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Equine Herpesviruses: Antigenic Relationships and Deoxyribonucleic Acid Densities

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Equine herpesviruses with a deoxyribonucleic acid density of 1.716 to 1.717 g/cm^3 were compared with one another by the plaque-reduction test and by the rate of development of cytopathic effect as indicated by plaque size in rabbit kidney cultures. Of the 19 isolates studied, the 9 which had already been tentatively labeled equine abortion viruses were serologically similar to one another; each of them grew more quickly than did any of the other 10 isolates although the mean plaque sizes formed a series of gradations with no clear hiatus which would permit the unequivocal delineation of the abortion viruses from the slowly growing strains. The 10 slowly growing isolates showed antigenic heterogeneity even though complement was present; the neutralizing capacity of an antiserum against the heterologous strains was, in most instances, markedly less than against the homologous strains, the range of the 50% endpoints being much greater than that observed among the equine abortion viruses, or among isolates of herpes simplex type 1. There was no cross neutralization between the equine abortion viruses and any of the 10 slowly growing isolates. An extra band of deoxyribonucleic acid, at 1.723 to 1.725 g/cm^3, was present in two of the slowly growing strains when originally grown in rabbit cells, but was no longer present after passage in cat cells. This band occupied the same position as one reported in the hamster-passaged strain of equine abortion virus, and had a density similar to that of the equine genital herpesvirus. Although the taxonomic demarcation of the equine abortion viruses and the slowly growing herpesviruses from one another is still open to question, they can be conveniently labeled equine herpesviruses 1 and 2, respectively; the genital virus would be termed equine herpesvirus 3.

More than one herpesvirus has been isolated from the horse. Equine abortion virus (6), variously known as equine rhinopneumonitis virus (5) or equine herpesvirus 1 (20), is responsible for respiratory disease and abortion in its host. A number of isolations have been made of another equine herpesvirus which grows much more slowly in tissue culture but which has the same deoxyribonucleic acid (DNA) density (1.716 to 1.717 g/cm^3) as the equine abortion virus; the slowly growing isolate seems to show no cross neutralization with the type 1 virus (19, 20). This virus, which is sometimes termed the equine cytomegalovirus (4, 8, 13) or equine herpesvirus 2 (20), is of unknown clinical significance but is commonly isolated from the respiratory tract, from circulating leukocytes or from kidney cultures of the horse. A third equine herpesvirus, the coital exanthema virus, is transmitted venereally, grows rapidly in tissue culture, and has a DNA density of 1.725 g/cm^3, which indicates that it is unequivocally distinct from the other two equine herpesviruses (16).

Preliminary data have indicated that the slowly growing equine herpesviruses (i.e., the "cytomegaloviruses"; the type 2 virus) do not form an antigenically uniform group (4, 14, 15, 19, 23). In two instances the serological differences were sufficient to cause the authors to label their virus isolates as distinct serotypes (13, 14). The criteria for the delineation of the equine herpesviruses from one another have thus become unclear, and the confusion has become accentuated by the isolation of equine herpesviruses with speeds of growth, as indicated by rate of development of cytopathic

effect (CPE), seemingly intermediate between those of the equine abortion and slowly growing strains hitherto described; the similarity in the densities of the DNAs of these viruses further brings into question the validity of the demarcation between them.

We have, therefore, studied the equine herpesviruses in the following respects: (i) the degree of serologic heterogeneity, as demonstrated by the plaque-reduction test, among 10 isolates of equine herpesviruses which appear to replicate slowly in tissue cultures; complement was added to the neutralization system because of its reported ability to reduce the antigenic heterogeneity among strains of human cytomegalovirus (10); (ii) the degree of heterogeneity among nine isolates of "equine abortion virus"; (iii) the plaque size of these viruses and its use as a criterion for distinguishing the abortion viruses from the slowly growing strains; (iv) the DNA densities of the various equine herpesviruses; this became of particular interest because the DNA of two of the slowly growing isolates contained an additional band of more dense DNA similar to an additional band already recognized (G. A. Gentry, personal communication and data presented at Cold Spring Harbor Workshop on Herpesviruses, 1972) in the DNA of the hamster-passaged strain of equine abortion virus, and similar to the reported (16) density of the DNA of the equine genital herpesvirus. The genital virus was studied only from the point of view of its DNA density; no antigenic or plaque studies were done.

