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Comparison of Four Horse Herpesviruses

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Four equine herpesviruses (equine abortion virus, equine herpesvirus types 2 and 3, and equine cytomegalovirus) were compared. The equine abortion virus did not cross-neutralize with any of the other viruses, but the other three did show varying degrees of cross-neutralization among themselves. Equine abortion virus grew more quickly in tissue cultures than did the others, and attained higher titers of infectivity in the culture fluid; it also formed plaques in a wider range of tissue culture species, although the other three were not specific for one tissue culture system only, in that they would multiply in rabbit and cat kidney cultures. The densities of the deoxyribonucleic acids of all four viruses were in the range 1.716 to 1.717 g/ml (a guanine plus cytosine content of 57 to 58%). Taxonomic separation, as a distinct serotype, of equine abortion virus from the other herpesviruses seems to be justified. The other three are closely related to one another. They should perhaps be regarded as separate viruses and termed horse herpesviruses types 2, 3, and 4, although an alternative view would be to regard them as variants of a single virus type. The question of whether types 2, 3, and 4, or any other herpesviruses, should be placed in a phylogenetically distinct subgroup, known as cytomegaloviruses, is a moot point.

Four horse herpesviruses have been described in the literature. The equine abortion or rhinopneumonitis virus, e.g., Doll et al. (1), is an etiological agent of the clinical conditions indicated by its names. The virus was shown by electron microscopy to be a member of the herpes group (4). A serologically distinct herpesvirus was isolated from the respiratory tract of a colt with a runny nose and a cough (4). It was designated horse herpesvirus type 2; equine abortion virus was therefore referred to as type 1. A third horse herpesvirus was isolated from equine kidney tissue cultures and designated type 3 (3). A so-called horse cytomegalovirus was recently detected in equine kidney cultures (2), though the reasons for calling this, or any other virus, cytomegalovirus are a little obscure.

We have attempted to clarify the relationship of the four viruses by comparing their behavior in tissue cultures, their guanine plus cytosine content, and their serological relationship. They will, in this paper, be tentatively referred to as types 1, 2, 3, and 4, which correspond, respectively, to the equine abortion virus, the horse type 2 of Plummer and Waterson (4), the horse type 3 of Karpas (3), and the cytomegalovirus of Hsiung et al. (2).

MATERIALS AND METHODS

Viruses. Horse herpesvirus type 1 was isolated by and obtained from the late E. R. Doll of the University of Kentucky. The horse type 2 virus was the LK isolate described by Plummer and Waterson (4). The type 3 isolate and the cytomegalo-isolate were obtained from A. Karpas and G. D. Hsiung, respectively.

Growth curves. An inoculum of less than 1 plaque-forming unit (PFU) per cell—1,000 PFU per 60 mm of tissue culture dish—was used. Replicate dishes were inoculated to make possible titration of cell-associated virus as well as extracellular virus. Culture fluid was directly titrated for extracellular virus; the titers expressed in the growth curves are per ml of culture fluid. For cell-associated virus, a cell sheet was washed, scraped into 1 ml of sterile maintenance medium, homogenized in a Ten-Broek grinder, and then clarified by low-speed centrifugation. The titers plotted on the growth curves represent the virus titer in the resulting supernatant fluid and therefore reflect the amount of virus in the total cell sheet.

Preparation of antisera. Adult rabbits were inoculated intramuscularly and intraperitoneally with virus suspension. They received similar virus inoculations 3 weeks later and then at weekly intervals for another 5 weeks. The animals were bled 10 days after the final inoculation.

Determination of deoxyribonucleic acid (DNA) density. Virus was grown in primary rabbit kidney

cultures; after the development of advanced cytopathic effect, the DNA was extracted from the cells by the method of Russell and Crawford (5). The mixture of viral and cellular DNA was centrifuged to equilibrium in cesium chloride, and scanner tracings were made. All centrifugations were for 24 to 48 hr at 44,000 rev/min and 25 C, initial density at 1.70 g/ml, in a double-sector, aluminum-filled Epon cell of a Spinco analytical ultracentrifuge (model E) equipped with monochromator, ultraviolet absorption optics, and electronic scanner. Two distinct peaks, one viral and one cellular, were obtained with each viral-cell mixture. The density of the cell peak, determined in a separate run by mixing rabbit DNA with *Clostridium perfringens* DNA (1.691 g/ml), was 1.699 g/ml. The positions of peaks were determined with a DuPont Curve Resolver (model 310). In this way the error of locating even such a broad peak as occurs with mammalian DNA corresponds to density differences of less than ±0.0008 g/ml.

