THE USE OF BINARY ETHYLENIMINE (BEI) FOR THE INACTIVATION OF FOOT-AND-MOUTH DISEASE VIRUS PRODUCED BY DIFFERENT SEMI-INDUSTRIAL TECHNIQUES

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SUMMARY

Foot-and-mouth disease vaccines produced with antigens grown in BHK cells, by the Frenkel method and in unweaned rabbits were tested. They were inactivated with binary ethylenimine (BEI) and tested in cattle. The potency of the vaccines was satisfactory as measured by the mouse protection test using the cattle sera. No significant differences were observed between inactivation with BEI or N-acetylethylenimine (AEI).

INTRODUCTION

The use of binary ethylenimine (BEI) for the inactivation of foot-and-mouth disease (FMD) virus produced in BHK cells was described by Bahnemann (*2*), who also compared the immunogenicity of vaccines with antigens inactivated by BEI and by N-acetylethylenimine (AEI) in guinea pigs and cattle (*3*).

In the present study virus suspensions obtained by the Frenkel method, by the inoculation of unweaned rabbits and by BHK cells were inactivated with BEI. Vaccines formulated with these antigens were stored for up to a year at 4°C and tested for their immunogenicity.

MATERIALS AND METHODS

Virus suspensions

Infectious viral suspensions were produced according to routine procedures of this laboratory.

Cell cultures in roller bottles

Two-liter roller bottles were seeded with 300 ml of BHK21 C 13 cell suspensions with a cell concentration of ± 2.5 x 10^5 cells/ml. These cells were grown in suspension with growth medium consisting of Eagle's medium supplemented with 8% bovine serum and 10% tryptose phosphate broth (*10*). The cultures were held at 36°C during 72 hours in a roller apparatus at 8 rotations/hr; during this period a compact cell layer was formed. The growth medium was removed, and maintenance medium[2] containing the seed virus was added. This seed virus was previously adapted to cell cultures by 2-3 passages in monolayer culture in roller bottles. The optimal multiplicity of infection was previously established for each of the virus strains. The volume of maintenance medium was adjusted up to 2.0 x 10^6 cell/ml. In 18-22 hours the cytopathic effect was complete, with destruction of the cell layer. The virus suspension harvests of each bottle were pooled and the pH adjusted to 7.6 with glycocol buffer[3] and then stored at 4°C.

Suspension cell cultures

BHK21 C 13 cells were cultured in 90- and 220-liter stainless steel fermentors with magnetic stirring. Seed suspension cell concentration was 6 x 10^5 cells/ml. At 40-48 hours a cell concentration of 1.8 to 2.0 x 10^6 cells/ml was obtained. The culture was then cooled to 4°C and the stirring discontinued to permit cell sedimentation. After 16-18 hours the supernatant fluid containing less

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[2] BHK21 medium without bovine serum with 30% Tris Buffer 0.2M at pH 7.6.

[3] Glycocol buffer: NaOH - 50.5 g; NaCl 104.0 g; Glycocol - 158.0 g; Dist. water enough to complete - 1,000 ml; pH = 10.0.

than 10^5 cells/ml was discarded and replaced by maintenance medium containing the seed virus. The optimal multiplicity of infection was previously determined for each of the virus strains.

Seed viruses came from monolayers in roller bottles; they did not require any special adaptation to suspension cultures. After 18-24 hours, when the cytopathic effect was nearly complete (viable cell counts less than 10^5 cells/ml), the virus suspension was cooled to 4°C.

Frenkel method

The method used for the production of virus in surviving tongue epithelium is basically as decribed by Frenkel (*5*) and Frenkel and Dunne (*7*), with culture medium as described by Frenkel (*6*).

Tongue epithelium was obtained at a slaughterhouse near the laboratory immediately after slaughter of the cattle, and was always used within 6 hours after collection.

Seed viruses were obtained by 3-8 passages in 2.5-liter Frenkel cultures under constant shaking at 36°C. The vaccine batch was produced in 25-liters vessel of the type described by Frenkel and Dunne (*7*).

The culture process lasted 20-22 hours, followed by cooling to 4°C, trituration of the epithelium into the own culture medium, and clarification by centrifugation of the virus suspension.

Unweaned rabbits

Virus suspensions were prepared as described (*1*) by the inoculation of 3-5 day old rabbits, using carcasses and thoracic viscera.

