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SOME PROPERTIES OF AVIRULENT COLD VARIANTS OF AFRICAN HORSE SICKNESS VIRUS (*)

By

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SUMMARY. In order to obtain strains of African horse sickness virus avirulent for mice, a wild strain and a neurotropic vaccine strain, both of Asian type 9, were propagated serially in a monkey cell line at decreasing temperatures. The 2 mouse avirulent strains obtained in this way were unable to induce detectable antibody in horses. These strains produce more interferon than the normal virulent strains. There is evidently a relationship between the neurovirulence of African horse sickness virus (AHSV) for mice and its immunogenicity for horses.

The present investigations were initiated to develop strains of African horse sickness virus (AHSV) that are avirulent for mice, and to study the immunological characteristics of such avirulent viruses. For this purpose a wild strain and a neurotropic vaccine strain, both of Asian type 9, were propagated serially in a monkey kidney cell line at various temperatures.

MATERIALS AND METHODS

Virus Strains

Viruses employed in this study were the wild strain S 28 isolated in 1960 from the blood of horses showing symptoms of the acute cardiac form of the disease in Khorram-Abad (Iran), and strain S 2, also isolated from the blood of a sick horse in Shiraz (Iran). The latter became neurotropic after 100 intracerebral passages in mice and is already used as a vaccine strain (Hazrati and Taslimi, 1963). Both viruses are related antigenically to the type 9 of AHSV described by Howell (1962).

After isolation, the strain S 28 was passed 4 times in mice, then 8 times in primary hamster kidney cells (HKC) at 36°C., twice in a monkey kidney stable cell

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line (MS) at 36°C., 31 times in MS cells at 30°C. and finally, 10 times in MS cells at 25°C. This passage history can be summarised as follows: mice 4 HKC 8 (36°C.) MS 2 (36°C.) MS 31 (30°C.) MS 10 (25°C.). After 100 intra-cerebral passages in mice, the neurotropic strain S 2 was first adapted to MS cells by 5 passages at 36°C., then 22 times in MS cells at 30°C., and 10 times in MS cells at 25°C.: Mice 100 MS 5 (36°C.) MS 22 (30°C). MS 10 (25°C.).

Cells

HKC were prepared according to the technique previously described (Mirchamsy and Taslimi, 1962, 1963). The cell strain MS has been used continually in this laboratory for the last 2 years, and consists of a fairly homogenous population of cells growing a uniform monolayer in 160 mm. × 16 mm. tubes. The media used for the growth and maintenance of MS cells have been described already (Mirchamsy and Taslimi, 1964).

In order to obtain enough virus at various temperatures and at different passage levels of MS cells, 10 culture tubes were used in each passage and for each virus. After 3 to 6 days the degeneration of cells was almost complete, when the fluids were harvested and pooled. Each passage was made with 0.1 ml. of a 10^{-1} dilution of the pooled fluid from the previous passage, which dilution as allowed to adsorb for 30 to 45 min. at 25°C. before 0.9 ml. of the maintenance medium was added to each tube. The pooled fluid of each passage was lyophilized and kept at —40°C.

Infectivity Test

The infectivity of the cultures was tested in mice. A volume of 0.03 ml. of each dilution of lyophilized virus was injected intracerebrally into each of a group of 8 to 10 adult mice. Mortality was recorded daily and the end-point determined after 2 weeks observation, by using the method of Reed and Muench (1938).

Tissue Culture Titration

The tissue culture infective dose 50 (TCID50) of viruses, at various passage levels and during the adaptation of viruses to growth at 30°C. and 25°C., was determined in MS cells. The titration was done at 36°C. and 2 MS tubes were used for each virus dilution.

Horse Sera

The experimental horses used were 2 to 3 years old and were free from AHSV antibodies. Serum neutralization tests of the horse sera, before and after immunization, were performed in MS cells according to the technique described in previous reports (Mirchamsy and Taslimi, 1964).

