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MONOVALENT LIVE-VIRUS HORSE-SICKNESS VACCINE (*)

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Since ALEXANDER (1) reported the development of polyvalent mouse neurotropic horse-sickness vaccine, it has been widely used for many years in Africa and Asia. Vaccine prepared from 6 or 7 different African types of horse-sickness virus have been used in the Middle East and Asia since 1960.

These vaccines provided protection for equines against the local strains, serologically resembling type 6 but later classified as a separate type (No. 9).

There were two main reasons for using polyvalent vaccines in the Middle East and Asia during epizootics since 1959.

Firstly no single attenuated strain capable of immunizing equines against local strains was available when severe outbreaks occurred. Secondly it was not known how many different types of horse-sickness virus were involved.

However, recent investigations by the Authors (3) and SHAH (19) showed that all the strains isolated from natural cases of horse-sickness in the Middle East and Asia were serologically idential with type 9 virus.

Serial intracerebral passages of an Iranian strain of horse-sickness virus in Swiss albino mice were carried out at the Razi Institute. After 100 passages, this strain, S2, was found to be fully attenuated for equines while maintaining its immunizing properties (2).

The S2 strain was successfully adapted to monkey kidney stable (MS) cells by OZAWA and HAZRATI (13) and standard procedures for preparing horse-sickness tissue-culture vaccines have been established (14, 15). The immunizing properties of tissue-culture adapted viruses have been confirmed by MIRCHAMSY and TASLIMI (8, 9) and by OZAWA and al. (15).

This paper extends previous observations (15) and presents the results of stability tests of these vaccines under various conditions and results of comparative

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safety and potency tests of monovalent mouse-brain and tissue-culture vaccines in horses and donkeys.

MATERIALS AND METHODS

Virus. — The 102nd intracerebral passage in mice of the attenuated Iranian strain (S2) of horse-sickness virus was used to prepare mouse brain and tissue-culture vaccines. Two additional passages in mice were made to prepare monovalent mouse-brain vaccine. The 6th passage of the same virus strain in MS cell cultures was used for tissue-culture vaccine. The 4th passage in suckling mice of an Iranian strain (10/60) (5, 17) served as challenge virus.

Infected mouse brains were stored at — 20 °C, and virus fluids at 4 °C throughout the experiment.

Cell Cultures. — The monkey kidney stable cell line, commonly known as MS cells, was obtained from the National Institute of Health, Japan. The method of cultivating MS cells has been described in previous papers (13, 15). The growth medium consisted of Earle's balanced salt solution with addition of 0.5 percent lactalbumin hydrolysate, 0.005 percent yeast extract, 0.0015 percent phenol red, 10 percent calf serum heated at 56°C for 30 minutes, 100 units/ml penicillin, 100 percent gm/ml streptomycin, and sodium bicarbonate to adjust the pH to 7.2 The maintenance medium had a composition similar to that of the growth medium but with calf serum reduced to 2 percent. Monolayers of MS cells were formed approximately 3 days after seeding with a cell suspension containinx 1.5×10^5 cells/ml of growth medium.

Preparation of Vaccine. — The method of producing monovalent mouse-brain vaccine was basically the same as that established by the Onderstepoort Veterinary Institute (1) and modified by other workers (6, 11). A 10 percent brain suspension in chilled distilled water, without centrifugation, was mixed with an equal volume of lactose-peptone-phosphate buffer solution (11) at pH 7.4.

To prepare monovalent tissue-culture vaccine, the 6th passage in MS-cell cultures of the S2 virus was centrifuged at approximately 500 g. for 10 minutes at 4 °C. The supernatant fluid was removed aseptically avoiding contamination with cell debris. One volume of the supernatant virus fluid was mixed with 10 volumes of freeze-drying diluent (*) and 9 volumes of sterile distilled water.

(*) Freeze-drying diluent was prepared as follows :

Solution (*a*) : 200 g lactose, and 40 g peptone were disolved in 1,000 ml of distilled water.

