

Inactivation of Foot-and-Mouth Disease Virus with Ethylenimine

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Foot-and-mouth disease virus (FMDV) was inactivated by ethylenimine (EI) at three concentrations and two temperatures. Comparison of inactivation kinetics and the antigenic and immunogenic potency of EI and *N*-acetylethylenimine (AEI)-inactivated FMDV indicates that EI has nearly optimal characteristics as an inactivant for FMDV vaccine preparation. Although AEI-inactivated FMDV has proved to be a potent specific immunogen, an equivalent percentage of EI inactivated FMDV at substantially faster rates and produced an equally potent immunogen. In addition, EI inactivated FMDV at rates that were essentially linear throughout the loss of nearly all measurable infectivity.

Inactivation of foot-and-mouth disease virus (FMDV) by ethylenimine (EI) derivatives for vaccine preparations has been described by several investigators (2, 6, 8, 10). Most recently, a number of experiments were described (R. E. Warrington, H. R. Cunliffe, and H. L. Bachrach, *Amer. J. Vet. Res.*, manuscript accepted for publication) that compare kinetic aspects of FMDV inactivation by EI derivatives, such as *N*-acetylethylenimine (AEI), as well as the immunogenic properties of their experimental products.

The present communication reports kinetic data for inactivation of FMDV by EI at two different temperatures and three concentrations. These data are compared with data from control samples of FMDV inactivated with AEI. In addition, antigenic and immunogenic properties of EI-inactivated FMDV are compared with those of AEI-inactivated FMDV.

MATERIALS AND METHODS

Virus preparations. FMDV, type O, subtype 1, strain Campos (FMDV, O Campos) was selected for study. Virus from the field sample was passed serially through six primary cultures of calf kidney cells (BK) grown in 4-oz (0.118 liter) prescription bottles. Two additional passages in baby hamster kidney cells (BHK-21, clone 13) were made to produce a pool of seed virus which was stored in 1-ml portions at -20°C . When needed for inactivation, a portion of seed virus was passed in roller cultures of BHK-21 cells. Virus was collected in the fluid medium after 18 to 24 h of culture, clarified by high-speed centrifugation, and stored at 4°C . Infectivity titers assayed by plaque enumeration (see below) ranged from 7.8 to 8.5 logs.

One such pool of virus was stored at -120°C in 1-ml portions and was used for mouse assays of virus-neutralizing (VN) antibody. Cell culture media and other details have been described elsewhere (R. E. Warrington, H. R. Cunliffe, and H. L. Bachrach, *Amer. J. Vet. Res.*, manuscript accepted for publication).

Before addition of inactivants, 100-ml virus portions in screw-capped serum bottles were equilibrated to the desired temperature in a constant-temperature water bath.

Inactivants. A sample of EI was generously provided by H. G. Bahnemann, Chief, Research, Pan American Foot-and-Mouth Disease Center, Rio de Janeiro, Brazil. Portions of EI in 15-ml amber bottles with tin-gasketed screw caps were stored at -20°C . A small NaOH pellet was placed in each bottle to maintain alkalinity. The AEI was obtained from Burroughs-Wellcome Laboratory, London, England. Both EI compounds were always handled in a well-ventilated room or small biological hood. Other precautions recommended by Dermer and Ham (5) were observed whenever possible.

Small volumes of EI or AEI were delivered by microliter syringe directly to virus samples. In most experiments, samples were taken at 30-min intervals after addition of the inactivant. However, where 0.05% EI was used at 37°C , samples were taken at twofold time increments (0, 10, 20, 40, and 80 min). A minimum of four samples was taken, but as many as 10 samples were taken in selected experiments in 30-min increments (0, 30, 60, 90 or 0, 30... 270 min).

Plaque assay procedure. Except where indicated below, the plaque assay procedure was essentially the same as that described by Bachrach et al. (1). The 0-h sample in all experiments was taken immediately after thoroughly mixing the inactivant and virus sample. To prepare initial dilutions for plaque assay, virus samples were mixed with an appropriate volume

of ice-cold Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate (HLH) and 2% sodium thiosulfate to neutralize the inactivant. Serial 5-fold dilutions in HLH were made from the initial dilution to obtain the desired set for each assay. A 0.1-ml sample of each test dilution was pipetted onto each of two BK cell cultures, but at dilutions where minimal plaque counts were expected, 10, 20, or 40 replicates were prepared. After 30 min at 37 C to permit virus adsorption, the BK cultures were overlaid with 5 ml of a warm HLH-gum tragacanth fluid medium (7) containing 4% fetal calf serum. After 48 h of incubation at 37 C, approximately 5 ml of a 0.1% aqueous solution of crystal violet containing 20% Formalin was added to each culture. The fixative stain was allowed to react for about 20 min, after which it was discarded, and the cultures were rinsed once with tap water. Infectivity end points were computed from plaque counts made from stained cultures and were expressed as the common logarithm of plaque-forming units (PFU) per milliliter of test material.

