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IMMUNIZATION AGAINST AFRICAN HORSE SICKNESS WITH TISSUE-CULTURE-ADAPTED NEUROTROPIC VIRUSES

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The spread of African horse sickness into Middle-East countries during 1959 necessitated production of a large amount of all types of virus for vaccination of the several million fully susceptible horses in this area (Rafyi, 1961). To provide standard polyvalent mouse-adapted vaccine for Iran and for the neighbouring countries our laboratories have used, during the last three years, more than 300,000 Swiss mice from a local inbred colony as well as from foreign colonies. It was found that the production of vaccine could be seriously affected when mice could not be supplied on a large scale. Consequently, the discovery of new methods for production of large supplies of vaccine as well as for research into problems connected with immunology of this disease is of extreme importance.

Alexander (1935), who first developed the polyvalent mice-adapted neurotropic vaccine, later felt the necessity of developing new techniques for production of large amounts of virus by other methods. He adapted the horse sickness virus (HSV) to grow in developing chick embryos (1938), but he finally concluded that the method would not be suitable for vaccine production.

Erasmus (1963) cultivated HSV in monolayers of chicken embryo fibroblasts and demonstrated replication of the virus. Because of poor viral yield and absence of cytopathic effect this method could not be applied to the production and testing of vaccine.

Propagation of HSV in primary hamster kidney cells by Mirchamsy &

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Taslimi (1962), 1963) was a first step in developing a new method for the production of a high titre virus by means of tissue culture. The same authors (1964) have shown :

(1) The possibility of immunizing the horse with a viscerotropic virus propagated in tissue culture and modified by serial passages in primary baby hamster kidney cells.
(2) The neurotropic type 9 virus adapted to a hamster kidney cell line (BHK 21) or to a monkey kidney cell line (MS) would induce a high antibody response in horses immunized with this antigen.
(3) The complicated and expensive seroneutralization test previously done in mice could be replaced by a neutralization test in tissue culture, which is far simpler.

In the present communication preliminary results on the production of polyvalent live attenuated tissue-culture-prepared vaccine, its efficacy in the limited number of horses thus far tested, and the practical simplicity of the tissue culture technique for evaluation of antibody response are described.

MATERIALS AND METHODS

Virus

The seven neurotropic attenuated strains representing the South African types of HSV were used. These were supplied by the Onderstepoort Laboratories and in our Institute they are used as a routine in the production of standard mouse-adapted vaccine. The strains are : type 1 (A 501), type 2 (O D), type 3 (L), type 4 (VRY), type 5 (VH), type 6 (114) and type 7 (Karen). The local strain (S2-Shiraz), successfully attenuated by 100 intracerebral passages in adult Swiss mice by Hazrati & Taslimi (1961), and used as a monovalent vaccine, was also included. By seroneutralization test the latter strain was found antigenically identical to another local strain (S10/60) classified by Howell (1962) as type 9.

Strain S2 was adapted to BHK 21 cells (supplied by Professor M. Stoker, Glasgow) and to MS cells. The other seven strains were only adapted to MS cells. Details about the preparation of monolayers, growth media and maintenance media were given in our previous publications (Mirchamsy & Taslimi, 1962, 1963, 1964). A cytopathogenic effect was visible in both cell lines 24 hours after incubation. Infected cells lost their shape and became rounded. These changes increased on the second day, when in all parts of the infected sheet cytopathological changes were evident. At this stage the cytoplasm was granular and contracted and the nucleus was pyknotic and disintegrated.

Degeneration was complete on the third day for MS and on the fourth day for BHK 21 cells. Figs. 1 and 3 show the uninfected sheets and Figs. 2 and 4 the final stages of the infected cells. In Table 1 the passage levels and the titre of viruses used for vaccine production are given.

TABLE I

MOUSE AND TISSUE CULTURE TITRES OF HORSE-SICKNESS SEED VIRUSES AT 35°C

Type of virus	*Cell line*	*Passage level*	*Harvest day*	*Titres (neg. log.)*	
				Mice, i.c. 0·03 ml.	*$TCID_{50}$/ml.*
1	MS	7	2	6·39	7·0
2		8	2	6·48	
3		9	2	6·20	6·5
4		9	2	6·24	7·0
5		8	2	5·70	6·5
6		9	2	5·70	6·5
7		8	3	5·85	6·5
9		5	3	5·40	6·5
9		16	3	6·38	7·0
9	BHK21	8	4	4·75	5·0
9		20	3	5·35	5·5

Vaccine production

MS cells were grown in serum bottles using the L.A.Y.E. medium of Franklin, Rubin & Davis (1957) with 10 per cent heated calf serum. The same medium with 5 per cent serum was used as maintenance medium. The final number of cells per bottle was approximately 40,000 in 2 ml. growth medium.