Production of Interferon

Interferon was prepared according to the method described by Wagner (1961) as follows: 3 sets of 20 tubes of MS cells were infected with 0.1 ml. of a

10^{-1} dilution of the 2 cold variants S 28 [Mice 4 HKC 8(36°C.) MS 2(36°C.) MS 31(30°C.) MS11 (25°C.)], and S 2 [Mice 100 MS 5(36°C.) MS 22(30°C.). MS 11(25°C.)], prototype strain 9 [S2 MS 19 (36°C.)] respectively. Five to 6 days later, when the CPE was almost complete in all tubes, the fluids of each set were harvested, lightly centrifuged and then subjected to three 2-hr. cycles of centrifugation at 100,000 g to free it from virus. The supernatant fluid was stored at + 4°C. for interferon assay.

The infectivity of this fluid was first tested in MS cells. No CPE was detected when undiluted fluid was assayed. One sample of each interferon was dialyzed overnight in phosphate buffer before interferon assay. The interference was not reduced by dialysis.

Plaque Reduction Method for Interferon Assay

The interfering activity of interferon was measured on monolayers of MS cells using as an end-point the reduction by 50% of the number pf plaques as suggested by Wagner (1961). For this purpose two-fold dilutions of the interferon to be titrated were made in a series of tubes containing 1 ml. of fresh maintenance medium without serum. A suspension of 1 ml. of AHSV type 9 neurotropic strain S 2 passed 20 times in MS at 36°C. from a lyophilized stock estimated to contain 180 to 200 plaque-forming units (PFU) was then added to each tube. Control tubes containing the same dose of virus without interferon were included in each experiment.

Plaque Assay

The plaque method described elsewhere (Mirchamsy and Taslimi, 1966) was applied as follows: 0.3 ml. of each mixture of virus and interferon was plated on monolayer cultures of MS cells in 2 oz. prescription bottles. After 2½ hr. incubation at 36°C. with constant rocking of the bottles in a shaking machine, the fluids were removed and replaced by 6 ml. agar overlay containing 1% washed agar in maintenance medium without calf serum. A few minutes later the bottles were placed in an incubator with the agar layer uppermost. After 3 days incubation at 36°C. plaques were stained with 1/50,000 neutral red in maintenance medium for 1 hr. The plaques were then counted 12 and 36 hr. later.

Culture Protection—Interferon Assay

In the method described by Sreevalsan and Lochkart (1962), the immunity of MS cells, pre-treated with interferon to superinfection was tested. The medium of MS cultures using 3 replicate tubes per dilution in Pyrex tubes of 16 mm. was removed and replaced by a 1 ml. dilution of the interferon to be tested. The cultures were then kept overnight at 36°C. Next day they were infected with 100 TCID50 of AHSV type 9 wild strain 10/60 MS 9(36°C.). The total or partial absence of CPE in the presence of interferon, in comparison with controls showing complete CPE, provides a rapid and reliable assay for interfering activity.

RESULTS

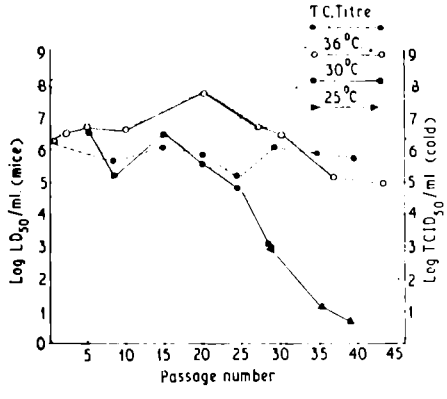
Changes in TCID50 at Different Temperatures

During the passages in MS cells at 36°C., the first cytopathic effect (CPE) was usually noticed 24 to 30 hr. after incubation. The CPE increased on the second day, when in all parts of the infected sheet the change was evident. Degeneration was complete on the third day and the TCID50/ml. was not significantly changed. In the first 3 passages at 30°C. the appearance of CPE was delayed until 48 hr. after infection, and total degeneration of cells occurred in 6 days. After the fourth passage, CPE was noticed 30 hr. post infection and the cell sheet had degenerated by 4 to 5 days. At this stage the TCID50/ml. had dropped by almost 1 log. The adaptation of both viruses to growth at 25°C. was apparently complete after 3 serial passages at this temperature. Although a slight decrease was noticed at this stage in TCID50/ml., this was not consistent and varied from one sample of the lyophilized virus to another.