All inactivation data were computed by least squares regression methods, and results were tested for first- and second-degree polynomial regression by analysis of variance. Such computation data were obtained with a Hewlett-Packard no. 9810 calculator (Hewlett-Packard Co., Loveland, Colo.) by using their standard program for polynomial regressions.

Antigen and antibody assays. Complement fixation (CF) methods, described by Cowan and Trautman (3), were used to assay 140S virus particle antigen. Preliminary block titration CF reactions between serum from guinea pigs hyperimmunized against FMDV O Campos and its purified 140S antigen were used to determine an optimal serum dilution for titration of 140S antigen in selected, crude virus preparations.

The VN antibody activity of sera obtained from guinea pigs immunized with EI- or AEI-inactivated virus was assayed in mice by using methods previously described (4). VN antibody end points were expressed as the common logarithm of the reciprocal of serum dilutions computed to protect 50% of the mice inoculated with test mixtures containing 100 mouse mean lethal doses (LD_{50}) of FMDV O Campos.

Vaccine safety tests. For use in vaccine studies, portions of virus were reacted with EI or AEI for 48 h at 37 C or for 72 h at 25 C. For safety tests, 1-ml samples were taken at 24 h and at the end of scheduled inactivation periods. Each of 10 BK cultures was inoculated with 0.1 ml of sample. Fresh HLH was added to each culture after a 1-h adsorption period at 37 C. These cultures were incubated for 48 h and observed for a cytopathic effect (CPE) ascribable to FMDV. Positive cultures were immediately subcultured in BK cells, and identity of the recovered virus as FMDV was established by CF and VN procedures. If CPE was not observed by 48 h in the preliminary test, pooled fluids were passed into BK cell cultures and observed at 72 h for CPE. Absence of CPE in the second passage was accepted as confirmation that all FMDV O Campos infectivity had been inactivated. All tests on samples taken at 48 and 72 h were negative, although one 24-h sample (Table 1, experiment 1) was positive for FMDV O Campos.

TABLE 1. *Effect of temperature and EI concentration on inactivation rate of FMDV*

Expt no.	Temp C	% EI	Inactivation rate ^a	
			log	mean log
1	25	0.01	0.35	0.40
2	25	0.01	0.45	
3	37	0.01	1.21	1.48
4	37	0.01	1.60	
5	37	0.01	1.64	4.13
6	37	0.02	2.47	
7	37	0.05	4.09	
8	37	0.05	4.18	
9	37	0.05	3.97	
10	37	0.05	4.27	

^a Common logarithm of virus infectivity inactivated per h which was calculated from slope values computed by first-degree polynomial regression.

Inactivated virus antigen was emulsified in an equal volume of Freund incomplete adjuvant, and 1-ml doses were injected subcutaneously into guinea pigs.

RESULTS

Table 1 summarizes results from 10 experiments designed to study inactivation kinetics of FMDV by using three concentrations of EI and two temperatures. These data indicate that 0.05% EI at 37 C inactivated about 4.13 logs of FMDV infectivity per h, whereas the values for 0.02 and 0.01% EI are 2.47 and 1.48 logs, respectively. The mean inactivation rate for 0.01% EI at 25 C (Table 1) was 0.40 logs per h.

Portions of the same FMDV used in experiments 4, 6, and 7 (Table 1) were inactivated with 0.05% AEI for comparison of kinetic data and antigenic properties because AEI is the most frequently used EI derivative for preparation of experimental FMDV vaccines. The mean FMDV infectivity inactivation rate with 0.05% AEI at 37 C was 1.21 logs per h, which agrees with the value given by Graves and Arlinghaus (6) for FMDV inactivation by AEI under these conditions. Therefore, the data reported here indicate that 0.05% EI at 37 C inactivated FMDV about 3.4-fold faster than AEI under the same conditions. Also at 37 C, 0.01% EI inactivated FMDV 1.22-fold faster than 0.05% AEI, whereas 0.02% EI inactivated FMDV 2.04-fold faster than 0.05% AEI.

Because most EI experiments assayed FMDV infectivity regression of less than 5 logs, it was of interest to study inactivation kinetics for evidence of divergence from linearity throughout the loss of nearly all measurable FMDV infectivity. Thus, experiment 5 (Table 1) was de-

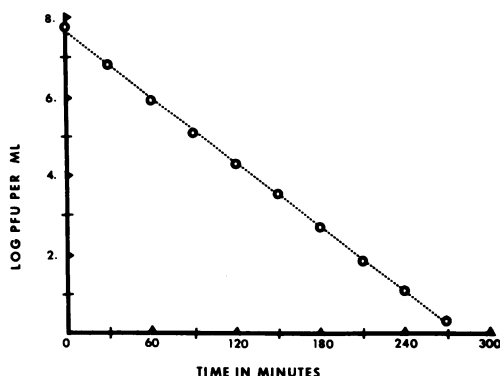


FIG. 1. Data from experiment 5 showing the inactivation of FMDV with 0.01% EI at 37 C ($b = -0.0274$ log units/min).

signed to assay FMDV inactivation by 0.01% EI at 37 C at 30-min intervals down to a desired level of about 1 PFU/ml. Figure 1 is a plot of these data showing a very close fit to linear regression from the zero-time sample (log 7.76 PFU/ml) to a final index representing 2 PFU/ml.