MATERIALS AND METHODS

Viruses. The nine isolates of equine abortion virus were labeled EAa to EAi. EAa was isolated by E. R. Doll from the respiratory tract and had been passed extensively in equine and rabbit cultures. EAb was isolated from an equine fetus by I. A. Schipper of North Dakota State University; it had been passed three times in tissue cultures. EAc was the EH39 strain isolated in Australia from the respiratory tract (22, 23). EAd, a fetal strain isolated in the U.S.A., had been passed more than 600 times in hamsters and was obtained from G. A. Gentry. EAe to EAi were isolated in Maryland from aborted fetuses by S. K. Dutta; each was passed five or six times in horse cells and twice in rabbit kidney cells.

The isolates of slowly growing equine herpesviruses used in this study are labeled 1 through 10 (corresponding to the decreasing order of their average plaque diameter). The origin of each was as follows: isolate number 1 was the LK strain obtained from the equine respiratory tract (19, 20); isolates 2 and 7 came from equine kidney cultures (14 and 13, respectively); isolate number 3 was recovered from horse leukocytes (B. Toma, personal communication); isolates 4, 5, 6, 8, 9, and 10 correspond to strains EH1-57, EH86, EH32B, EH1-141, EH2-141, and EH3-141, all isolated from the respiratory tract (22, 23). These viruses were plaque-picked prior to their use in the neutralization test.

The genital herpesvirus was the equine coital exanthema virus of Pascoe et al. (18).

Plaque test. The equine abortion viruses and the slowly growing viruses were titrated in primary cultures, in Falcon dishes (60 by 15 mm), of rabbit kidney which had been grown on 199 and fetal bovine serum. For the plaque overlay, the medium was rendered semisolid with 3.7% methyl cellulose (1500 centipoise; Fisher Scientific Co., N. J.). Plaques of the slow viruses were counted under the low power of the inverted microscope after 6 or 7 days; those of the equine abortion viruses were usually counted with the naked eye after 4 days, the tissue culture having been stained with methylene blue.

DNA densities. DNA densities were determined by the method described elsewhere (9); the viruses were grown in either rabbit or kitten kidney cultures, except for the genital herpesvirus which, because of its cell specificity, was grown in a continuous line of equine kidney cultures.

Antisera. The antisera used in these studies were prepared in young adult New Zealand white rabbits by a series of intramuscular and intraperitoneal inoculations of virus grown in rabbit kidney cultures; four inoculations were given at weekly intervals, followed by a fifth 10 days after the fourth; the animals were bled 10 days after the last inoculation. Some antisera were prepared by using antigen mixed with complete Freund adjuvant, but this seemed to have little influence on the antibody titers.

Neutralization tests. Doubling dilutions of the rabbit antisera were made in 199 containing 1% lamb serum. Each dilution was then mixed with an equal volume of fresh guinea pig serum, already diluted 1/8, as the source of complement; each of these mixtures was then mixed with an equal volume of challenge virus so that the final reaction mixture would contain between 50 and 100 plaque-forming units (PFU)/0.2 ml. The final dilution of guinea pig serum in each mixture was thus 3%; in a few selected experiments it was employed at a concentration of 4%, but that did not seem to alter the results. The enhancing effect, if any, of the presence of complement, as opposed to no complement, on the cross-neutralizations presented in this investigation was not determined. A control consisting of diluent, guinea pig serum (final concentration 3%), and virus was included. All mixtures were incubated for 1 h at 37 C, with agitation at 15-min intervals. Two-tenths milliliter of each mixture was then plated into primary cultures of rabbit kidney cells in 60-mm petri dishes, absorbed 15 min, and overlayed with medium 199 containing methocel.

