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The use of acetylethyleneimine in the production of inactivated foot-and-mouth disease vaccines

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INTRODUCTION

The virucidal properties of formalin are widely employed in the production of inactivated vaccines against numerous virus diseases, although the uncertainty which accompanies its use for the *complete* inactivation of viruses is well known. This uncertainty was tragically emphasized by one of the earlier vaccines prepared against poliomyelitis. Since Vallée, Carré & Rinjard (1926) demonstrated that the virus of foot-and-mouth disease (FMD) could be inactivated with dilute formalin to give a product which would immunize cattle, this agent has been used extensively for the production of inactivated vaccines against the disease. In most laboratories concerned with the production of FMD vaccines the virus is adsorbed on to aluminium hydroxide gel before incubation with dilute formalin for periods up to 72 hr. (Waldmann & Köbe, 1938). Doubts have been expressed about this procedure because of the possibility that traces of residual virus might remain undetected owing to its combination with the adjuvant, and there is evidence that infective virus is present in vaccines prepared in this way (e.g. Moosbrugger, 1948; Schneider, 1955). Wesslen & Dinter (1957) showed clearly that complete inactivation of FMD virus is likely to be a prolonged process and some of our early inactivation experiments, carried out in the absence of aluminium hydroxide, showed that infective virus could be detected (by intradermal inoculation of cattle) after prolonged periods of incubation with 0·05 % formalin. It seemed important, therefore, to examine alternative methods of inactivation which would be more certain to yield regularly a non-infective product.

Preliminary experiments showed that one strain of FMD virus could be inactivated extremely rapidly with 0·05 % acetylethyleneimine (AEI) at 37° C. with the production of an effective vaccine for guinea-pigs (Brown & Crick, 1959). These findings have now been extended to other strains of the virus, grown in tissue culture, with the aim of producing vaccines for cattle.

MATERIALS AND METHODS

Virus production

Virus of strains 997 (type C) and Pak 1 (type Asia 1) was grown in cultivated pig kidney cells (Sellers, 1955) or in surviving bovine tongue epithelium fragments (Frenkel, 1951). The pig kidney cells were grown as monolayer cultures in Roux

flasks and inoculated with virus after 6–8 days. Virus was harvested when the monolayers were completely destroyed and the cell debris then removed by centrifugation. The production of virus in surviving tongue epithelium cells has been described in detail by Frenkel and his colleagues. In the present experiments, virus was harvested 18 hr. after infection.

Virus titration

Infectivity titrations were carried out by the intraperitoneal inoculation of groups of 7-day-old mice with tenfold serial dilutions of the virus (Skinner, 1951).

Virus inactivation

(*a*) Inactivation with AEI was carried out with 0·05% (v/v) solutions at pH 7·6 and 37° C. and the reaction was stopped after the appropriate interval by the addition of $\frac{1}{10}$th volume of 20% (w/v) sodium thiosulphate.

(*b*) For inactivation with formalin, virus suspensions containing 0·05% (v/v) formalin were incubated at pH 7·6 or pH 9 for periods varying from 48 to 144 hr. at 26° C. The formalin was then neutralized with excess sodium bisulphite. For the preparation of vaccines similar to those available commercially, an equal volume of aluminium hydroxide gel was added to the virus suspension before incubating with 0·05% (v/v) formalin at 26° C.

Innocuity tests

All the vaccines used in the guinea-pig experiments were tested for innocuity by intraperitoneal inoculation of 7-day-old mice (10–50 animals for each vaccine) and by intradermal inoculation of guinea-pig pads. In the cattle experiments innocuity tests were made by inoculating 0·1 ml. samples at twenty separate sites on the tongues of each of six steers. The significance of these tests has been discussed by Henderson (1952).

Potency tests

Groups of guinea-pigs or steers were inoculated subcutaneously with dilutions of vaccine. Those vaccines which were tested for innocuity in the absence of aluminium hydroxide were mixed with this adjuvant before inoculation. Blood samples were taken at 21 days and the animals were then challenged by intradermal inoculation of one hind pad (guinea-pigs) or of ten sites on the tongue (cattle) with 10,000 ID 50 of virus. The animals were then examined daily for the appearance of lesions typical of foot-and-mouth disease. In one experiment the cattle were not challenged but the virus neutralizing antibody levels of their sera were determined at intervals.

Serum neutralization tests

Specific neutralizing antibody was estimated by two methods:

(*a*) Dilutions of the homologous virus were mixed with an equal volume of 1/10 serum and the mixtures inoculated intraperitoneally into mice. The depression in virus titre produced by the serum was taken as the neutralization index of 1/20 serum (Skinner, 1953).