Solution (*b*) : 250 ml of 0.2 M Trishydroxymethylamino methane was mixed with 207 ml of 0.2 M HCl and 543 ml of distilled water. This gave a final pH of 7.4.

The two solutions were mixed. Four hundred units/ml penicillin and 400 micro g/ml. streptomycin were added to the mixture which was sterilized by filtration.

Both mouse-brain and tissue-culture vaccines were dispensed in 1-oz. penicillin bottles in volumes of 5 ml liquid vaccine which was then freeze-dried overnight in a Stokes freeze-drying machine. Dried vaccines were vacuum sealed and stored at 4 °C. The method of preparing freeze-dried horse-sickness tissue-culture vaccines has been described in detail (15).

Reconstitution of Vaccine. — All freeze-dried vaccines were reconstituted by adding sterilized physiological saline to dilute the virus to the required concentration.

Titration of Virus. — Serial tenfold dilutions of virus were prepared in maintenance medium. The viscerotropic challenge virus, strain 10/60 was titrated in 5 to 6 week-old mice by injecting intracerebrally 5 mice per dilution each with 0.05 ml of inoculum. The inoculated mice were kept under close observation for 2 weeks. S2 virus adapted to tissue cultures was titrated in MS-cell tube cultures inoculating 4 tubes per dilution with 0.1 ml of inoculum as described previously (13, 15). The final reading was made 10 days after inoculation. Virus LD50 in mice and TCID50 were calculated by the REED and MUENCH (18) method.

Antibody Titration.— The serum neutralization titers were tested in MS tube cultures against S2 virus. All serums were heated at 56 °C for 30 minutes.

Serial tenfold dilutions of the virus were prepared in maintenance medium. Three tenth ml. of each dilution were mixed with an equal volume of the inactivated serum to be tested. The mixtures were incubated at 37 °C for 60 minutes.

Four tubes were inoculated with 0.1 ml of each virus dilution mixed with serum in equal parts. They were incubated at 37 °C for 30 minutes before 1.5 ml of the maintenance medium was added to each tube. The final reading was made 7 days after inoculation. The neutralization index (N I) was expressed as the logarithm of the difference in titer between virus mixed with normal serum and serum under test.

Horses and Donkeys. — Horses were obtained from an isolated village in a mountainous area near the Caspian Sea. All horses used were approximately 12 months old and had not been vaccinated against horse-sickness.

Donkeys were obtained from villages around the Razi Institute. Their ages ranged from 6 to 18 months. They had not been vaccinated against horse-sickness and no clinical case of the disease had been observed in the area during their lifetime.

All vaccinated and control animals were kept in an insect-proof stable. The body temperature of each animal was recorded daily at 9 a.m. and 4 p.m. Any clinical signs of abnormality were recorded.

RESULTS

Stability of the Vaccine. — The stability of virus titers of monovalent mouse-brain and tissue-culture vaccines were tested under various conditions.

Titers of freeze-dried vaccines of 4 different batches were examined before and after storage at 4° C. The results are shown in Table I. There was no decrease in titer of freeze-dried mousebrain or tissue-culture monovalent vaccine during storage for 6 months at 4 °C.

Data concerning stability of these vaccines at 37 °C are given in Table II.

The stability of reconstituted monovalent S2 vaccine was also examined. Six bottles of freeze-dried vaccine prepared either from infected mouse-brains (Table III) or tissue-culture virus (Table IV) were reconstituted in 60 ml of sterile saline.

TABLE I

Stability of freeze-dried monovalent horse-sickness vaccines at 4° C

VACCINE	(BATCH No.)	STORAGE PERIOD	VIRUS TITER
Mouse-brain	(batch 1)	0 month	4.8 (*)
		12 months	4.5
Mouse-brain	(batch 2)	0 month	5.0
		6 months	5.3
Mouse-brain	(batch 3)	0 month	4.8
		4 months	4.8
Tissue-culture	(batch 1)	0 month	5.5 (**)
		6 months	5.6

(*) Log. LD_{50}/ml.
(**) Log. $TCID_{50}$/ml

TABLE II

Stability of freeze-dried monovalent horse-sickness vaccines at 37° C

VACCINE	(BATCH No.)	STORAGE PERIOD	VIRUS TITER
Mouse-brain	(batch 2)	0 day	5.3 (*)
		3 days	4.8
Mouse-brain	(batch 3)	0 day	4.9
		3 days	4.3
Tissue-culture	(batch 1)	0 day	5.5 (**)
		7 days	5.0

(*) Log. LD_{50}/ml.
(**) Log. $TCID_{50}$/ml.