RESULTS

Plaque size. The mean plaque diameters attained after 6 days in rabbit kidney cultures by the 10 isolates of slowly growing equine herpesviruses are shown in Table 1 along with 4 isolates of equine abortion virus. Four other strains of equine abortion virus (EAf to EAi)

TABLE 1. *Comparison of 14 strains of equine herpesviruses*

Virus isolate	Country of origin	Site of original isolation	Plaque diameter (in mm) at 6 days postinoculation[a]
1	England	Respiratory tract	1.0
2	France	Kidney	0.9
3	France	Leukocytes	0.9
4	Australia	Respiratory tract	0.9
5	Australia	Respiratory tract	0.8
6	Australia	Respiratory tract	0.8
7	U.S.A.	Kidney	0.8
8	Australia	Respiratory tract	0.6
9	Australia	Respiratory tract	0.5
10	Australia	Respiratory tract	0.5
EAa	U.S.A.	Respiratory tract	2.2
EAb	U.S.A.	Fetus	2.1
EAc	Australia	Respiratory tract	1.8
EAe	U.S.A.	Fetus	1.4

[a] The plaque sizes represent the average of the maximum diameters of at least 30 plaques, well isolated from neighboring plaques, in primary rabbit kidney cultures. No plaque size is shown for the hamster passaged strain (EAd) because it produced no CPE in rabbit kidney cultures.

produced plaques with mean diameters of 2.0 to 2.1 mm at 6 days postinoculation. A plaque size in rabbit cultures cannot be given for the hamster-passaged EAd strain because of its apparent inability to replicate in rabbit cells. Although the plaques of none of the equine abortion viruses were as small as those of any of the "slow" viruses, there is no clear-cut and unequivocal demarcation on this basis. Growth curves, presented elsewhere (19), do indeed indicate that the equine herpesviruses which form small plaques replicate more slowly, with longer eclipse periods, than do those which form large plaques.

During passage in cell cultures there has not been observed selection from the slow equine herpesviruses of variants with plaque sizes equal to those of the equine abortion viruses. Isolate 1, which has the largest plaque size of the slow viruses, has indeed been passaged more than the other isolates, but passing this virus a further 20 times in cell cultures produced no increase in plaque size. Similar passage of isolate 2 yielded no alteration in its plaque size.

Serologic study of the slowly growing viruses. The cross-neutralization, in the presence of complement, among isolates 1 through 10 is summarized in Table 2, where both the 50% and 100% endpoints are shown. A considerable degree of heterogeneity is evident. The range of variation among the 50% endpoints exceeds that among the 9 isolates of equine abortion virus (see below) or among 26 isolates of herpes simplex virus type 1 or 17 isolates of herpes simplex virus type 2 tested against the same type 1 antiserum or the same type 2 antiserum, respectively (Plummer, unpublished data); in these latter instances the range of variation among the 50% endpoints was slightly over fourfold. The antisera against the slowly growing viruses were particularly inefficient in bringing about 100% neutralization of heterologous strains, something which we have not observed with the equine abortion viruses or with the herpes simplex viruses.

The 50% neutralizing titers of the four antisera documented in Table 2 were in every instance less than $^1/_4$ when tested against the different strains of equine abortion virus, including EAe.

Serologic studies of the equine abortion viruses. The nine strains of equine abortion virus appeared to be very similar to one another when tested in the plaque-reduction test against EAa antiserum, although the Australian isolate, EAc, seemed to show a consistent fourfold difference in its endpoint against this antiserum (see Fig. 1). Even strain EAd, passed over 600 times in hamsters, and strain EAe, the slowest of the equine abortion viruses where plaque development was concerned, were similar to the other equine abortion viruses in the plaque-reduction test.

The 50% neutralizing endpoint of the antiserum against equine abortion virus (the antiserum used in Fig. 1) was in every instance $< ^1/_4$ against each of the 10 isolates of the slow equine herpesviruses.

DNA densities. Despite their serologic heterogeneity, all the slowly growing isolates 1 to 10 had a similar DNA density, which was in the range 1.716 to 1.717 g/cm^3 (Fig. 2). We believe the DNA peaks observed at this density to be herpesviral for a number of reasons. The band of DNA was seen when each virus was grown in either rabbit cells or cat cells. And of the many DNA densities we have now determined in the analytical ultracentrifuge by using nucleic acid from rabbit or cat cells infected with herpesviruses, we have never encountered a DNA band with density 1.716 to 1.717 g/cm^3 from a contaminant. Furthermore, Russell and Crawford (21) have also determined the DNA density of isolate number 1; their estimate was 1.715 g/cm^3, a figure very close to ours.