RESULTS

Behavior in tissue cultures. All four viruses grew in primary rabbit kidney cultures. They could, in fact, be reproducibly titrated in this cell system. Plaques were formed under 199/lamb serum medium rendered semisolid with methocel. The type 1 plaques developed quickly, reaching a diameter of about 2 to 3 mm in 3 days. They could easily be counted when the methocel overlay was sucked off and the cell sheet was stained with 1/10 Loeffler's methylene blue. Plaques of types 2, 3, and 4 developed much more slowly. After 3 days, no plaques could be seen under the low power of an inverted microscope; even after 10 or 11 days the plaques were barely half the size of the type 1 plaques at 3 days. They were therefore counted under the low power of a microscope. As a background beneath the petri dish, we used a petri dish lid onto which a grid had been scratched; the cell sheet was stained with methylene blue prior to counting.

The comparative growth curves of the four viruses are shown in Fig. 1. The inoculum in each

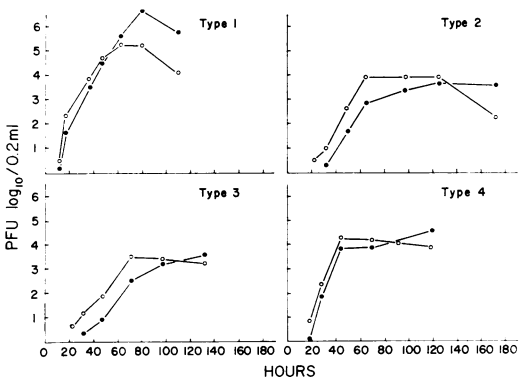

FIG. 1. *Growth curves of the four horse herpesviruses.* ○, *Cell-associated virus;* ●, *virus in the culture fluid.*

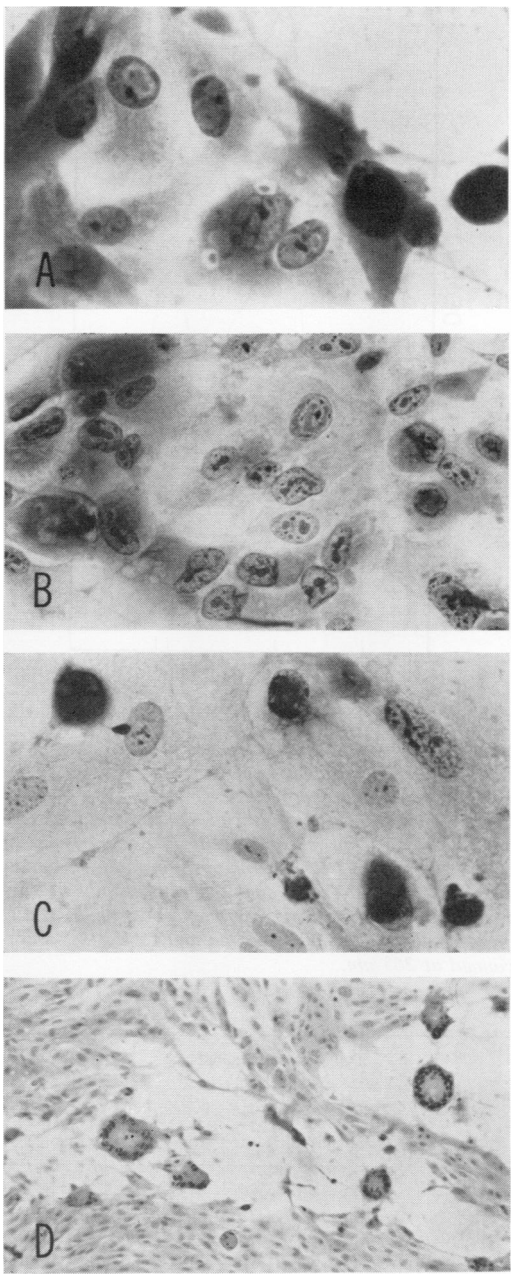

FIG. 2. (*A, B, and C*) *Primary rabbit kidney cultures infected with horse herpesvirus types 1, 3, and 4, respectively.* × *300.* (*D*) *A continuous line of rabbit cells infected with horse herpesvirus type 2.* × *40. H and E stained.*