The growth medium for BHK 21 was one containing 0.5 per cent lactalbumin hydrolysate in Earl's saline and 5 per cent heated calf serum. The maintenance medium was the same. The final number of cells per bottle was approximately 300,000 in 2 ml. of growth medium. The pH of all media was adjusted to 7.1-7.2 by adding 1.4 per cent sodium bicarbonate. One hundred units penicillin, 100 g. streptomycin and 100 units mycostatin per millilitre were added before use.

Monolayers were generally complete in three to four days. Bottles were inoculated with 0.2 ml. of 10^{-1} of lyophilized viruses restored in sterile distilled water. Thirty to 45 minutes were allowed for viral adsorption at 35 °C. Maintenance medium (1.8 ml.) was then added to each bottle. The whole

culture of each type was harvested by pooling cells and fluid when the cytopathogenic effect was complete. This suspension was immediately frozen at -40 °c and thawed. After centrifugation at 3,000 rev./min. for 30 minutes at 2°c the supernatant fluid was separated. For the polyvalent vaccine equal parts of the fluids of eight types of virus were pooled, dispensed into 1 ml. ampoules and lyophilized. Monovalent type 9 vaccine was prepared in the same manner with fluid of the corresponding culture.

The LD 50/ml. of lyophilized polvalent vaccine for adult mice was approximately 3.3×10^7 Ten months after lyophilization and storage in -40°c this vaccine kept its original titre for mice.

Experimental horses

Horses were bought from Khorassan, north-east Iran, where no previous outbreak of the disease had been recorded. The animals were two to three years old. The serum of all horses was screened before inoculation as well as eight weeks after immunization, in order to assay the neutralizing antibodies against all different types. It was ascertained that before immunization all sera were free of antibodies for the agents tested.

Each horse was subcutaneously inoculated with 1 ml. of lyophilized polyvalent or monovalent vaccine suspended in sterile distilled water. The temperature of all horses was recorded twice a day for eight weeks. No thermal rise was recorded and no untoward reaction was observed during the entire period of observation.

In vitro seroneutralization test

Sera were inactivated at 56°C for 30 minutes. Twofold dilutions were made of each serum from 1 :4 to 1 :1,024. Two-tenths ml. of each serum dilution was mixed with 0.2 ml. of the test virus containing 100 media tissue culture infective dose (TCID 50) per 0.1 ml. and the mixture was incubated for two hours at room temperature. A control of normal horse serum and a virus titration were included with each test. After incubation, 0.1 ml. of each serum-virus mixture was inoculated into two MS tissue culture tubes and incubated at 35°c. The tubes were read each day for the cytopathogenic effect, and the readings on the day that the control virus titration showed 100 TCID 50 of virus to be present were used as the final readings. All serum titres were expressed as the reciprocal of the highest dilution of serum that completely inhibited the cytopathogenic effect in both tubes inoculated.

Challenge of immunized hourses

In the ninth week of immunization with type 9 vaccine, the immunized horses as well as a non-immunized control were challenged with $10^{7.5}$ mice LD 50/ml. of homologous virus (S10/60) by intravenous injection. The virulent virus was prepared from an 8 per cent suspension of suckling mouse brain, collected when the mice were *in extremis*. The suspension was centrifuged for 15 minutes at 2,500 rev./min. at 2°c before inoculation.

RESULTS

(a) Monovalent vaccine

Horses immunized with 1 ml. of virus suspension, strain S2 of type 9, adapted to BHK 21 or to MS cell lines, responded with a high antibody titre (Table II).