Changes in Virulence of Virus for Mice with Subcultures at Different Temperatures

The virulence of viruses was tested by intracerebral inoculation in mice after each 5 passages in MS cells. Figs. 1 and 2 show the decrease of virulence of both viruses following serial passages. In the case of neurotropic strain S 2 there was first an increase and then a slow decrease in virulence when virus was propagated serially at 36°C. The LD50/ml. of the starting material was $10^{6.2}$, and was reduced after 44 passages to $10^{5.0}$. The loss of virulence was accentuated when the virus was adapted to 30°C., the LD50/ml. of the strain S 2 dropping to $10^{4.76}$ after 25 passages at 30°C. An avirulent strain was obtained finally when the virus, adapted already, to 30°C., was passed 10 more times in MS cells at 25°C.

The virulence of the wild strain S 28 dropped in the same fashion. While the LD50/ml. of this virus was $10^{5.5}$ after 44 passages in Ms cells at 36°C., it dropped to $10^{2.33}$ when the virus was passed 40 times in the same cells at 30°C. and the pathogenicity for mice was lost after 10 more passages at 25°C.



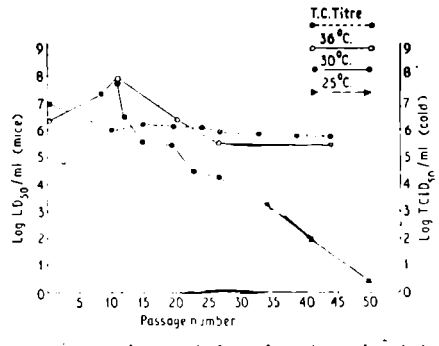


FIG. 1. Decrease of neurovirulence for mice and variation of tissue culture titre after serial passages of AHSV type 9, vaccine strain S2 in MS cell line at various temperatures.

FIG. 2. Decrease of neurovirulence for mice and variation of tissue culture titre after serial passages of AHSV type 9, wild strain S 28 in MS cell line at various temperatures.

Antigenic Characteristics of the Cold Adapted Virus in Horses

Three groups of 5 horses were immunized subcutaneously with a single dose of the following viruses: Group 1, 1 ml of cold adapted wild strain S 28; Group 2, I ml. of cold adapted neurotropic strain S 2; Group 3, 1 ml. of a 1 in 10 dilution of the standard vaccine strain S 2 passed 5 times in MS cells at 36°C. Each injection dose contained 10^5TCID50 of the corresponding virus. Horses were under close veterinary control. No thermal reaction was observed during the 8 weeks of observation. The neutralizing antibody was assayed 8 weeks after immunization. The results are presented in Table 1.

From data presented in this table it is evident that while the standard vaccine strain induced a significant antibody response in horses, the cold variants were unable to initiate antibody formation at a measurable level.

TABLE I

VACCINE TITRE ESTIMATES IN HORSES FOR TYPE 9, TC ADAPTED VACCINE STRAIN OR COLD VARIANTS OF AHSV BY NEUTRALIZATION TESTS IN A MONKEY KIDNEY CELL LINE

Horse No.	Virus-passage level	Titre of virus (Neg. log) LD_{50}/0·03 ml. i.c. mice	Titre of virus (Neg. log) $TCID_{50}$/ml.	Neutralizing antibody (titre)
1	Wild type S 28, mice			<4
2	4 HKC 8(36°), MS 2(36°),	<1	5	<4
3	MS 31(30°), MS 10(25°)			<4
4				<4
5				<4
6	Vaccine strain S 2, mice			<4
7	100 MS 5(36°), MS 22			<4
8	(30°), MS 10(25°)	<1	5	<4
9				<4
10				<4
11	Vaccine strain S 2, mice			512
12	100 MS 5(36°)			>1024
13		6·5	5	128
14				512
15				512

In another experiment, not recorded here, 5 horses were immunized with 2 separate doses of the cold variant S 2 used in the previous experiment at an interval of 4 weeks. In this experiment also no detectable antibody was found 4 weeks after the injection of the second dose of virus.