case was 1,000 PFU per plate. These curves do do not represent single-step growth cycles, and the resulting eclipse periods are not of the minimal duration that could be obtained with higher inocula. Type 1 had an eclipse period of 12 hr, and the other three had eclipse periods of about 20 hr. The type 1 virus attained higher titers than the other three viruses and destroyed the cell sheet more rapidly.

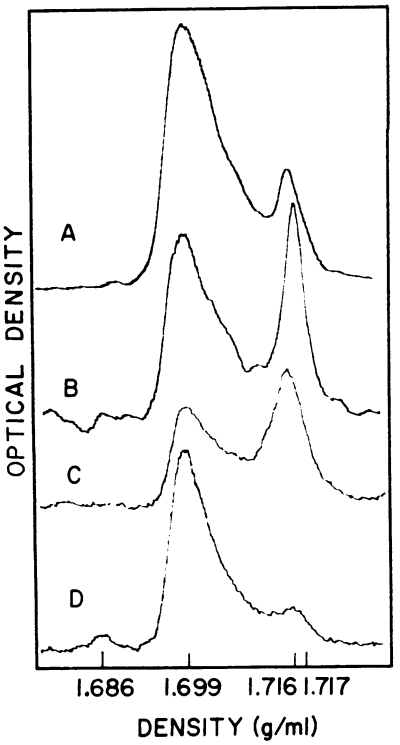

FIG. 3. *Density gradient analyses of DNA extracted from rabbit kidney cells infected with four horse herpesviruses (curves A to D are types 1 to 4, respectively). The peak at 1.699 g/ml is cell DNA; the peak at 1.716 or 1.717 g/ml is viral DNA. The small peak in curve D at 1.686 g/ml is at the position to be expected of mycoplasma DNA and may, therefore, indicate the presence of a contaminant. Samples were run in CsCl for 24 to 48 hr at 44,000 rev/min and 25 C; they were scanned at 265 nm.*

The inclusion bodies formed in rabbit kidney cultures were examined, particularly in view of the suggestion of Hsiung et al. (2) that their isolate should be regarded as a cytomegalovirus. Infected primary rabbit kidney cells are shown in Fig. 2(A to C). The intranuclear inclusions of type 1 were not easy to discern, probably because the virus grows quickly; a few inclusions which were found are shown in Fig. 2A. The intranuclear inclusions of types 2, 3, and 4 were similar to one another; type 3 inclusions are shown in Fig. 2B. A search was made for "cytomegalotype" cell configurations. A very small number were found in cultures infected with types 2, 3, and 4. A field from a type 4-infected culture, showing two such cells, is shown in Fig. 2C; The cytoplasmic inclusions are, however, very faint and not as easy to discern as they are in cells infected with human cytomegalovirus. The cytopathic effect produced by type 2, 3, and 4 frequently included what appeared to be swollen or cytomegalic cells; staining revealed these to be not single, swollen cells but small, rounded syncytia containing a dozen or more nuclei arranged in a circle or an oval. Such syncytia were particularly common when types 2, 3, and 4 were grown in a continuous line of rabbit epithelial cells (*see* Fig. 2D).

In addition to rabbit kidney cultures, all four viruses formed plaques in cat kidney cultures. Only the type 1 virus formed plaques in primary dog or mouse embryo cultures and in the continuous lines MK2, VERO, BSC, and 3T3; no attempts were made, however, to detect viral multiplication without production of cytopathic effect in these cell systems.

