

## INACTIVATED AFRICAN HORSE SICKNESS VIRUS CELL CULTURE VACCINE ( \* )

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**Summary.** Immunogenic killed vaccine against African Horse Sickness can be prepared from a neurotropic vaccine strain or a virulent strain of virus, type 9, grown in a monkey kidney stable cell line. Virus was inactivated with either formaldehyde in a final concentration of 1:8000 or  $\beta$ -propiolactone at 0.2 per cent. In order to enhance the immunogenicity of the product, aluminium hydroxide was added to the vaccine as an adjuvant.

After inoculation of a single dose of either vaccine, neutralizing antibodies developed within 4 weeks, and all horses resisted challenge with homologous virulent virus. When two injections of inactivated vaccine were administered at an interval of 4 weeks, much higher neutralizing antibodies were present in sera and 6 months later all horses were still resistant to a challenge dose of virulent virus.

Under the conditions of these experiments a significantly higher degree of antigenicity was demonstrated with formalin-inactivated vaccine than with  $\beta$ -propiolactone-inactivated vaccine.

### INTRODUCTION

African Horse Sickness (AHS) is a culicoides-borne virus disease which since 1959 has caused severe epizootics among horses, mules and donkeys in the Middle East, North Africa and Spain. Nine serologically different types of virus are already recognized (Howell, 1962). Because of its originally limited sporadic occurrence as an infection of equidae in South Africa, the disease was carefully studied by South African scientists. Immunoprophylactic measures were developed for prevention of the disease. For example the mouse-adapted live neurotropic virus vaccine was successfully employed by Alexander, Neitz and Du Toit (1936). This vaccine was not without untoward reactions and many cases of post-vaccinal complications were observed (Nobel and Neumann, 1961; Pavri and Anderson, 1963; Shah, 1964). The cell culture live vaccine developed by Mirchamsy and Taslimi (1964a, b) was largely used in the Middle East, Tunisia, Algeria, Morocco and Spain without producing any untoward signs or symptoms. However, there is always concern regarding a possible return of virulence during serial passage of the virus and many countries free from the disease would not accept a live vaccine as an immuno-pro-

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phylactic agent. Efforts were therefore directed toward developing a killed vaccine. Unfortunately studies by several workers (Whitworth, 1929; Du Toit and Alexander, 1930; Walker, 1931; Du Toit, Alexander and Neitz, 1933a, b; Kind, 1934) demonstrated a poor antigenicity for formal killed vaccine. This was normally prepared in the field as a 20 per cent suspension of spleen of horses dead from the disease and was inactivated by 1:1000 to 1:4000 of formalin. Large amounts of this vaccine mixed with saponin (1:5000) were used in Iran (Rafyi, 1961) and in Turkey (Reid, 1961) with irregular results. The vaccine sometimes failed to protect. Recently Ozawa and Bahrami (1966) using up to 50 ml of a formalin killed cell culture vaccine were able to protect eight horses against challenge by virulent virus 5 weeks after immunization; however, the serological responses of these horses to the vaccine were very poor. A severe epizootic of AHS in the majority of North African countries and Spain in 1966 prompted efforts to prepare such a killed cell culture vaccine, as would be acceptable to those countries already free from the disease and reluctant to use the live vaccine in their territories.

The present report is concerned with the development of methods for the preparation and assay of a killed cell culture AHS vaccine which is safe and immunogenic for equidae.

## MATERIALS AND METHODS

### *Virus*

The mouse-adapted neurotropic strain S2 used at the Razi Institute for the preparation of live vaccine (Hazrati and Taslimi, 1963) and the virulent strain 10/60 both of type 9 were used. The vaccine strain had been through 101 serial passages in mouse brain and seven passages in a monkey kidney stable cell line (MS). The virulent strain 10/60 was first passed six times in baby mouse brain and eight times in MS cells. The stock viruses were lyophilized and stored at  $-40^{\circ}$  before use.

### *Cell culture*

Line MS of monkey kidney cells were grown in a medium consisting of Earle's solution with 0.5 per cent lactalbumin hydrolysate, 0.5 per cent Difco yeast extract and 10 per cent calf serum inactivated for 30 minutes at  $56^{\circ}$ . In the maintenance medium the calf-serum was reduced to 5 per cent. One hundred units of penicillin and 100  $\mu$ g of streptomycin per millilitre were added.

