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VENEZUELAN EQUINE ENCEPHALITIS VIRUS: HORSE VIRULENCE OF P-676 AND MF-8 SMALL AND MINUTE PLAQUES*

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Abstract. The P-676 and MF-8 epizootic strains of Venezuelan equine encephalitis (VEE) virus were found to contain a minute plaque (MP), different from the predominant small plaque (SP) present in these virus strains. The MP and SP were stable after passages in Vero cells, mice, or horses. Equines were inoculated with the SP or MP of the P-676 and MF-8 strains. Inoculation of either P-676 SP or MP into horses induced high fever and viremia but no signs of encephalitis or death. Four horses infected with MF-8 SP became very ill, with high fever and viremia; three of the inoculated animals died. Four horses were infected with MF-8 MP; only two showed viremia but appeared asymptomatic and afebrile. Neutralization tests with immune sera from the infected equines showed that the P-676 SP and MP appear distinct, while a clear difference cannot be observed with MF-8 SP and MP.

During the course of studies to investigate the possibility of chronic infection of horses infected in utero as one possible survival mechanism of epizootic Venezuelan equine encephalitis (VEE) virus in nature during interepizootic periods, we found that explant cultures from fetuses of mares infected with epizootic VEE strain P-676 yielded two plaque types. One was a minute plaque (MP), 0.3 to 0.5 mm in diameter; the other a small plaque (SP), 1.5 to 2.0 mm in diameter, which was the predominant plaque type in the parental pool.

The finding of a P-676 MP variant led us to search for minute plaques in the MF-8 epizootic strain. We found and cloned a similar MF-8 MP variant. In order to characterize these P-676 and MF-8 plaque variants we conducted the equine virulence studies which this report describes.

MATERIALS AND METHODS

VEE virus

The MF-8 and P-676 epizootic VEE virus strains, which have been characterized as members of subtype I-ABC of the VEE complex have been described elsewhere.[1] The MF-8 strain was originally isolated from a human in Honduras during the 1969 epizootic and the P-676 strain from mosquitoes in Venezuela in 1963. The P-676 strain used to infect the equines had one passage in mice, two in Vero cells, and one in primary fetal equine kidney cells. The MF-8 strain used had one passage in mice, one in Vero cells, and one in primary fetal equine kidney cells. Explant cultures from fetuses of mares infected with P-676 were the source of the small and minute plaque virus. Minute plaques were picked and inoculated into tubes of Vero cell cultures; upon development of cytopathic effect (CPE) the fluid was harvested, serially diluted, and inoculated into Vero 6-well panels. This procedure was repeated until the MP were cloned.

P-676 SP were also selected, diluted directly with phosphate-buffered saline (PBS) plus 0.5% gelatin, and inoculated into 6-well panels. This procedure was repeated until the small plaque type was cloned.

The MF-8 strain was inoculated into Vero panels and the small and minute plaques were selected and cloned. P-676 and MF-8 plaque variants were confirmed as VEE by complement fixation (CF) tests and proved to be stable after several passages in Vero cells and newborn mice. All SP and MP virus pools were prepared in Vero cells as follows: the cell cultures were inoculated and upon appearance of CPE, the cells were frozen and thawed, and the fluid was collected and cen-

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TABLE 1

Patterns of viremia in horses inoculated with Venezuelan equine encephalitis virus, small (SP) and minute (MP) plaque types of strain P-676

Days after inoculation	Virus titers in plasma following inoculation with									
	P-676 SP*						P-676 MP*			
	211†	242	178	198	217	260	190	257	255	259
1	4.4‡	5.5	4.3	5.9	6.2	6.8	1.7	5.3	5.2	7.3
2	NT§	5.7	3.2	5.4	4.1	4.4	1.7	5.2	3.9	5.2
3	5.4	5.2	2.9	4.3	2.5	4.3	1.7	5.2	2.2	2.1
4	3.0	4.0	1.9	2.2	0	3.8	0	4.2	0	0
5	0	0	0	0	0	0	0	0	0	0

* All animals survived.
† Horse number.
‡ Titers expressed as dex/ml.
§ NT, not tested.

trifuged at 2,000 × *g* for 30 min. The supernatant fluid was then diluted 1:2 with PBS containing 15% sucrose and 4% hydrolyzed gelatin, and was dispensed in vials and frozen at −70°C.

Horses

Local crossbred mares (*Equus caballus*) 4–10 years old were used. Animals were free of neutralizing antibodies against VEE and eastern equine encephalitis virus. With the exception of P-676 SP, which was inoculated into six horses, we inoculated each of the other plaque clones into groups of four horses. Equines were housed in an insect-proof stable located in a pasture land 900 meters above sea level. The area is free of VEE virus activity.