TABLE 1. *Cross-neutralization tests*

Serum dilution	Type 1 serum against				Type 2 serum against				Type 3 serum against				Type 4 serum against			
	Type 1 virus	Type 2 virus	Type 3 virus	Type 4 virus	Type 1 virus	Type 2 virus	Type 3 virus	Type 4 virus	Type 1 virus	Type 2 virus	Type 3 virus	Type 4 virus	Type 1 virus	Type 2 virus	Type 3 virus	Type 4 virus
1/4	0	48	80	93	84	0	0	11	58	0	0	3	46	0	0	0
1/8	0	55	75	110	70	0	1	20	58	4	0	11	60	1	0	0
1/16	0	44	94	123	68	0	9	32	59	9	0	12	63	5	1	0
1/32	0	54	76	99	85	0	8	50	73	15	0	24	73	13	7	0
1/64	0					0	10				0	38	64		24	3
1/128	0	47	106	113	92	8	20	71	69	43	3	60	64	35	42	49
1/256	0					31	42				10					110
1/512	14	43	94	104	95	38	54	75	65	54	22	56	71	59	58	131
1/1024	56					49	64				49					127
Control	87	49	94	110	98	51	68	65	81	52	58	58	69	67	76	120

Guanine plus cytosine content of DNA species. The density of DNA extracted from cultures infected with the four viruses is shown in Fig. 3. The positions of the viral DNA peaks were determined by their distance from the rabbit DNA peak, the density of which was 1.699 g/ml, as calculated in previous experiments with bacterial DNA of known density. A peak of DNA in the density range 1.716 to 1.717 g/ml was present in the gradients containing DNA from cells infected with each of the four viruses. These peaks were absent from the DNA extracted from uninfected control cultures. It is likely that these peaks represent viral DNA; those of type 1 and type 2 occupy the same position as in the earlier experiments of Russell and Crawford (5). In the case of the type 4 virus, an additional small peak with a density of 1.686 g/ml was present. This component was present in DNA extracted from cells infected with either plaque-purified virus or virus passed through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.). We do not know whether this is a viral component or a contaminant such as mycoplasma.

Based on the DNA densities, and calculated by the method of Schildkraut et al. (6), the guanine plus cytosine contents of the four viruses would be 57, 58, 57, and 58%, respectively. We cannot be sure that there are, in fact, differences among them; running mixtures of the DNA would not help because the density differences are too small to be resolved.

Serological studies. Antisera were prepared in rabbits by inoculation of virus grown in rabbit kidney tissue cultures; the serum used in the maintenance medium of these cultures was obtained by prior bleeding of the rabbits eventually to be used for the antiserum preparation.

Cross-neutralization tests were done by mixing doubling dilutions of antiserum with virus suspension, incubating for 1 hr at 37 C, and inoculating into petri dish cultures of rabbit kidney. This type of test, which is more suited for demonstrating slight cross neutralization, was used rather than neutralization curves, which are best suited for demonstrating slight differences between viruses.

Each neutralization test was repeated several times, and with more than one antiserum; the results were reproducible. Representative results are summarized in Table 1; the actual plaque counts per plate are given. Each serum completely neutralized the homologous virus to a dilution of between 1/64 and 1/256. The heterologous tests indicate varying degrees of cross neutralization between types 2, 3, and 4, which was evident even when the sera were heated to 56 C for 0.5 hr. The reduction in PFU of type 4 virus by types 2 and 3 antisera is a little difficult to interpret, but in view of the other cross neutralization it may well represent specific neutralization. Type 1 virus does not seem to be involved in cross neutralizations with the other three viruses. None of the antisera caused any neutralization of infectious bovine rhinotracheitis virus or of herpes simplex virus type 2. The preinoculation sera did not neutralize any of the horse viruses.

DISCUSSION

It is difficult to decide, on the basis of the results presented in this paper, where one horse herpesvirus "species" ends and another begins. Even more difficult is the question of whether horse herpesvirus types 2, 3, and 4 should be regarded as cytomegaloviruses. In essence this comes down to the question of whether a distinct cytomegalovirus-subgroup of the herpesviruses exists phylogenetically. The present criteria for regarding a virus as a cytomegalovirus are exceedingly vague. Until criteria which are a little more definitive become available, we suggest that new herpesviruses not be referred to as cytomegaloviruses even if they do grow slowly in tissue cultures. We also suggest that new names not be invented for new herpesviruses, but that they should simply be referred to as type 1, type 2, etc., herpesviruses of a given host, provided, of course, that they show sufficient serological differences.

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