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DEVELOPMENT OF NEW AFRICAN HORSESICKNESS CELL CULTURE KILLED VACCINES (*)

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Introduction

The possibility of developing a killed vaccine against African horsesickness (AHS) from the virus propagated in cell cultures was explored by Ozawa and Bahrami (9), and by Mirchamsy *et al.* (6, 7). The immunity in horses vaccinated with this vaccine was promising but, nevertheless, indicated that further studies on the different means of inactivation of the virus and use of additional adjuvants were needed to improve the quality of the killed vaccine and to enhance its immunological value.

In the present paper two inactivated cell culture AHS vaccines, incorporated in 4 different adjuvants are compared.

Materials and Methods

Virus strain. Neurotropic virus strain S2, type 9, of AHS virus was used in this study. The virus was isolated during 1959 outbreak of the disease in Iran and was attenuated through 100 intracerebral passages in mice (1). The attenuated virus strain was passed 8 times in monkey kidney stable (MS) cell line and kept at – 70°C until use.

Preparation of virus suspension. The virus was grown in MS cell culture as previously described (8). The $TCID_{50}$ of the final pool was $10^{7.5}$ /ml.

Formalin inactivation. The pH of the virus suspension was adjusted to 7.5 before formalin was added to a final concentration of 1:3,000 at room temperature. The treated virus suspension was then mixed and kept in a 32°C water-bath for 3 days. During the inactivation period samples were removed at various intervals for residual virus determination.

Merthiolate inactivation. To the virus suspension adjusted to pH 7.5, a solution of 10% merthiolate (Thiomersal-BDH) was added to a final concentration of 1:10,000. After mixing with the content of the bottle it was left in a 35°C water-bath for 5 days with occasional shaking and removal of samples at various times after incubation for virus determination.

Residual virus assay. 0.025 ml of undiluted sample to be tested was

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inoculated intracerebrally into each of a litter of 5–6 suckling mice. When residual virus was still present in the sample the animal showed signs of encephalitis and died in 4–7 days. When signs of illness were absent and mice resisted the inoculation, the suspension of virus was supposed to be free of residual live virus.

Preparation of the Vaccines

1. *Iron-oxide adjuvant vaccine.* The adjuvant was prepared with powdered Fe2O3(BDH) according to the technique described by Warren *et al.* (11, 12). To prepare the antigen, 100 mg of sterile iron-oxide was added to 500 ml of formalin- or merthiolate-inactivated AHS virus suspension. The mixtures were shaken at room temperature then centrifuged at 1,000 rpm for 20 min. The supernatant was discarded and the precipitate was resuspended in an appropriate amount of phophate-buffered saline (PBS), pH 7.5. The final concentration of Fe2O3 in this suspension was 10 mg/ml.

2. *Freund's incomplete vaccine.* 85 ml of refined paraffin was slowly mixed with 15 ml of Arlacel A (incomplete Freund's adjuvant) and the mixture was sterilized at 115°C for 30 min. To 450 ml of inactivated virus suspension, 50 ml of sterile Freund's incomplete vaccine was added slowly when a magnetic stirrer was stirring the virus suspension. This antigen was vigorously shaken before inoculation.

3. *Aluminium hydroxide adsorbed vaccine.* The antigen was prepared, as described before (10), and to l,ooo ml of inactivated virus 400 ml of double-concentrated aluminium hydroxide was added. After shaking, the vaccine was kept in cold room until use.

4. *Saponin added vaccine.* A solution of 56 mg/ml of purified saponin (BDH) in distilled water was prepared; the pH was adjusted to 7.5 The solution was first clarified by Seitz filtration and then autoclaved at 108°C for 45 min. This solution was added to either of the inactivated antigens to a final concentration of 1 ml/ml antigen.

5. *Immunization of horses.* Two horses were inoculated subcutaneously with 10 ml of each vaccine. The body temperature of each horse was recorded twice a day. A second injection of 10 ml of the same vaccine was given subcutaneously 4 weeks after the first vaccination. In one experiment 6–9 months foals born from immune mares were used. The sera of all foals has shown a trace of maternal neutralizing antibody. In the other experiment horses of 3–4 years, bought from North of Iran, were used. These horses were free of neutralizing antibodies for the virus type tested.

6. In vitro *neutralization test.* All sera collected and stored at –40°C were inactivated at 56°C for 30 min immediately before use. To evaluate the

neutralizing titers, the serum samples obtained from each horse before immunization and after 1st and 2nd inoculations were tested against 100 TCID 50 of 8th MS passage of strain S2. Details of the neutralization technique have been given before (4).

Results

Reactions to the vaccines. The local reaction to different vaccines consisted of swellings, observable for a few days in case of aluminium hydroxide and iron salt, but lasting for several weeks following the use of saponin-added vaccine. The reaction was more persistent, lasting 10–12 weeks when antigens were incorporated in Freud's incomplete vaccine. The edema regressed without leaving any purulent lesion.