IMMUNITY IN HORSE IMMUNIZED WITH MONOVALENT TISSUE-CULTURE-ADAPTED TYPE 9 STRAIN S2 (NEUROTROPE) OF HORSE-SICKNESS VIRUS

Horse no.	*Cell line/ passage level*	*Titre of virus (neg. log.)*		*Neutralizing antibody titre*	*Reaction to challenge*
		LD_{50} 0·03 *i.c. mice*	$TCID_{50}$/*ml.*		
1	MS 5	5·40	6·5	1024<	N
2				512	N
3	MS 16	6·38	7·0	512	N
4				256	N
5	MS3 BHK8	4·75	5·0	1024<	N
6				128	N
7	MS3 BHK20	5·36	5·5	128	N
8				512	N
9 control				4>	Died

N = Normal

Although the LD 50/ml. for mice and TCID 50/ml. are about 1 log higher for vaccine produced in MS cells, there was no significant difference

between the antibody responses. The horses immunized in this manner resisted challenge with virulent homologous virus, while the control died from horse sickness 12 days after challenge, with typical symptoms of the disease. The prevalent type of HSV recognized since 1959 in the Middle-East countries is type 9 and the monovalent tissue culture neurotropic type 9 vaccine may therefore be expected to give satifactory results in immunizing the horse population of the area.

(b) Polyvalent vaccine

It was interesting to study, from the practical point of view, the immunogenic effect of combining several types of HSV adapted to tissue culture. The results of immunizing ten horses inoculated with 1 ml. of the eight mixed viruses are shown in Table III.

TABLE III

ANTIBODY RESPONSE BY 10 HORSES INOCULATED WITH 8 TYPES OF NEUROTROPIC TISSUE-CULTURE-ADAPTED HORSE-SICKNESS VIRUSES

Horse no.	*Antibody titre to:*							
	Type 1 A 501	*Type 2* OD	*Type 3* L	*Type 4* VRY	*Type 5* VH	*Type 6* 114	*Type 7* Karen	*Type 9* S2-Shiraz
1	512	512	128	4>	512	16	512	512
2	32	512	512	8	1024<	128	32	512
3	128	512	512	32	512	512	512	512
4	32	128	1024<	4>	1024<	128	512	128
5	32	512	128	32	1024<	512	128	1024<
6	128	512	128	32	128	128	128	512
7	512	1024<	1024<	8	1024<	128	512	1024<
8	32	512	512	4>	512	512	32	512
9	512	512	512	4>	512	512	512	512
10	32	512	512	64	512	1024<	512	128

It is worthy of note that the antibody response for all types was satisfactory except for type 4. Forty per cent of horses showed no detectable antibody for this type and the rest responded inadequately. The poor antigenic quality of this type might be attributed to the strain VRY and by selection of other strains one might obtain a better response for type 4.

PLATE I

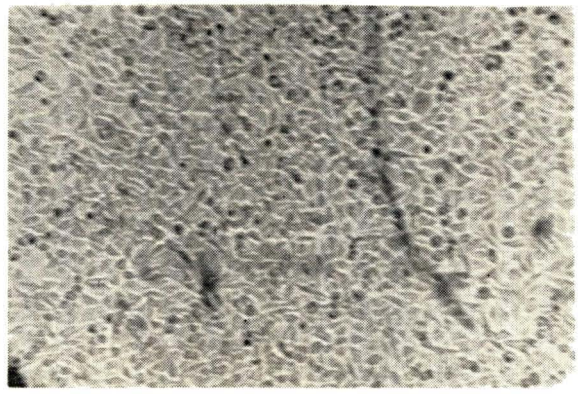

Fig. I Uninfected control of MS cells grown on L.A.Y.E. medium with 10 per cent calf serum culture of seven days (×240).

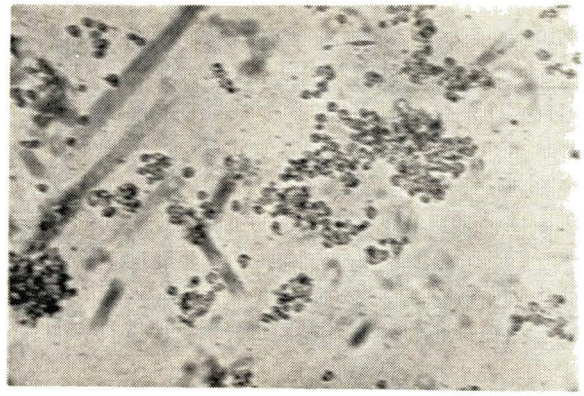

Fig 2. Same MS culture as in Fig. I, 60 hours after infection, with 10^{-1} of HSV type 2, strain OD from sixth passage in MS (×240).