(*b*) In the second method, twofold dilutions of serum were mixed with 100 ID 50 of the homologous virus and the mixtures tested for residual virus in suspensions of cultivated pig kidney cells (Martin & Chapman, 1961). The end-point was the highest dilution of serum which prevented the growth of virus.

RESULTS

Inactivation of FMD virus with acetylethyleneimine and formalin

The experiments described by Brown & Crick (1959) showed that virus suspensions in tris buffer, pH 7·6, prepared from guinea-pig pad epithelium could be made non-infective for mice and guinea-pigs by incubating with 0·05 % AEI for 4 hr. at 37° C. As virus for the large-scale production of FMD vaccines is most conveniently produced in cultivated cells, the inactivation of virus grown in tissue culture cells was first examined. Whereas 4 hr. was sufficient for the inactivation of virus grown in guinea-pig pad epithelium and suspended in tris buffer, a rather longer period of reaction was required for complete inactivation of both bovine tongue epithelium and pig kidney culture viruses (as tested in mice). This increase in time is probably caused by reaction of the AEI with some of the constituents of the medium in which the cultured cells are maintained; for example, the concentration of amino acids is much greater in tissue culture virus suspensions than in pad epithelium extracts. Dialysis of tissue culture viruses against tris buffer before addition of the AEI increased the subsequent rate of inactivation but the losses of infective virus by adsorption on to the dialysis sac were considered large enough to outweigh the advantages of a shorter inactivation period (see below). On the basis of several experiments, 6 hr. was finally chosen as the inactivation time required for the production of a vaccine which would be innocuous for mice and guinea-pigs. In order to obtain a product which was innocuous for cattle, longer periods of inactivation were required. In five experiments, using six cattle in each experiment, so that a total of 12 ml. of each vaccine was tested, it was found that infective virus was still present after 8 hr. incubation but could not be detected after 12 hr. The results are summarized in Table 1.

The inactivation of FMD virus with 0·05 % formalin at pH 7·6 and 26° C. proceeds rapidly in the initial stages of the reaction, but the rate later decreases and the product is innocuous for mice only after periods greater than 50 hr. In some experiments virus could be detected in mice even after 72 hr. incubation. By adjusting the virus suspension to pH 9 with glycine buffer before adding the formalin, the required incubation period could be reduced to 48 hr. These findings are in general agreement with those of Wesslen & Dinter (1957), who used calf kidney tissue culture cells for detecting the residual virus. However, in all the experiments in which the vaccines were tested for innocuity by intradermal inoculation of cattle tongues, infective virus was detected in suspensions which had been incubated with 0·05 % formalin for periods up to 144 hr., either at pH 7·6 or at pH 9·0. Prior filtration of the virus suspension through a Seitz EK filter had no effect on the result. These experiments are summarized in Table 1.

If the virus is first added to aluminium hydroxide, as in the commercial process, and then incubated with 0·05 % formalin, the product is apparently innocuous for

cattle after 48 hr. It seems likely, from the results described in the previous paragraph, that infective virus will survive this procedure unless the adsorption of FMD virus to aluminium hydroxide renders it more sensitive to the action of formalin.

Table 1. *Inactivation of FMD virus with AEI and formalin*

Virus strain and infectivity (log ID50/ml.)		Inactivation procedure	Proportion of cattle reacting	No. of reaction sites	Interval between inoculation and reaction (days)
997	7·3	0·05% formalin, pH 7·6, 26° C., 114 hr.	1/6	1/120	4
997	7·8	0·05% formalin, pH 7·6, 26° C., 121 hr.	2/6	2/120	3 and 4
997	7·5	0·05% formalin, pH 7·6, 26° C., 144 hr.	2/6	3/120	2 and 5
997	7·5	0·05% formalin, pH 7·6, 26° C., 120 hr.	3/6	4/120	3, 3 and 3
		EK filtered. Then as above	2/6	3/120	3 and 3
997	7·0	0·05% formalin, pH 8·9, 26° C., 72 hr.	6/6	10/120	1, 2, 2, 2, 2, 2
997	6·5	0·05% formalin, pH 8·9, 26° C., 144 hr.	3/6	3/120	3, 4 and 5
997	7·0	0·05% formalin, pH 8·9, 26° C., in presence of $Al(OH)_3$, 72 hr.	0/6	0/120	—
PAK 1	7·4	0·05% formalin, pH 8·9, 26° C., in presence of $Al(OH)_3$, 72 hr.	0/6	0/120	—
997	7·5	0·05% AEI, pH 7·6, 37° C., 8 hr.	3/6	13/120	3, 5 and 5
997	7·0	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—
		0·05% AEI, pH 7·6, 37° C., 18 hr.	0/6	0/120	—
PAK 1	7·4	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—
PAK 1	5·4	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—

Comparison of the potency of AEI and formalin vaccines for guinea-pigs

Before proceeding to experiments in cattle, it was desirable to ascertain whether AEI-inactivated tissue culture vaccine was effective in guinea-pigs. In preliminary experiments it was found that inactivation of the tissue culture virus proceeded more rapidly if it was first dialysed against tris buffer, but this process

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led to the loss of about 0·5 log of virus infectivity, presumably as a result of adsorption on to the dialysis casing. This loss of virus was reflected in the loss of potency of the vaccine prepared from the dialysed virus (see Table 3). In subsequent experiments the dialysis step was omitted and the longer inactivation period (6 hr.) was used.