Treatment of virus suspensions

The virus suspensions produced in cell cultures or Frenkel culture, following cooling at 4°C, were treated with 1% (v/v) chloroform and strongly shaken for 2 hours. The mixture was left to sediment at +4°C and the supernatant was separated from the precipitated formed by undisolved chloroform and cellular debris. After clarification by filtering through a Seitz K2 filter, the virus suspension was ready for inactivation. Viral suspension obtained from unweaned rabbits was treated twice with chloroform, centrifuged and clarified through Seitz K2.

Characteristics of the virus suspensions

The characteristics of the virus suspensions used in the present study are described in Table 1.

Inactivation

In preliminary tests with virus suspensions from the 3 production systems (BHK cells, Frenkel and unweaned rabbits) the kinetics of inactivation were found to be similar for each of the virus suspension under comparable conditions of pH, molarity and temperature (Bahnemann, unpublished data). Similarly, previous adsorption to aluminum hydroxide for virus produced in unweaned rabbits did not alter inactivation kinetics.

Thus, the virus suspensions used in this study were all inactivated as described by Bahnemann (*2*). A solution of 0.1M bromohydrate of bromoethylamine (BEA) in 0.2N sodium hydroxide solution was kept in a waterbath at 37°C for one hour, during which the formation of BEI (bromoethylenimine) took place by the cyclization of the BEA. The solution of BEI was added to each of the virus suspensions previously heated to 37°C, in a final concentration of 1% (v/v) to obtain final concentration of 0.001M BEI. The inactivation process continued for 24 hours at 37°C under continuous mixing.

The cell culture and Frenkel virus suspensions were inactivated as trivalent mixtures before being adsorbed to aluminum hydroxide. The trivalent virus suspension produced in unweaned rabbits was adsorbed through aluminum hydroxide before being inactivated.

For comparison a portion of the trivalent virus mixture produced in BHK cells was inactivated with 0.05% (v/v) AEI (0.006M) at 37°C for 24 hours.

After inactivation and innocuity testing, 1% (v/v) of a solution of 1M sodium tiosulfate was added to the virus suspension to neutralize the residual BEI or AEI.

TABLE 1. *Caracteristics of the virus suspensions*

Origin of virus suspension	Virus strain	No. of passages: In monolayer	No. of passages: In suspension	CF[a] 3 μC' 90'	ITCC[b] 50%/ml	ITSM[c] 50%/ml
(suspension)	O1 Campos	2	1	1/25	$10^{7.7}$	–
BHK (monolayer)	A24 Cruzeiro	4	–	1/25	$10^{8.0}$	–
(suspension)	C3 Resende	2	1	1/25	$10^{8.0}$	–
		In Frenkel				
	O1 Campos	8		1/15	$10^{7.7}$	–
Frenkel	A24 Cruzeiro	6		1/10	$10^{7.4}$	–
	C3 Resende	10		1/10	$10^{7.6}$	–
		In unweaned rabbits				
	O1 Campos	6		1/12	$10^{7.75}$	$10^{9.5}$
Unweaned rabbits	A24 Cruzeiro	3		1/15	$10^{8.0}$	$10^{9.5}$
	C3 Resende	6		1/8	$10^{8.3}$	$10^{8.8}$

[a]Complement fixing - 3 units C' - 90 minutes.
[b]ITCC = Infective titer 50% for cell culture/ml.
[c]ITSM = Infective titer 50% for suckling mice/ml.

Innocuity

The innocuity of the inactivated virus suspensions was tested by the inoculation of 48-hour old cultures of BHK_{21} C 13 cells in Roux bottles. These cells had been grown with growth medium containing 10% bovine serum free of FMD antibodies (*10*). This growth medium was replaced by maintenance medium. To each of 5 bottles, 3 ml of inactivated virus suspension with or without previous addition of aluminum hydroxide were added. The cultures were kept at 36°C for 48 hours and then examined for cytopathic effect. The bottles were then shaken to remove as much as possible the cells from the glass wall, and 5 ml of the culture were used to seed the next series of 5 bottles. This procedure was repeated after 48 hours for 3rd passage. After 48 hours no cytopathic effect was observed and the pool of the culture fluid obtained at this 3rd passage level was submitted to the complement fixing test with negative results.