Each of the reconstituted vaccine, which had pH values of 7.3, was distributed into 3 small bottles fitted with rubber stoppers. They were stored at 3 different temperatures, 4°, 22°, 37 °C. Samples for titration were taken immediately after reconstitution and thereafter at given intervals. The titration results of reconstituted vaccines are shown in Tables III and IV.

Tests in Horses. — Safety and potency tests of both mouse-brain and tissue-culture vaccines were made in a total of 12 horses. Horses 1 to 7 were inoculated with monovalent mouse-brain vaccines containing different concentrations of virus (see Table V). Horses 8, 9, and 10 were inoculated with monovalent tissue-culture vaccine containing 125,000 TCID50 of S2 virus. Horses 11 and 12 remained unvaccinated, and were kept in the same stable. No significant temperature responses or adverse effects due to vaccination were recorded.

TABLE III

Stability of reconstituted monovalent mouse-brain vaccine at 3 different temperatures

Storage temperature	Hours of storage			
	0	9	24	72
4 °C	6.0 (*)	6.0	5.9	5.6
22 °C	6.0	6.0	5.5	5.0
37 °C	6.0	5.5	5.2	4.5

(*) Log. LD_{50}/ml.

TABLE IV

Stability of reconstituted monovalent tissue-culture vaccine at 3 different temperatures

Storage	Hours of storage			
	0	3	9	24
4 °C	5.6 (*)	5.6	5.5	5.5
22 °C	5.6	5.5	5.5	5.0
37 °C	5.6	5.5	4.5	4.2

(*) Log. $TCID_{50}$/ml.

The vaccinated horses were bled 7 weeks after vaccination in order to investigate antibody responses. Antibody titers were measured by neutralization tests using prevaccination serum as a control in each case. The results are shown in Table V. The day after the last bleeding, all 12 horses were subjected to intravenous challenge with infectious mouse-brain virus suspension (strain 10/60) containing 6.3 $\times$ 10^6 LD50/ml of virus for suckling mice. It has not been possible to determine the infectivity titer of the challenge virus in horses. Horses 1 to 3 were inoculated with 1 ml, horses 4 to 7 with 2 ml, and horses 8 to 12 with 1 ml of the above suspension.

The body temperatures of both control horses rose 4 days after the inoculation of challenge virus. Horse 11 died of horse-sickness showing predominant cardiac symptoms 2 weeks after inoculation. Postmortem findings and isolation of horse-sickness virus from various tissues confirmed that the cause of death was horse-sickness. Horse 12 also suffered from horse-sickness for several days showing symptoms of the disease such as swelling of supraorbital fossae and high fever. This animal, however, recovered and developed neutralizing antibodies (N I = 3.0) 2 months after challenge injection.

All horses vaccinated either with mouse-brain vaccine or with tissue-culture vaccine developed immunity, and no significant temperature response was recorded for 5 weeks following inoculation with challenge virus.

TABLE V

Antibody titers in horses vaccinated with horse-sickness monovalent mouse-brain and tissue-culture vaccines

House No.	Type of vaccine used	Virus titer of inoculum	Virus mixed with prevaccination serums	Virus mixed with post-vaccination serums	N I
1	Mouse-brain	63,000 (*)	6.3 (**)	2.0	4.3
2	Mouse-brain	126,000	6.0	2.0	4.0
3	Mouse-brain	126,000	6.3	2.0	4.3
4	Mouse-brain	315,000	6.3	1.3	5.0
5	Mouse-brain	315,000	6.0	2.5	3.5
6	Mouse-brain	1,260,000	6.0	2.5	3.4
7	Mouse-brain	3,150,000	6.0	2.0	4.0
8	Mouse-brain	125,000	6.5	2.0	4.5
9	Tissue-culture	125,000	6.3	2.0	4.3
10	Tissue-culture	125,000	6.6	4.4	2.2

(*) LD_{50} or $TCID_{50}$.
(**) Log $TCID_{50}$/ml. (Titers of S2 virus mixed with respective serums.)