Prior to plaque purification, isolates 3 and 6 contained an additional DNA peak with a density of 1.723 to 1.724 g/cm^3 (see Fig. 3), although this was observed only when the DNA was harvested from rabbit cultures, and not

TABLE 2. *Neutralization of 10 isolates of slowly growing equine herpesviruses by antisera against isolates 1, 2, 7, and 9*

Virus isolate	Antiserum against							
	Isolate 1		Isolate 2		Isolate 7		Isolate 9	
	100%	50%	100%	50%	100%	50%	100%	50%
1	256[a]	2048	4	64	8	128	4	32
2	8	128	128	1024	16	64	NT[b]	NT
3	4	128	4	128	8	128	<4	64
4	8	256	<4	8	4	16	<4	32
5	8	256	<4	128	4	128	<4	32
6	4	128	4	64	4	64	<4	32
7	4	128	<4	32	128	1024	<4	16
8	<4	16	<4	<4	<4	<4	<4	<4
9	<4	8	<4	<4	<4	<4	256	2048
10	<4	4	<4	<4	<4	<4	128	2048

[a] Titers expressed as the reciprocal of the highest dilution of serum causing 100% or 50% neutralization of the virus during 1 h of incubation at 37°. A "<" sign means the titer is less than 1:4, the lowest dilution tested.
[b] NT, not tested.

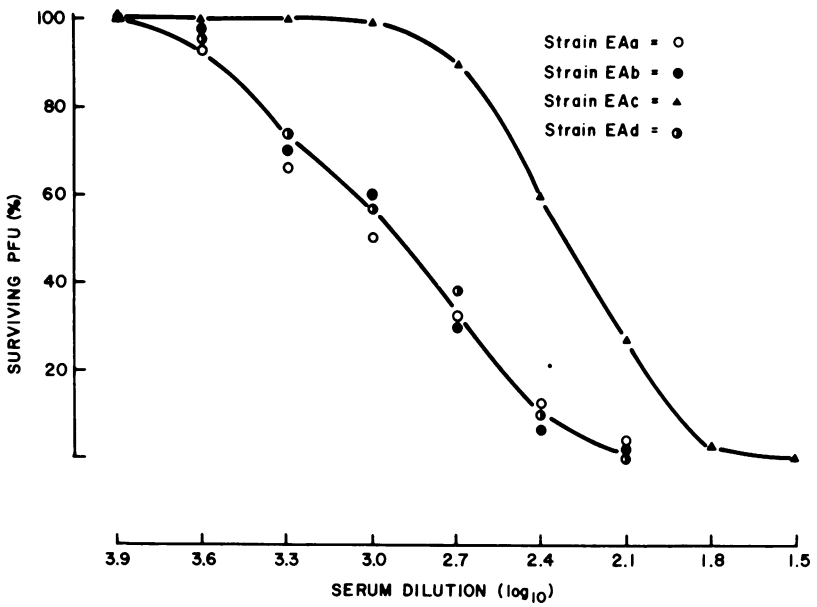

FIG. 1. *Neutralization of four strains of equine abortion virus by antiserum prepared against strain EAa. The results are expressed as the percentage of PFU surviving 1 h/37 C of incubation with doubling dilutions of serum.*

when harvested from cat cultures; when these two viral strains were passed three times in cat kidney cultures and then grown in rabbit cultures, the more dense peak was no longer present. It was also absent from stocks prepared from single plaques in rabbit cells. The DNAs of these two viruses free of the heavy band are shown in Fig. 2.