Inoculation and collection of specimens

Equines were inoculated subcutaneously with 1.0 ml of inoculum containing 1,000 to 10,000 suckling mouse intracerebral median lethal dose units of virus ($SMICLD_{50}$), except those animals inoculated with MF-8 MP, which received between 10 and 100 $SMICLD_{50}$. Rectal temperatures were taken once daily from day 1 preinoculation to day 12 postinoculation. Heparinized blood was collected from each horse from day zero (day of inoculation) through day 12. Plasma/serum was separated, dispensed in vials, stored in liquid N_2 and transported to the laboratory.

Titrations of plasma were made by intracerebral inoculation (0.02 ml) of 10-fold serial dilutions of the plasma into mice 2–3 days old. Only

TABLE 2

Patterns of viremia in horses inoculated with Venezuelan equine encephalitis virus, small (SP) and minute (MP) plaque types of strain MF-8

Days after inoculation	Virus titers in plasma following inoculation with							
	MF-8 SP				MF-8 MP*			
	191†	183	225	256	228	233	210	253
1	5.3‡	4.3	5.2	4.3	0	0	0	0
2	7.3	6.4	6.2	5.2	0	0	0	0
3	8.1	6.4	7.3	5.2	3.6	0	0	0
4	7.2	3.2	6.7 (D)§	5.2	4.3	0	0	0
5	4.0	<1.7		<1.7	4.5	3.2	0	0
6	2.3 (D)	0		0	3.2	0	0	0
7		(D)		(S)¶	0	0	0	0

* All animals survived.
† Horse number.
‡ Titers expressed as dex/ml.
§ (D), died.
¶ (S), survived.

TABLE 3

Patterns of antibody in sera from horses infected with Venezuelan equine encephalitis virus, strains P-676 and MF-8, small (SP) and minute plaque (MP) types

Mare no.	Days after infection	Reciprocal serum or plasma titers to VEE virus strains employed: P-676 (P)*	P-676 (SP)	P-676 (MP)	MF-8 (P)	MF-8 (SP)	MF-8 (MP)
P-676 (SP)							
211	5	16†	128	8	16	<4	16
	7	64	1,024	64	64	16	64
242	5	4	64	4	4	<4	4
	7	64	128	64	64	4	16
P-676 (MP)							
255	5	4	4	64	<4	<4	<4
	7	128	32	256	8	16	32
257	5	4	4	64	4	<4	<4
	7	16	64	128	64	64	64
MF-8 (SP)							
191	5	16	32	128	16	16	32
	7	128	≥1,024	512	512	64	512
256	5	>4	4	32	16	16	4
	7	32	256	≥1,024	1,024	256	128
MF-8 (MP)							
210	12	<4	8	8	4	4	8
	31	4	16	16	8	16	256
228	12	16	256	256	32	64	256
	31	64	256	512	64	128	256

* (P), parental strain.
† Reciprocal serum titer inhibiting 90% of the test dose.

mice which became sick or died within the first 3 days were considered for the study. Suspect isolates were confirmed as VEE virus by CF test.

Serological tests

Plaque reduction neutralization tests were carried out in Vero cells as reported elsewhere,[2] with overnight incubation of plasma/serum-virus mixtures. Antibody titers were recorded as the highest dilution of serum giving 90% reduction of 60–80 plaque-forming units (pfu) of the test dose.

Titrations of crude suckling mouse brain CF antigen were performed using a microtechnic,[3] with four to eight antibody units.

RESULTS

A group of six horses was infected with P-676 SP and another group of four with P-676 MP (Table 1). All equines infected with either P-676 SP or MP developed fever but no other clinical signs, and none died. Fever was as high as 40.9°C in some animals and lasted from day 1 through day 7 after inoculation. Viremia was high in all six horses infected with P-676 SP. In five of the horses the viremia lasted as long as 4 days. Viremia was also high in three of four horses infected with P-676 MP; but the other, mare No. 190, had barely detectable levels of virus in its blood. Viremia lasted 3 days in three of the mares; mare No. 257 was still viremic on day 4.

Three of four mares infected with MF-8 SP died, but all four horses infected with MF-8 MP survived (Table 2). All equines inoculated with MF-8 SP were severely ill with fever which persisted in some cases for 7 days and in some instances reached 41°C. Viremia was high in all four animals and was present for 5 days. Mares No. 191 died on day 6, No. 183 on day 7, and No. 225

which died on day 4, had a peak viremia above 7.0 dex/ml. Mare No. 256 was the only survivor of the MF-8 SP group.