TABLE VI

Antibody titers in donkeys vaccinated with monovalent horse-sickness vaccines

Donkey No	Type of vaccine used	Virus titer of inoculum	Neutralization indices of serums	
			Days after vaccination	
			60 days	115 days
1	Mouse-brain	60,000 (*)	3.5	4.5
2	Mouse-brain	500,000	3.5	4.0
3	Tissue-culture	300,000	2.0	2.5
4	Tissue-culture	6,000,000	3.5	4.5
5	Tissue-culture	6,000,000	2.5	3.0
6	Tissue-culture	30,000,000	3.5	3.5
7	Tissue-culture	50,000,000	3.5	4.0
8	Control	0	0	0

(*) LD_{50} or $TCID_{50}$.

Tests in Donkeys. — In the same manner as in horses, the safety and potency of both mouse-brain and tissue-culture vaccines were tested in a limited number of donkeys. Two donkeys were vaccinated with a mouse-brain vaccine and 5 donkeys with a tissue-culture vaccine containing various concentrations of S2 virus (Table VI).

Antibody titers 60 days and 115 days after vaccination were titrated by neutralization tests, using prevaccination serum as a control in each case. Neutralization indices of all donkey serums are shown in Table VI. There was no antibody response in a control donkey, but all vaccinated donkeys developed antibodies against the vaccine virus.

No significant rises in body temperature nor any obvious local or general reactions due to vaccination were recorded.

All the donkeys were challenged with infectious mouse-brain suspension of strain 10/60, 5 months after vaccination. Each challenge dose contained approximately 10^6 LD50 of the virus titrated is suckling mice. One week after inoculating the challenge virus, the control donkey had a high fever, and was distressed for about one week, but eventually recovered. All vaccinated donkeys appeared normal, and no significant temperature responses were recorded.

Field Trials of Vaccines. — The first trial was designed to compare the post vaccination reactions in donkeys vaccinated with monovalent and polyvalent mouse-brain vaccines. Two groups of susceptible donkeys were used in this experiment.

Group 1 consisted of 61 donkeys. They were vaccinated with monovalent S2 mouse-brain vaccine and kept under close observations for 45 days. Group 2 comprised 78 donkeys. They were vaccinated with polyvalent mouse-brain vaccine which contained 6 serologically different neurotropic vaccine strains (A 501, OD, L, Vryheid, VH, and 114). These strains were obtained from the Veterinary Institute in Onderstepoort. Such polyvalent vaccines have been widely used in the Middle East and Asia.

According to a report received from the Veterinary Department, the donkeys of group 1 showed no severe general reactions and no death occurred. Three of these donkeys (approximately 5 percent) showed a mild reaction. However, more severe reactions were observed in donkeys of group 2 vaccinated with polyvalent vaccine. More than 8 donkeys (approximately 10 percent) of this group showed very severe general reactions, and 4 of them (about 5 percent) died. Some of other vaccinated donkeys of group 2 showed mild reactions towards the end of the observation period. Since we have not observed these donkeys ourselves, it is not possible to give a more detailed description of the clinical picture.

The 2nd trial was made to investigate the safety of monovalent tissue-culture vaccines in donkeys as well as in horses and mules. Approximately 450 donkeys, 45 horses, and 50 mules in the field were vaccinated with monovalent tissue-culture vaccine which contained approximately 6.32×16^6 TCID50 of S2 virus per dose. No adverse effect due to vaccination was observed during a 6-month observation period (KAWEH and SIADAT, personal communication). Polyvalent tissue-culture vaccines have not been tested in the field.