It seemed desirable that the action of the AEI should be stopped after the appropriate incubation period; it was essential, however, that the agent used for 'neutralizing' the AEI should have no adverse effects on the potency of the vaccine.

Table 2. *Neutralization of FMD virus inactivating agents*

Mixture	Infectivity log (mouse ID 50/ml.)
Virus only, 37°/4 hr.	7·0
Virus + 0·05 % AEI, 37°/4 hr.	None detected
Virus + (0·05 % AEI + 2 % sodium thiosulphate), 37°/4 hr.	7·1
Virus only, 26°/48 hr.	5·1
Virus + 0·05 % formalin, 26°/48 hr.	1·0
Virus + (0·05 % formalin + sodium bisulphite), 26°/48 hr.	4·8

Table 3. *Comparison of AEI and formalin FMD vaccines (strain 997) in guinea-pigs*

	Guinea-pigs protected by			Neutralization index of 1/20 sera (log ID 50)		
					Dialysed	
Virus content log (mouse ID 50/dose)	AEI vaccine + thiosulphate	Dialysed AEI vaccine + thiosulphate	Formalin vaccine + bisulphite	AEI vaccine	AEI vaccine	Formalin vaccine
6·0	5/5	5/5	5/5	—	—	—
5·5	5/5	2/5	5/5	2·0	1·5	1·9
5·0	5/5	1/5	3/5	2·0	1·0	1·4
4·5	0/5	0/5	0/5	—	—	—

Sodium thiosulphate (2 %) was shown to completely 'neutralize' 0·05 % AEI (Table 2) and it also had no effect on the potency of vaccines prepared by inactivation with AEI (Table 4). Vaccines prepared with formalin were neutralized with excess sodium bisulphite after the inactivation period. The bisulphite had no effect on vaccine potency (Table 4) and is effective in stopping the reaction of formalin with virus (Table 2).

Vaccines were prepared from pig kidney culture virus (strain 997) by inactivation with 0·05 % AEI at 37° C. for 6 hr. or by incubating with 0·05 % formalin for 96 hr. at pH 7·6. An AEI vaccine was also prepared from a sample of the virus which had been dialysed against tris buffer. Inactivation was stopped with sodium thiosulphate and sodium bisulphite respectively, and each vaccine was tested for innocuity in both mice and guinea-pigs. Dilutions of each vaccine were then mixed with aluminium hydroxide and inoculated subcutaneously into guinea-pigs. The

results of the subsequent challenge of the guinea-pigs with virus at 21 days are shown in Table 3, from which it can be seen that the 6 hr. AEI vaccine is slightly superior to the 96 hr. formalin vaccine prepared at pH 7·6. In another experiment with a different virus suspension, the effect of adding thiosulphate and bisulphite to the two vaccines was tested and these results are shown in Table 4. The results of the experiments with the AEI vaccine were considered sufficiently encouraging to warrant their extension to cattle.

Table 4. *Effect of thiosulphate and bisulphite on the potency of FMD vaccines (strain 997) in guinea-pigs*

Virus content log (mouse ID50/dose)	Guinea-pigs protected by			
	AEI vaccine	AEI + thio-sulphate	Formalin vaccine	Formalin + bi-sulphite
6·0	10/10	10/10	10/10	10/10
5·5	10/10	10/10	9/10	10/10
5·0	7/10	9/10	7/10	8/10
4·5	5/10	7/10	4/10	3/10

Comparison of the potency of AEI and formalin vaccines for cattle

As indicated above, it has not been possible in the present experiments to obtain a formalin vaccine which was innocuous for cattle (by intradermal inoculation) unless aluminium hydroxide was added. In consequence, all comparisons in cattle between AEI and formalin vaccines have been made with formalin vaccines prepared in the presence of aluminium hydroxide. The results of one experiment with virus of strain 997, produced in cultivated pig kidney cells, indicate that an AEI vaccine inactivated for 18 hr. is as effective as the conventional formalin vaccine (Table 5).