### *Preparation of the virus suspension*

Twenty-four Roux bottles of 1 litre of MS cells were inoculated, each with approximately 10,000 50 per cent tissue culture infectivity doses (TCID<sub>50</sub>) of either strain. The virus was left to adsorb for 2 hours at  $36^{\circ}$  with occasional shaking, and then 35 ml of maintenance medium was added. The bottles were re-incubated at  $36^{\circ}$  for 36 hours. By this time more than 50 per cent of the cell sheet normally showed a specific cytopathic effect (CPE). The fluid was then removed and stored at  $+2^{\circ}$  and 15 ml of an isotonic 0.05 M borate buffer of pH 9.0 was added. After 30 minutes at  $36^{\circ}$  the borate buffer containing large amounts of virus was removed from the bottles and kept at  $+2^{\circ}$ . By applying this method,

suggested by Hallauer and Kronauer (1965), a large amount of intracellular virus was released into the fluid medium without disturbing the cell sheet. Another 25 ml of maintenance medium, pH 7.2, was then added. After a further 24 hours of incubation at 36° the CPE was almost complete. At this time the cell sheet was scraped into the fluid and the contents of all bottles of the same virus were pooled. After two cycles of rapid freezing and thawing, cell debris was removed by slow centrifugation and the fluid was pooled with the two previous harvests; samples were examined for bacterial sterility and stored at -40° for later titration. On the basis of several experiments the TCID<sub>50</sub> of the final pool would be 10<sup>6.5</sup> to 10<sup>7.5</sup>/ml. Four lots of each strain were prepared. Two lots were inactivated by formalin and another two lots were inactivated by β-propiolactone. The homologous lots were mixed after residual live virus had been proved to be absent.

#### *Formalin inactivation*

Inactivation of virus was effected by the addition of formalin (HCHO, 37 per cent) to a final concentration of 1:8000 formaldehyde. The bottle containing the mixture was shaken vigorously and immersed in a water bath at 25°. The inactivation was allowed to proceed for 48 hours during which eight samples were removed and dialysed against two changes of cold phosphate buffer solution (PBS) for 12 hours before being used for residual virus titration. The bottle of virus suspension was then kept another 5 days at 4° before the final test for viable virus was done.

#### *β-Propiolactone inactivation*

The inactivation by β-propiolactone (BPL) was carried out according to the method described by Logrippo and Hartman (1955) as follows: The solution of BPL (COH<sub>2</sub>CH<sub>2</sub>-C:O—Eastman organic chemicals) prepared in ice cold water was slowly mixed with the suspension of virus at a final concentration of 0.2 per cent. The pH of the medium was adjusted to 8.0 with 1 M disodium hydrogen phosphate before the addition of BPL. The pH of the mixture was brought to 7.4 after 15 minutes incubation in a 36° water bath. Samples were taken every 5 minutes and stored at -40° for testing for viability of virus.

#### *Adjuvant*

Aluminium hydroxide was produced according to the technique described by Rafyi and Mirchamsy (1956). The final product was adjusted to pH 6.6 and autoclaved 45 minutes at 121°.

#### *Preparation of the vaccine lot*

To 1 litre of each inactivated virus lot 400 ml of aluminium hydroxide was added. Aluminium hydroxide was double concentrated before use by removal of 50 per cent of its total volume from the clear supernate. Merthiolate was also added to a final concentration of 0.01 per cent.

After vigorous shaking each lot of vaccine was distributed into vials of 20 ml, sealed and labelled and kept at +2°.

#### *Immunization of experimental horses*

Each horse was inoculated subcutaneously with 15 ml of one vaccine. The

temperature of all horses was recorded twice a day for 4 weeks. A second injection of 15 ml of the same vaccine was given subcutaneously 4 weeks after the first.

Horses were bought from Khorassan, north east of Iran where no previous outbreak of the disease had been recorded. The animals were 2-3 years old. The serum of all horses was screened before vaccination as well as 4 weeks after each immunization, in order to measure neutralizing antibodies. It was ascertained that before immunization all horses were free of antibodies for the virus type tested.

*In vitro sero-neutralization*

This test was performed in MS cells as previously described (Mirchamsy and Taslimi, 1964a, b).