DISCUSSION

The results obtained from the limited numbers of animals thus far observed indicate that the monovalent mouse-brain and tissue culture vaccines used are safe in horses, donkeys and mules. Both monovalent vaccines produced sufficient immunity in vaccinated horses and donkeys to protect them against the homologous Asian type (No. 9) of horse-sickness virus. The antibody titers developed by vaccinated animals showed some individual variation.

Since all recoveries of horse-sickness virus made from Iran, Turkey, Pakistan and India were serologically identical (3, 4) the possibilities of using a monovalent vaccine against this strain were investigated. Neurological involvement has been observed by NOBEL and NEUMAN (10) and by PAVRI and ANDERSON (16) in horses following vaccination with polyvalent vaccines. They isolated type 2 (OD) virus from the brains and other organs of horses which died after vaccina-

tion. Cyprus and Iran have previously reported unfavorable reactions in donkeys following use of the polyvalent vaccine (20). HOWELL (7) reported that horses failed to develop specific antibodies to certain components of the polyvalent vaccine in spite of repeated vaccination and that African horse-sickness occurred from time to time in vaccinated equines. This may have been due to poor antigenicity of the respective components as HOWELL suspected or to interference between certain virus types incorporated in the vaccine. *In vitro* interference between horse-sickness viruses was demonstrated by OZAWA (12) in tissue culture of monkey kidney cells.

The possibility cannot be disregarded that, theoretically new virus types may be formed by genetic recombination after simultaneous inoculation of various attenauted strains into susceptible hosts; however, it could be conversely discussed from the experience following the wide use of polyvalent vaccine in the Middle East that its use may have been a factor in suppressing the appearance of new virus types of providing a broad antibody spectrum.

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SUMMARY

Monovalent live-virus horse-sickness vaccine against Asian strains of horse-sickness virus was prepared by using the mouse-adapted neurotropic Iranian strain, S2. The strain was attenuated by 102 intracerebral passages in adult mice while maintaining its immunizing properties. This attenuated strain was used in both mouse-brain and tissue-culture vaccines. Monovalent tissue-culture vaccine was prepared after 5 or 6 additional passages in MS cells.

The effect of storage under various conditions was studied. There was no decrease in titers of freeze-dried vaccines during a storage period of 6 months at 4 °C. Virus titers of reconstituted vaccines were fairly stable when stored at 4 °C.

Horses and donkeys vaccinated with either mouse-brain or tissue-culture monovalent vaccines developed immunity against an Asian type viscerotropic strain of horse-sickness virus. The safety of both monovalent vaccines has been tested in the field with no adverse effects being observed among vaccinated equines.

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RESUME

Un virus-vaccin vivant monovalent contre les souches du virus de la Peste

équine africaine a été préparé en utilisant la souche iranienne S2 neurotrope adaptée à la souris.

La souche a été atténuée par 102 passages effectués par voie intracérébrale chez la souris adulte tout en maintenant ses propriétés immunisantes. Cette souche atténuée a été utilisée aussi bien sous forme de vaccins préparés au moyen de cerveau de souris que de cultures de tissu.

Un vaccin de culture de tissu monovalent a été préparé après que 5 ou 6 passages supplémentaires eurent été effectués en cellules MS.

L'effet de la conservation dans différentes conditions a été étudié. Il n'y eut pas de diminution du titre des vaccines lyophilisés après une période de conservation de six mois à 4 °C. Les titres en virus des vaccins reconstitués se sont montrés remarquablement stables lorsqu'ils ont été conservés à 4 °C.

Les chevaux et les ânes vaccinés avec les vaccins monovalents préparés soit avec le cerveau de souris soit avec la culture de tissu ont été immunisés contre une souche asienne de type viscérotrope du virus de la Peste équine. L'innocuité de ces deux vaccins monovalents a été éprouvée dans la pratique. Aucune réaction défavorable n'a été observée chez les équidés vaccinés.

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