Each of the strains of equine abortion virus, EAa to EAd, had a DNA density of 1.716 to 1.717 g/cm^3. Gentry (personal communication and data presented at Cold Spring Harbor Workshop on Herpesviruses, 1972) has observed an extra band of DNA at 1.723 to 1.724 g/cm^3 in strain EAd when harvested directly from serum of infected hamsters; we did not observe the

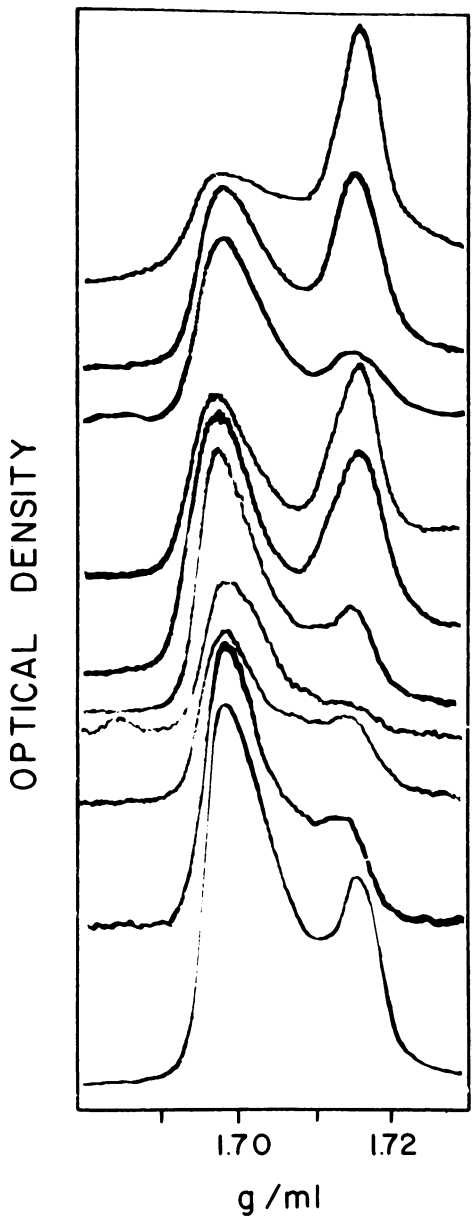

FIG. 2. *Density gradient analyses of DNA extracted from cat or rabbit cell cultures infected with 10 isolates of equine "cytomegalovirus". The upper five tracings are of DNA from infected cat cells, and the bottom five are DNA from infected rabbit cells. From top to bottom, the isolates are: 1, 2, 3, 5, 6, 4, 7, 10, 9, 8. Isolates 3 and 7 appear to have a small peak at 1.685 g/cm³, probably due to mycoplasma DNA. All centrifuge runs were at 44,000 rpm, 25 C, and tracings were taken at 265 nm after equilibrium was reached.*

extra band when the virus strain was passed three times in cat cells since it was obtained from Gentry.

We do not know the nature of this additional peak of DNA seen in isolates 3 and 6, and reported in EAd prior to passage in cat cells, but it has a density similar to that of the equine venereal herpesvirus (Fig. 3). We have not attempted to further characterize the source of the extra DNA band.

DISCUSSION

The antigenic heterogeneity which we have observed among the slowly growing herpesviruses of the horse may be similar to the heterogeneity among the cytomegaloviruses of man (1, 2, 3, 7, 12, 17, 24) and of the vervet monkey (11). But the diversity among these equine viruses does not seem to reflect clear and distinct serotypes despite certain of the differences being as great as those between herpes simplex viruses type 1 and type 2, or between the herpes simplex viruses and B virus. Although the heterogeneity among the various strains of human cytomegalovirus has been recognized for many years, the absence of clearly designated subtypes suggests a similar situation where they are concerned. The various data now available do indeed beg the general question of whether the slowly growing, cytomegalo-type viruses show more antigenic diversity than do the rapidly growing herpesviruses, although the data of Haines et al. (10) indicate that the presence of complement in