## RESULTS

### (a) INACTIVATION CURVE of AHS VIRUS WITH FORMALIN AND BPL

All samples removed during the course of inactivation with formalin or

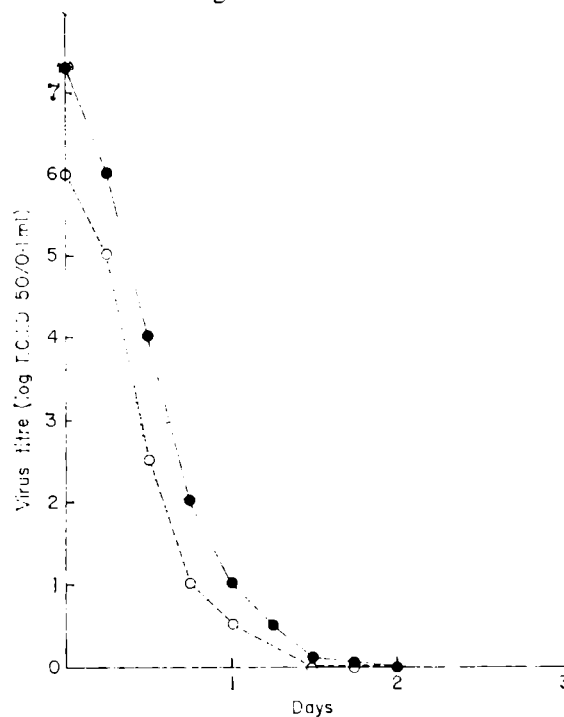


FIG. 1. Residual infectivity of African Horse Sickness virus suspension during inactivation by formaldehyde of final concentration 1:8000, at 25° and pH 7.2. S2 (—), neurotropic virus strain S2, type 9; S10/60 (---), virulent virus strain S10/60, type 9.

BPL were titrated for infectivity in MS culture. Titration was carried out by inoculating 0.1 ml of the undiluted and of each of a series of ten-fold dilutions in four MS tubes. CPE was recorded daily in each tube for 6 days and the TCID<sub>50</sub> of residual virus for each sample was determined. Based on our preliminary study, 0.1 ml of undiluted samples representing 30 minutes of inactivation with BPL or 2 days inactivation with formalin were considered as fully inactivated and were inoculated into each of twelve MS tubes. All twenty-four MS tubes inoculated with these samples were negative for CPE during 8 days of observation.

When the tests were completed, curves of the course of inactivation were drawn in which log residual virus was plotted against time (Figs. 1 and 2). This titration revealed that the inactivation time with BPL at a concentration of 0.2 per cent and at 36° was 30 minutes, the time for inactivation with formalin at a final concentration of 1:8000 formaldehyde at 25° was 2 days.

#### (b) POST-VACCINAL AND POST-CHALLENGE REACTIONS

The temperature of all horses immunized with each vaccine was recorded twice a day for 4 weeks. No rise was observed and no abnormal reaction was

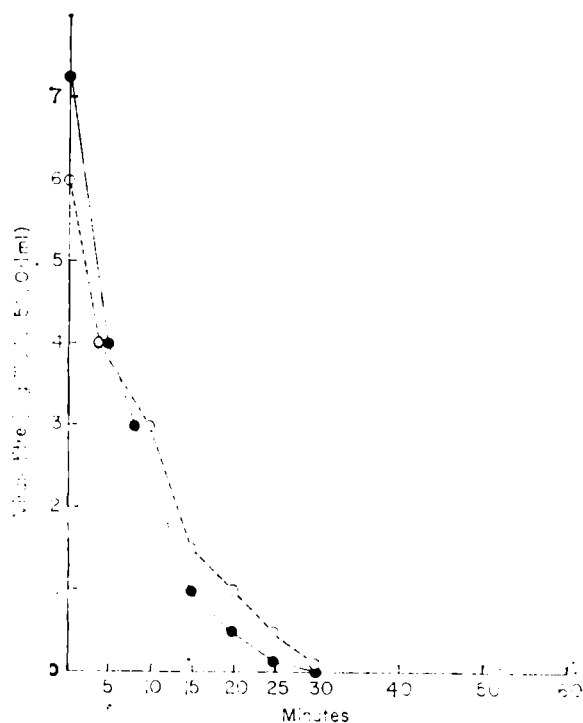


FIG. 2. Residual infectivity of African Horse Sickness virus suspension during inactivation by  $\beta$ -propiolactone of final concentration 1:500, at 36° and pH 7.4. S2 (—), neurotropic virus strain S2, type 9; S10/60 (---), virulent virus strain S10/60, type 9.

noted during the entire period of observation. Twenty-five per cent of horses showed a local swelling of various sizes, due to inoculation of aluminium gel; this reaction faded 3—4 weeks after immunization.

A few days after challenge with virulent virus a rise of temperature to not exceeding 40.7° was recorded in some horses immunized with BPL vaccine; this hyperthermia was soon over and no other signs of illness were noticed. The controls died from the disease with specific pathological changes in various organs. The virus was also isolated from their blood.