An attempt was made to re-adapt the cold variants, (*i.e.* those with 10 passages in MS cells at 25°C.) to the same cells at 36°C. After 8 serial passages for re-adaptation at 36°C. no rise of virulence for mice was found and 5 horses immunized with 1 ml. of undiluted tissue culture virus had no significant antibody rise, 8 weeks after immunization.

Interferon Production

The results of interferon assay by plaque and CPE methods are reflected in Table II. It can be seen that the highest interferon yields were obtained in cultures infected with cold variants. Similar results were consistently obtained in 4 experiments.

DISCUSSION

The problem of production of AHSV vaccine in tissue culture being solved (Mirchamsy and Taslimi, 1964), it becomes necessary to direct attention to the stability of antigenic characteristics of tissue-culture-adapted viruses. All 7 mice-

TABLE II

COMPARATIVE TITRATION OF INTERFERON PRODUCTION BY AHSV TYPE 9

Virus (passage level)	Interferon dilution	Plaque reduction assay		CPE method
		Average of plaque count/ml. (a)	Reduction in plaque number %	Immunity to CPE% (b)
Avirulent wild strain S 28	Undiluted	40	78	100
mice 4 HKC 8(36°) MS 2(36°),	1 : 2	53	70	100
MS 31(30°), MS 11(25°)	1 : 4	73	59	100
	1 : 8	133	26	50
	1 : 16	170	6	20
	1 : 32	183	0	0
Avirulent vaccine strain S 2	Undiluted	60	66	100
mice 100 MS 5(36°), MS 22(30°),	1 : 2	63	65	100
MS 11(25°)	1 : 4	77	57	75
	1 : 8	133	26	25
	1 : 16	183	0	10
	1 : 32	180	0	0
Virulent vaccine Strain S 2	Undiluted	85	53	50
mice 100 MS 5(36°)	1 : 2	140	22	25
	1 : 4	185	0	0
	1 : 8	179	0	0
	1 : 16	182	0	0
	1 : 32	182	0	0
	0	181	0	0

(a) Four bottles were used for each dilution. (b) Immunity to CPE: 100 = no degeneration, 75, 50 and 25 = approximately 25%, 50% and 75% degeneration, respectively. 0 = complete degeneration.

adapted African types and the mice-adapted Asian type of this virus are easily adapted to tissue culture and produce, after 5 to 20 serial passages, a high yield of virus; they also induce a significant rise of neutralizing antibodies in horses, comparable with that induced by mouse-brain vaccine.

It is not known, however, how long these viruses can be passed in different host cells without changing their genetic characteristics.

To answer this question the present studies were started. It was especially

important to find the correlation between the passage number at various temperatures in a given host cell, the virulence of the virus for mice, and its immunogenic ability for horses. From data presented in this report it is evident that there is a relationship between neurovirulence of the virus for mice and its antigenicity in horses, virus avirulent for mice being unable to induce immunity in horses.

Another characteristic of the cold variants of AHSV was the increase in interferon yield. Difference in interferon production between strains of the same virus have been reported by several investigators. De Mayer and de Somer (1962) found that attenuated variants of both measles and polio viruses produced more interferon than the normal strains. Sellers (1963) reported that attenuated strains of foot-and-mouth disease virus produced more interferon than did the virulent virus.

A practical result we can assume from the present study is that the continuous passage in tissue culture of AHSV strains used for live vaccine production may affect the immunogenicity of the virus for horses and should be avoided.

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