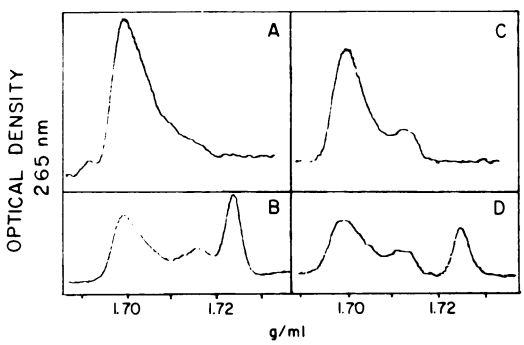

FIG. 3. *Density gradient analyses of DNA extracted from rabbit kidney cultures infected with isolate 6 prior to plaque purification (Fig. 3B); from healthy, uninfected rabbit cultures (Fig. 3A); from horse kidney cultures infected with equine genital herpesvirus (Fig. 3D); and from uninfected horse cultures (Fig. 3C). The band of equine "satellite" DNA can be seen in Fig. 3C and 3D.*

the neutralization test reduces the apparent heterogeneity among the cytomegaloviruses of man. A further point which should, perhaps, be raised when considering cross-neutralization data is the possible variation in the quantities of antigen and nonviable virions in the challenge virus. Even though the number of PFU in different challenge viruses may be the same, the antigenic mass may be much greater in some instances, particularly where "cytomegaloviruses" are concerned; such nonviable antigen could block antibody and thus reduce the neutralizing capacity of an antiserum.

The serologic differences between certain of the slow equine herpesviruses are almost as pronounced as the difference between them and the equine abortion viruses. The clear discrimination from one another of the equine abortion viruses and the slowly growing equine herpesviruses should perhaps be questioned, especially in view of the similarity in the densities of their DNAs. Although it is true that none of the slow equine herpesviruses which we studied grew as rapidly as any of the abortion viruses, and there is an absence of cross-neutralization, hybridization studies between their genomes would be of considerable interest. Even though the line of delineation between these viruses is somewhat unclear, it is probably best for the moment to group all the slowly growing herpesviruses of the horse, antigenically heterogeneous though they may be, together as "equine herpesvirus 2"; the word "cytomegalovirus" should be avoided because of the vague and ambiguous nature of the criteria for the designation of any herpesvirus as a cytomegalovirus. The more rapidly growing abortion-rhinopneumonitis viruses would continue to be known as "equine herpesvirus 1". Subsequent studies may, of course, show that all these viruses should be grouped under one name.

Because of its markedly distinct DNA density there seems little doubt that venereal herpesvirus of the horse is a taxonomically separate agent, and could best be referred to as "equine herpesvirus 3". We hesitate from drawing a parallel with the human herpesviruses and calling it type 2, because it would be unwise to set the precedent of always reserving "type 2" for the genital herpesviruses, and in any case "type 2" has long been in use for the slowly growing herpesviruses of the horse.

The presence in the DNA from two of the slowly growing isolates of a contaminating DNA with a density of 1.723 to 1.724 g/cm^3 was of interest because of the similar band in the equine abortion strain which had been passed repeatedly in hamsters; Gentry (personal communication and data presented at Cold Spring Harbor Workshop on Herpesviruses, 1972), who observed the band in the DNA from infected hamster serum, found it to be still present in filtered, gradient-purified virus, suggesting that if a contaminant were the cause then the contaminant was probably a virus, the DNA of which occupied that position in the gradient. The absence of this extra DNA band, whether in the hamster-passaged abortion virus or in the two slow equine herpesviruses, after passage in cat cells, tends to suggest a viral contaminant. Although the DNA density of the venereal herpesvirus of the horse is similar to that of the contaminating band, and its presence as a contaminant could provide the explanation, this seems unlikely because of the apparent specificity of this virus for equine cells; we were unable to grow the Pascoe strain of coital exanthema virus in rabbit cells, and Bryans (4) has reported his strain to be specific for equine cells. There may, however, be more than one equine virus with a DNA density of 1.723 to 1.725 g/cm^3. The additional band of host cell DNA, "satellite" DNA, which we found in both uninfected and infected horse cells, had previously been reported by Ludwig et al. (16). The centrifuge tracings of virus strain 6 grown in rabbit cells (Fig. 3B) and equine coital exanthema virus in horse cells (Fig. 3D) look very similar to one another. But this is entirely coincidental; the lightest band in each tracing is host cell DNA; the intermediate band is, in one case, equine herpesvirus 2, in the other case, horse satellite DNA; the heaviest band is either "contaminant" DNA (Fig. 3B) or DNA from the equine genital herpesvirus (Fig. 3D).

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