#### (c) IMMUNOLOGICAL RESPONSE OF THE HORSE TO ONE DOSE OF INACTIVATED AHS VACCINE

A comparison was made of the immunogenic response induced in two groups of five horses previously vaccinated with formalin-treated or BPL-treated vaccine. All horses were bled 4 weeks after immunization and tested for neutralizing antibodies. Two weeks later these horses and one control were challenged with virulent virus (S10/60) by intravenous injection. The virulent virus was prepared from an 8 per cent suspension of fresh suckling mouse brain, collected when the mice were *in extremis*. The suspension was centrifuged for 15 minutes at 2500 rev/min at 2° before inoculation. Each horse was inoculated with 4 ml of challenge virus. Horses immunized with formalin-treated vaccine had higher levels of serum antibodies; however both groups were resistant to intravenous challenge with virulent virus. The control died with respiratory symptoms 17 days after inoculation of virus and the virus was recovered from its blood (Table 1).

TABLE 1  
IMMUNITY IN HORSES IMMUNIZED WITH A SINGLE DOSE OF INACTIVATED AFRICAN HORSE SICKNESS VACCINE

Horse No.	Type of vaccine	Antibody titre 6 weeks after immunization	Reaction to challenge
1	Formalin-treated	128*	N
2		512	N
3		64	N
4		64	N
5		128	N
6	BPL-treated	4	N
7		4	N
8		16	N
9		4	N
10		16	N
11	Control	---	Died

\* Reciprocal of the dilution of serum which neutralized 100 tissue culture of infective doses (50 per cent).

N. Horse remained well; no signs of infection; BPL,  $\beta$ -propiolactone.

#### (d) IMMUNOLOGICAL RESPONSE OF HORSES TO TWO DOSES OF INACTIVATED AHS VACCINE

Two groups of five horses were immunized, one group with two doses of

15 ml of formalin-treated vaccine, and one with BPL-treated vaccine at an interval of 4 weeks. Test bleeding was performed 4 weeks after each injection and 6 months after the last injection and just before challenge with virulent virus.

The antibody response was much higher to formalin-treated vaccine than to BPL-treated vaccine (Table 2). Six months after immunization the remaining antibodies in horses immunized with formalin-treated vaccine were of higher titre than in horses immunized with BPL-treated vaccine. However, both groups resisted challenge with homologous virus.

TABLE 2  
IMMUNITY IN HORSES IMMUNIZED WITH TWO DOSES OF INACTIVATED AFRICAN HORSE SICKNESS VACCINE

Horse No.	Type of vaccine	Antibody titre			Reaction to challenge 6 months after second immunization
		4 weeks after first immunization	4 weeks after second immunization	6 months after second immunization	
1	Formalin-treated	64*	512	64	N
2		32	64	8	N
3		32	128	32	N
4		128	512	128	N
5		64	256	32	N
6	BPL-treated	16	64	16	N
7		4	16	4	N
8		4	8	<4	N
9		4	8	<4	N
10		4	16	<4	N
11	Control	—	—	—	Died

N, Horse remained well: no signs of infection; BPL,  $\beta$ -propiolactone; <4, less than 4; —, not done.

\* Reciprocal of the dilution of serum which neutralized 100 tissue culture infective doses (50 per cent).

## DISCUSSION

The recent spread of severe epizootics of African Horse Sickness in North Africa and specially in Spain is a real source of danger for equine populations of many European countries, where fully susceptible animals are not protected by any immuno-prophylactic agent. The live cell culture vaccine successfully used during 1965 in Tunisia, Morocco, Algeria and Spain is not likely to be used in Europe because of the traditional fear of the dangers of all live virus vaccines. There is no doubt that the attenuated live monovalent vaccine produced in cell culture against AHS type 9 was effective in preventing the disease in the above mentioned countries. The vaccine virus did not spread from vaccinated to non-vaccinated animals by contact. Could the attenuated virus be transmitted serially in equidae by culicoides, the natural vectors and is there any chance of reversion of the virus to virulence? To the doubts raised by these questions one should add the record of unpleasant reactions attributed to live vaccine. Blindness and fatal colic have been observed recently in Tunisia by EL Fourgi (personal communication) among equidae immunized with polyvalent live mouse brain vaccine, especially in those animals which had no rest after immunization.

The present study has provided the opportunity to compare and evaluate the immunogenic capacity of formalin- and  $\beta$ -propiolactone-inactivated vaccines. Both antigens were mixed with aluminium hydroxide as adjuvant. Formalin-

inactivated vaccine prepared from a cell line of monkey kidney, infected with vaccine or virulent strains of type 9, was safe and induced a high level of neutralizing antibodies in horses.

The serological response of horses to BPL-inactivated vaccine was lower but in both groups neutralizing antibodies persisted for 6 months and vaccinated animals resisted challenge with virulent homologous virus.

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