Table I. Antibody response by foals, 6–9 months old (the progeny of immune mares) to AHS killed vaccines

Horse No.	Virus inactivated by	Adjuvant	Antibody titer		
			preimmunization	4 weeks after 1st injection	4 weeks after 2nd injection
1		aluminium	8≥ [1]	16	128
2		hydroxide	8≥	8	24
3		saponin	8≥	8	64
4			8≥	16	128
	merthiolate				
5		freund's	8≥	8≥	32
6		incomplete adjuvant	8≥	8	128
7		Fe_2O_3	8≥	8≥	8≥
8			8≥	8≥	8≥
9		aluminium	8≥	8≥	16
10		hydroxide	8≥	8≥	16
11		saponin	8≥	8≥	48
12			8≥	8	128
	formalin				
13		freund's	8≥	8≥	32
14		incomplete adjuvant	8≥	8	64
15		Fe_2O_3	8≥	8≥	16
16			8≥	8	32

1 Reciprocal of the dilution of serum which neutralizes 100 $TCID_{50}$ of strain S2, type 9, AHS virus

Immunological Responses

Formalin-inactivated vaccine. Data presented in table I show that the response of foals, which had detectable neutralizing antibody before immunization, to the first dose of vaccine was poor regardless of the type of adjuvants used. However, following a 2nd injection of the same vaccine, a 4-fold or more rise of neutralizing antibodies (NA) was recorded. The response of horses free of NA to the first injection of formalin-inactivated vaccine was remarkable, the lower response was recorded for the antigen adsorbed on aluminium hydroxide. By the second injection a 2- to 4-fold increase in NA titer was observed (table II).

Table II. Antibody response by horses, 3–4 years old and free of neutralizing antibody, to AHS-killed vaccines

Horse No.	Virus inactivated by	Adjuvant	Antibody titer		
			preimmunization	4 weeks after 1st injection	4 weeks after 2nd injection
17	merthiolate	aluminium hydroxide	0	256[1]	512
18			0	16	64
19		saponin	0	4⩾	16
20			0	16	64
21		freund's incomplete adjuvant	0	4⩾	4⩾
22			0	256	256
23		Fe_2O_3	0	4⩾	16
24			0	256	256
25	formalin	aluminium hydroxide	0	16	64
26			0	32	32
27		saponin	0	64	256
28			0	256	256
29		freund's incomplete adjuvant	0	256	1,024
30			0	256	256
31		Fe_2O_3	0	12	96
32			0	256	256

1 Reciprocal of the dilution of serum which neutralizes 100 $TCID_{50}$ of strain S2, type 9, AHS virus.

Merthiolate-inactivated vaccine. The response of foals with preimmunization NA to the first injection of the merthiolate-inactivated vaccine was

generally better than that of formalin-inactivated vaccine and, following the 2nd inoculation of the same vaccine, a 4-fold increase in NA titer was recorded from all foals, irrespective of the nature of adjuvant used (table I); the use of fluid antigen also impairs the onset of immunity.

The first and the second response of horses free of preimmunization NA, to merthiolate-inactivated vaccine was not different from those recorded in the case of formalin-inactivated vaccine (table II).

Discussion

Although a cell culture vaccine of an excellent potency, comparable to the South African mouse brain attenuated vaccine, had been produced with 9 types of AHS virus-attenuated strain in this laboratory (2, 4, 5, 8), the introduction of the live vaccine or even importation of horses immunized with live vaccine is not authorized in hitherto AHS free countries; it was, therefore, of interest to produce a killed vaccine giving protection to the horses imported from infected area to the free zones. Such a vaccine had been discussed in previous reports (6, 7) giving protection for at least 6 months following two injections of vaccine.

The purpose of this study was to determine whether change of method of inactivation of virus or introduction of new adjuvants would make a better vaccine.

In this study the previous vaccine, prepared by inactivation of AHS virus with 1:3000 formalin at 32°C in 3 days and adsorption of killed virus on aluminium hydroxide, was compared with a vaccine prepared by inactivation of AHS virus by merthiolate in 5 days and adsorption of inactivated virus on the aluminium gel. Formalin- or merthiolate-inactivated viruses were also separately incorporated with incomplete Freund's adjuvant, saponin or iron-oxide (Fe_2O_3) used as adjuvants, and were applied for immunization of horses.

Data presented in this report indicated that the procedure of inactivation by formalin or merthiolate had little effect, if any, in the elaboration of NA in horses free of type-specific antibody or with a trace of maternal antibody when two injections of vaccine are made. The addition of a suitable adjuvant to the antigen is essential in order to enhance the immunity. Another point of interest in the present study is that the group of adjuvants used had little effect on the primary immune response when a trace of maternal NA was still present; on the contrary, they all exert a marked effect on primary immune response in horses free of NA. Regarding the type of adjuvant to be used, one should evaluate the possible undesirable side-effect of potent adjuvant and its potential to initiate a high titer. In the present study Freund's incomplete adjuvant and saponin used as adjuvant stimulated production of high titers

of NA in horses in the primary immune response, but in large-scale immunization we do not yet know the percentage of possible severe long-term side-effects which may follow utilization of these adjuvants and the difficulties of using a water-in-oil emulsified antigen.

The iron-oxide salt is a mild adjuvant with slight reaction and ability to induce high titer of NA but its use, on a large scale, cannot be recommended. Thus, it appears that aluminium gels will be the adjuvant of choice, easy to produce and with minimum side-effect.

The study of the stability of antigenic potency under different storage conditions and the duration of immunity of two batches of formalin- or merthiolate-inactivated vaccine adsorbed on aluminium hydroxide are in progress and the results will be presented later.

However, from data so far recorded it seems that merthiolate-inactivated antigen induces a longer immunity in horse. This finding is in accordance with the observation of Masuno *et al.* (3) who have shown a long-lasting NA in children immunized with merthiolate-inactivated measles vaccine.

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