Vaccines

The vaccines were formulated as follows: 75% of the trivalent antigen suspension OAC was mixed with 25% aluminum hydroxide[4] at 2% of Al_2O_3.

The pH was adjusted to 8.0 with glycocol buffer and the antigen was adsorbed under continuous shaking. The suspension was then left to sediment and concentrated by the elimination of the supernatant liquid, to the extent that each 5 ml trivalent dose (after addition of glycerin, saponin solution and merthiolate) would correspond to the following composition: suspension virus O: 2 ml; suspension virus A: 2 ml; suspension virus C: 2 ml; $Al(OH)_3$: 2 ml; glycerin: 0.25 ml; saponin[5]: 1.0 mg/ml; merthiolate[6]: 1:30,000 final mixture, and glycocol buffer: enough to adjust pH 8.0.

This formulation was the same for all vaccines, independent of the virus suspension origin.

Vaccination and evaluation of the immunity

Each vaccine was tested in cattle at 45 and 350 days after the formulation. Lots of 9 to 10

[4]Prepared from ammonium hydroxide and aluminum sulphate.
[5]Saponin P3 - Food Industries Ltd. Bromborough Port, Wirval, Merseyside L42-4SU, Englad.
[6]Thimerosal Powder, N.F. Lilly, Elly Lilly & Co. Indianapolis, Ind. 46206, USA.

Hereford cattle 21-23 months old used in this experiment were maintained on a farm where FMD had not occurred during the last 12 years. Each vaccine was inoculated subcutaneously in 5 ml doses. The animals were randomly assigned to each of the vaccine groups. Serum was collected at 0 and 30 days post-vaccination. Antibody levels were assayed by the mouse protection test (*4*).

RESULTS

Table 2 presents results of the antibody tests, as the expected percentage of protection (EPP) and the arithmetic mean of the mouse protection indices (MPI). The EPP was higher than 90% in all vaccines after a 45-days storage except for the O antigen produced in unweaned rabbits. No differences were observed between the results of the vaccines produced in BHK cells inactivated either with BEI or AEI.

After 350 days of storage at 4°C, lower values were observed (Table 2) which still provided a reasonable level of protection with the exception of the O and C antigens produced in unweaned rabbits.

DISCUSSION

The BEI-inactivation efficiency was satisfactory with FMD virus suspensions produced with all 3 methods used. This result agrees with earlier non-published observations.

The vaccines were formulated similar to the industrial processes with antigen suspensions not especially selected or concentrated (2 ml virus suspension per valence). Results indicate that excellent protection levels could be obtained with recently formulated vaccines.

The results from the same vaccines after 350 days storage were somewhat lower (Table 2), particularly with the vaccines produced with antigens from unweaned rabbits.

In the present experiment no rapid deterioration of the immunogenicity of FMD vaccines as

TABLE 2. *Foot-and-mouth disease vaccines stored for 45 and 350 days*

		Mouse protection test					
		Virus O		Virus A		Virus C	
Origin of virus	Inactivant	45	350	45	350	45	350
Frenkel	BEI 0.001M	4.63[a]	2.48	4.68	2.48	4.62	3.38
		94%[b]	83%	97%	85%	98%	94%
Suckling rabbits[c]	BEI 0.001M	2.60	1.11	5.40	4.11	4.35	2.0
		78%	52%	99%	99%	93%	77%
BHK	BEI 0.001M	3.95	2.95	5.75	4.50	5.40	4.32
		96%	90%	99%	99%	99%	95%
BHK	AEI 0.006M[d]	4.02	2.41	5.60	4.20	5.11	3.56
		92%	80%	99%	97%	99%	91%

[a] Arithmetic mean of mouse protection indices.
[b] Expected percentage of protection. Gomes and Astudillo (*9*).
[c] Inactivated after adsorbed on Al(OH)3.
[d] Normal concentration for use.

described by other workers with different strains was observed (*8, 11, 12*).

No differences were observed between vaccines produced with these viruses and grown in BHK cells and inactivated with either BEI or AEI (Table 2), both in the recently produced vaccines and vaccine after 350 days storage. These results complement the observations of Bahnemann *et al.* (*2, 3*) with vaccines inactivated with BEI that were not stored before vaccination.

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