culture methods will remain the mainstay for prophylaxis against FMDV for some time and inactivation methods like the one described here may be useful in their production. We are studying the feasibility of this inactivation process for industrial use in the production of FMDV vaccines.

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REFERENCES


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