Table 2 summarizes CF data for 140S FMDV antigen before and after inactivation with EI or AEI. No substantial effects were shown by these assays, which could be attributable to degradation of 140S FMDV antigen by EI or AEI. It is of interest that in experiment 8 (Table 2), reactions with AEI and EI were allowed to continue at 37 C for 7 days. The CF data show a slight loss of 140S FMDV antigen for both inactivants, whereas the 37 C control virus showed about 50% loss of this antigen. A 4 C control sample showed no loss of 140S FMDV antigen when assayed in the CF test.

Table 3 summarizes results of assays for VN antibody in guinea pigs immunized with FMDV inactivated by EI or AEI. These data show that regardless of inactivation conditions, all FMDV antigen products contained sufficient immunogenic antigen to stimulate substantial levels of FMDV-neutralizing antibody in guinea pigs. It is especially noteworthy that EI- as well as AEI-inactivated FMDV was immunogenically potent after 7 days of treatment at 37 C (Table 3, experiment 9).

DISCUSSION

Results of these experiments indicate that FMDV inactivation by EI is essentially a linear reaction at rates substantially faster than with a similar percent solution of AEI. Although regression analyses detected very slight curvature of these data, at present the reaction is considered linear until more definitive experiments

TABLE 2. Effect of EI inactivation on FMDV complement-fixing antigenicity

Expt no.	Inactivant		Temp (C)	Cf titer ^a	
	% AEI	% EI		Before	After
2		0.01	25	48	64
4		0.01	37	48	64
4	0.05		37	48	64
8		0.05	37	64	48
8	0.05		37	64	48
8			37	64	64 ^b
8			4	64	64 ^c
9		0.05	37	56	56
9	0.05		37	56	56

^a Reciprocal of antigen dilution which fixed all but one of 5 CH₅₀ U of guinea pig complement. "Before" titer is the CF end point of antigen before addition of EI or AEI. "After" titer is the CF antigen end point after inactivation was complete as indicated by safety tests.

^b 37 C control.

^c 4 C control.

TABLE 3. Virus-neutralizing antibody in guinea pigs immunized with EI-inactivated FMDV

Vaccine expt no. ^a	Inactivant ^b	Days postvaccination		
		7	14	28
2	0.01% EI, 25 C	1.08 ^c	1.95	2.54
4	0.01% EI, 37 C	1.37	1.63	2.72
4	0.05% AEI, 37 C	1.11	1.84	2.91
8	0.05% EI, 37 C	0.68	1.08	2.60
8	0.05% AEI, 37 C	0.84	1.34	2.53
9	0.05% EI, 37 C	0.93	0.75	2.65
9	0.05% AEI, 37 C	1.12	0.89	2.55

^a Experiment numbers correspond to those shown in Table 2.

^b Treatments at 37 C were for 2 days, except in experiment 8, where treatment was extended to 7 days. At 25 C, treatment was for 3 days.

^c Protective serum dilution (PD₅₀) value: virus-neutralizing antibody activity is expressed as the logarithm of the reciprocal of serum dilution protecting 50% of mice inoculated with 100 mouse LD₅₀ FMDV. PD₅₀ values shown are means from groups of five guinea pigs.

establish precise regression characteristics for EI inactivation of FMDV.

Data published by Rowlands et al. (9) indicate that among the many strains of FMDV, some may be antigenically unstable to treatment with EI derivatives. Evidence of 140S FMDV antigen degradation due to EI or AEI treatments was not observed with the FMDV, O Campos used in the present study. Indeed, where EI and AEI treatments were continued at 37 C for 7 days, CF and guinea pig VN antibody data (Tables 2 and 3, experiment 8) show little

loss of 140S FMDV antigen or its immunogenicity. In those cases where FMDV antigen may be degraded by EI or its derivatives, prior stabilization with Formalin as described by Rowlands et al. (9) may prove to be of great practical importance in FMDV vaccine preparation.

Experiments reported by Bahnemann (H. G. Bahnemann, *Zentralbl. Veterinaermed.*, in press) also showed that EI inactivated FMDV at rates significantly greater than AEI. However, inactivation rates of FMDV with EI, AEI, and other EI derivatives reported by Bahnemann were substantially greater than those found in the present study. Presumably, such differences may be due to use of different virus preparations. Nevertheless, these data indicate that EI rapidly inactivates FMDV with a linear loss of infectivity, but little, if any, degradation of its antigenicity or immunogenicity.

Fairly wide use of EI and its derivatives in textile and other industries (5) has reduced its cost to a favorable range as an inactivant for FMDV. Additional evidence of its successful use at about one-fifth the concentration of AEI and its stability at room temperatures (H. G. Bahnemann, *Zentralbl. Veterinaermed.*, in press) indicate that EI has near optimal characteristics as an inactivant for FMDV vaccine preparation.

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