Table 5. *Comparison of AEI and formalin FMD vaccines (strain 997) in cattle*

Virus content log (mouse ID50/dose)	Cattle protected by vaccines	
	AEI	Formalin
6·5	5/8	3/8
7·0	7/8	7/8
7·5	8/8	6/8

In a second experiment, samples of virus of strain Pak 1 grown in surviving bovine tongue epithelium cells ($10^{7·4}$ mouse ID50/ml.) were inactivated by each method and the levels of specific neutralizing antibody produced in cattle were determined (Fig. 1). The cattle used in this experiment were not challenged because they were required for subsequent studies on the effects of re-vaccination with inactivated FMD vaccines (Hyslop, unpublished observations). The specific antibody levels resulting from the primary response to the two vaccines, however,

again demonstrated the efficacy of AEI as an inactivating agent for the preparation of FMD vaccines.

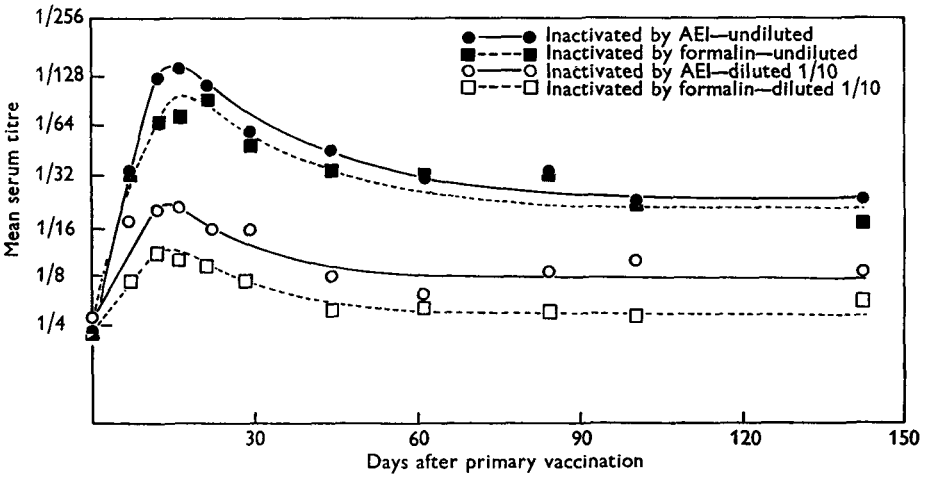

Fig. 1. Mean serum antibody titres of groups of 8 cattle inoculated with $10^{8\cdot6}$ or $10^{7\cdot6}$ ID_{50} of virus treated with AEI for 12 hr. or formalin for 48 hr.

DISCUSSION

The reaction of animal viruses with dilute formalin is a complex process. In common with other viruses, the initial rate of inactivation of FMD virus is extremely rapid but prolonged incubation periods are required to obtain a non-infective product. In normal practice FMD vaccines are produced by inactivation of virus which has been adsorbed on to aluminium hydroxide before or at the time of addition of the dilute formalin. Unless this process of adsorption renders the virus more sensitive to the action of formalin it is certain that these vaccines will contain infective particles at the end of the 48 hr. incubation period. Evidence has, in fact, been produced (Moosbrugger, 1948; Schneider, 1955) that vaccines prepared in the presence of aluminium hydroxide which were innocuous for cattle did contain infective virus particles. In our experiments we have failed to inactivate completely the virus for cattle by incubating the suspensions with 0·05% formalin for periods up to 144 hr.

These experiments also emphasize that vaccines which are to be used in cattle should not be tested for innocuity only in mice or tissue-culture cells. This difference in sensitivity of the three host systems towards formalin-treated virus is interesting in view of their equal sensitivity towards the untreated virus. In this connexion it may be significant that the virus detected by the intradermal inoculation of cattle with suspensions which have been incubated with formalin for long periods produces lesions as late as 5 days after inoculation, whereas the lesions produced by untreated virus generally occur within 24–48 hr. (Table 1).

Incubation with AEI instead of formalin is a more certain method of obtaining a vaccine free from infective virus and the results described above indicate that such vaccines are as potent antigenically as formalin vaccines of the same strain produced by methods used commercially.

SUMMARY

Suspensions of FMD virus treated with 0·05 % formalin at 26° C. for periods up to 144 hr. remained infective for cattle, although the infectivity could not be detected in the presence of aluminium hydroxide. Infectivity was detected in similar virus suspensions which had been treated with 0·05 % AEI at 37° C. for 8 hr. but not in suspensions treated for 12 hr.

Vaccines prepared from these suspensions were antigenically potent and serum neutralization tests demonstrated the development and regression of serum antibody. The AEI vaccines were at least as potent as the corresponding formalin vaccines.

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