None of the four mares infected with MF-8 MP developed fever or any other signs of disease but all developed antibodies. Only two animals showed viremia; No. 228 had a 4-day viremia which began 3 days after inoculation, and mare No. 235 had only a viremia with a low titer of 3.2 dex/ml on day 5. Viremic blood of equines infected with either plaque type of P-676 or MF-8 yielded only the type of plaque which had been inoculated into that horse.

Table 3 summarizes the serologic response of equines infected with cloned plaque variants of P-676 and MF-8. Only two animals from each of the experimental groups are listed, but they are representative of the pattern observed in the entire group of horses tested. Homologous antibody was first detected in most of the animals 4 days after inoculation, and by day 5 antibody titers were high enough so that specific differences between the virus plaque variants could be seen.

Mares No. 211 and 242 inoculated with P-676 SP and mares No. 255 and 257 infected wtih P-676 MP developed highly specific antibodies to the homologous virus variant by day 5 after inoculation, with 8- to 16-fold differences in titers. By day 7 the difference in antibody titer was no greater than 2-fold between the homologous and the other variants, with the exception of animals No. 211 and 255 which maintained a high specificity.

The sera taken on days 5 and 7 from the animals inoculated with MF-8 SP showed no difference in antibodies when compared to the other plaque variants. The P-676 SP and MP viruses were neutralized more avidly than the MF-8 SP homologous virus. The horses inoculated with MF-8 MP showed a delayed appearance of antibodies, probably due to the low virus dose inoculated. Animal No. 228, infected with MF-8 MP, showed no greater difference in antibody specificity among the virus variants, while No. 210 showed low antibody titers at 12 days after inoculation, but by day 31, the serum showed a high specificity to the homologous virus.

DISCUSSION

Horses infected with P-676 SP had a longer viremia than those inoculated with MP. Either the P-676 SP or MP alone appeared to be less lethal for equines than the parental pool, which was shown to cause 20–30% mortality.[4,5] We found (G. Justines, unpublished data) that two of four horses developed moderate neurological signs but none died after infection with an inoculum prepared to contain a proportion of 1 MP to 100 SP of P-676. We do not know whether the combination of the SP and MP works synergistically to increase the virulence of the P-676 strain for equines, but one is tempted to speculate that this is so.

The results obtained with the MF-8 strain are of particular interest since the MF-8 MP form infected equines and induced viremia high enough to infect vectors but appeared to be completely non-lethal for horses. This was in contrast to the MF-8 SP form, which was highly lethal for horses.

The serological results indicate that the P-676 SP and MP are antigenically distinct, while a clear difference cannot be observed with the MF-8 variants.

Several hypotheses have been proposed to account for the apparent absence of epizootic VEE virus during nonepizootic periods.[6] The finding in this study that two plaque types could be isolated from each of two epizootic VEE virus strains supports the assumption that epizootic strains are maintained in silent mosquito-vertebrate cycles.[7] The presence of a MP form, distinct from the predominant SP form in P-676 and MF-8 virus strains, shows that epizootic strains can be composed of a heterogeneous virion population. The finding in the epizootic viruses of virions which are less lethal for equines suggests that fluctuations in the proportion of virion types in the virus population may affect the pathogenicity of the virus for equines.

Martin et al. observed that mortality in horses varied in different areas during the 1969–1971 VEE epizootic in Central America.[8] It appears doubtful that factors such as conditions of terrain, equine health, food, and water supply could account totally for this difference.

Modulations in the proportion of virions with high and low lethality for equines in the virus population, as suggested by the MF-8 findings, could possibly result from virus passing in different hosts and vectors in the field.

After an epizootic strain emerges, its virulence as measured by mortality for equines might fluctuate due to modulations of its virion components, but probably it would not subside naturally to a low-virulence, enzootic state. Furthermore, it would have extended beyond its natural geo-

graphic range and its association with its pre-epizootic hosts, and probably would die out.

It seems possible that each epizootic strain must arise *de novo* from one or another naturally occurring enzootic strains which include in their makeup a subordinate proportion of epizootic virion types.

At present no epizootic virions have been found in enzootic (low-virulence) virus strains, but only enzootic strains from Central America have been examined.[9] Since past epizootics of VEE have all emerged in South America, we consider that enzootic strains from that area, especially Colombia and Venezuela, should be examined carefully for the presence of virions with epizootic characteristics.

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