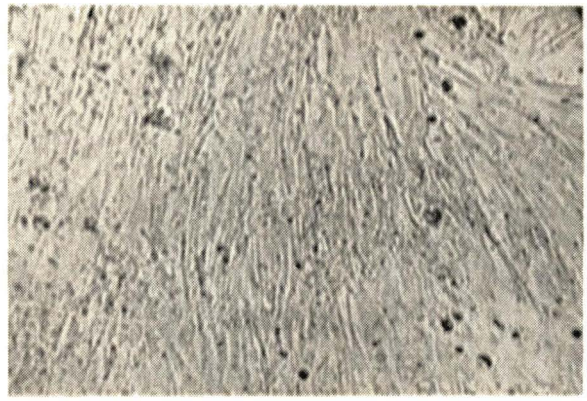

Fig. 3. Uninfected control of BHK 21 cells grown on 0.5 per cent lactalbumin hydrolysate in Earl's saline with 5 per cent calf serum.

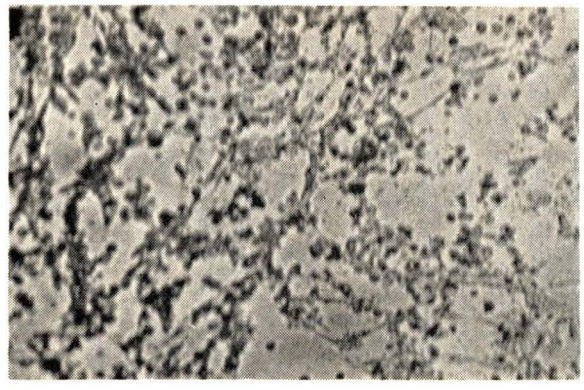

Fig. 4. Same BHK 21 culture as Fig. 3, 60 hours after infection, with 10^{-1} of HSV type 9, strain S 2 from eighth passage in BHK 21.

DISCUSSION

The production of horse sickness vaccine in tissue culture is a new step to combat African horse sickness radically in a newly infected area. Because of its novelty a short discussion of its advantages seems necessary.

It is well recognized that the preparation of mouse-adapted vaccine requires careful control to minimize hazards which may occur in the course of the production of vaccine. During the last four years our laboratory in charge of production of this vaccine faced great difficulties in supplying polyvalent vaccine for field use. On two occasions the inbred colony was infected by *Salmonella typhimurium* or by ectromelia and the colony had to be discarded and replaced by a new one.

Another difficulty is that large numbers of mice have to be used for the seroneutralization test currently used for control of horse-sickness antibodies. The problem is acute when antibodies against nine established types have to be detected in the sera of a group of horses.

The newly developed vaccine has the advantage of being simple and easy to produce. Our laboratory is currently providing several hundred Roux bottles with monolayer sheets of a selected cell line. The culture may be harvested in two to three days.

The seroneutralization test in tubes is also a rapid and simple technique. Twenty MS monolayer tubes are enough to perform a seroneutralization test, in five to six days, while a single *in vivo* neutralization requires a group of 60 healthy mice to be observed for a period of two to three weeks. The results of *in vivo* and *in vitro* neutralization tests are in good agreement.

From data presented above it is clear that there is a poor antibody response to strain VRY of type 4 virus, presumably due to the poor immunogenic quality of this virus. In the present work we deliberately sought to measure the results of combining eight different type viruses adapted to issue culture, without paying special attention to the selection of highly immunogenic strains. Howell (1963), by using the same VRY strain of type 4 in his polyvalent mouse-adapted vaccine, had a similar failure. A point of interest is the rapidity of growth of this strain in MS cells in comparison with other strains used in these experiments.

Although the present experiments are essentially preliminary, it seems reasonably well established that polyvalent vaccine produced in tissue culture may replace that produced in mice. From the practical point of view careful attention should be given to the selection of highly immunogenic strains of all nine types of HSV for vaccine production in tissue culture.

SUMMARY

The seven neurotropic types of African horse-sickness viruses, as well as type 9 which was isolated and mouse-adapted in Iran, were carried from five to 20 rapid passages in MS or in BHK 21 cultures. At this level these types showed a high titre of infectivity for tissue culture and mice without inducing any clinical symptom of the disease in horses. By seroneutralization test in MS culture it was demonstrated that the antibody response of horses immunized with a single dose of monovalent vaccine (type 9) or combined vaccine produced in tissue culture was satisfactory. The poor response with type 4, however, was evident and is discussed.

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