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Type 1 and Type 2 Herpes Simplex Viruses: Serological and Biological Differences[1]

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Forty isolates of herpes simplex virus were compared by means of cross-neutralization curves. The 11 oral isolates were serotype 1, and all 29 genital/anal isolates were serotype 2. The cytopathic effects of the two serotypes were consistently different. Passage of strains of type 1 and type 2 in mice and in rabbits yielded two variants, although the majority of the strains remained unchanged serologically and in their cytopathic effects. The two variants were derived from type 1 strains and differed from the parent strains in their cytopathic effects, each of them producing syncytia and enlarged plaques. They had, however, retained the serotypic properties and the deoxyribonucleic acid (DNA) densities of their parent strains. The Roizman syncytial/macroplaque strain of herpes simplex virus was also included in the study; the density of its DNA (1.727 g/ml) was typical of type 1 strains, and serologically it seemed to be basically a type 1 strain, although it was neutralized by type 2 antiserum slightly better than were other type 1 strains. Growth curves were performed of the two serotypes in rabbit kidney, human fibroblast, and mouse embryo tissue cultures. The type 2 strains attained lower titers of infectivity in these three cell systems; the levels of infectivity of type 2 virus in the culture fluid decreased much more rapidly after the maximum had been attained than did the levels of infectivity of the type 1 strains, due to the greater instability of the type 2 virus. Parallel titrations of different strains in tissue cultures and intracerebrally in mice indicated that the latter assay system was usually more sensitive for type 2 strains than it was for type 1 strains. The paralytic sequelae and inflammatory changes of lumbar ganglia and spinal cord in young rabbits inoculated extraneurally with strains of the two serotypes also indicate that the type 2 virus is more virulent in laboratory animals than is type 1 virus.

Herpes simplex virus isolates do not form a single, serologically uniform group; two distinct serotypes seem to have been established (7, 8, 11). Type 1 strains are mainly from mouth lesions and type 2 strains from genital lesions (1, 10). They also seem to differ in their behavior in tissue cultures, and perhaps also in their virulence for animals and for eggs (2, 3, 6, 9). Centrifugation of the viral deoxyribonucleic acid (DNA) in CsCl has indicated a slight difference in the densities of the DNA species from the two serotypes (4).

A phylogenetic division of herpes simplex viruses, correlated with the site of origin on the body of the host, is a little surprising. We have therefore done a number of further studies on differences between the two serotypes, including: (i) serological examination, by neutralization curves, of 11 oral isolates and 29 genital (or anogenital) isolates; (ii) a study of the effect on type 1 and type 2 strains of passage in tissue cultures and in animals; (iii) an examination of variants thereby obtained, from the point of view of serology, density of the DNA, and cytopathic effect; (iv) studies of the growth and serological properties of the two serotypes in different cell systems; (v) a comparison of the virulence in animals of type 1 and type 2 strains.

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MATERIALS AND METHODS

Virus strains. The L2 strain of type 1 (12) and the MS strain of type 2 (5, 8) were used as reference

strains for the preparation of antisera. Other strains used in this study were 01-011 (oral isolates), G1-G27 (genital isolates), G28 and G29 (anal isolates), and the Rodanus strain of type 1 herpes simplex virus obtained from S. Kibrick.

Preparation of antisera. Antisera were prepared in rabbits. Virus, grown in primary rabbit kidney tissue cultures, was inoculated intramuscularly into a hind leg and intraperitoneally. Similar inoculations were given 3 weeks later, and the animals were bled 10 days later.

Neutralization curves. The antisera were always diluted to give a suitable rate of neutralization; the required dilution was usually 1/20 to 1/40. The neutralization curves were done at 37 C. The sera were prewarmed and then mixed with a prewarmed virus suspension, previously diluted so that there would be 750 plaque-forming units (PFU)/0.2 ml of the final mixture. A similar control mixture was made of virus and pre-immunization rabbit serum diluted either 1/20 or 1/40. The control was titrated immediately and was regarded as the zero-minute sample; test samples were titrated at 5, 20, and 40 min. All titrations were done by the plaque technique by using primary rabbit kidney cultures and an overlay of medium 199/lamb serum hardened by methocel. In neutralization tests involving virus harvested directly from brain tissue, the virus was extracted by grinding about 1 cm^3 of tissue in 3 ml of sterile maintenance medium in a Ten-Broeck grinder. The extract was centrifuged, the supernatant fraction was passed through a Millipore membrane filter (pore size, 3 μm; Millipore Corp., Bedford, Mass.), and the filtrate was diluted 1/10. Samples were stored and titrated; appropriate dilutions were then made to produce a suitable challenge dose for the neutralization test.

Determination of DNA density. Methods for extracting DNA from infected cells and for determining the density of the viral DNA by analytical ultracentrifugation have been described (4).

Growth curves and stability. An inoculum of less than 1 PFU per cell (1,000 PFU per 60-mm 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 milliliter of culture fluid. For the cell-associated virus, a cell sheet was washed, scraped into 1 ml of sterile maintenance medium, homogenized in a Ten-Broeck grinder, and then clarified by low-speed centrifugation. The titers plotted in the growth curves represent the virus titer in the resulting supernatant fraction and therefore reflect the amount of virus in the total cell sheet. Because of the low inocula used in these experiments, no cell-associated virus could be detected during the eclipse.

Comparative stability studies were done with virus from the culture fluid and with cell-associated virus. The culture fluid and the cell extract were passed through Millipore membrane filters (pore size, 3 μm), and then diluted 10-fold in medium 199 containing antibiotics and 0.15% sodium bicarbonate, but no serum. The suspension was held in tightly stoppered containers at 37C and was titrated at intervals.

RESULTS

Serological comparison of oral and genital isolates. Four rabbit antisera prepared against an established type 1 strain (L2) and five rabbit antisera prepared against an established type 2 strain (MS) were used in this study. All of them were found to be satisfactory for distinguishing the viral serotypes. They were appropriately diluted, usually between 1/20 and 1/40, to give convenient speeds of neutralization.

All 11 oral isolates behaved in the neutralization curves as type 1 virus; the 27 genital isolates and the 2 anal isolates behaved as type 2 virus. The curves were similar to those for the L2 and MS strains, respectively (Fig. 1A, B, D, and E). No evidence was found of strains occupying a serologically intermediate position. In a few of the tests, the distinction between the neutralization curves was found to be less than in others, but repeat tests indicated that these were experimental variations rather than strain differences.

Effect of passage in tissue cultures and in animals. Two strains of type 1 virus (02 and 03) and two of type 2 virus (G4 and G5) were each passed 30 times in rabbit kidney cultures. Each strain retained its serotypic properties and also its type-characteristic cytopathic effect (see Fig. 2A and B for the characteristic cytopathic effects of type 1 and type 2, respectively).

Five strains of type 1 (01 to 05) and four strains of type 2 (G2, G4, G5, and G7) were each passed 10 times intracerebrally in mice. After the 10th pass, the viruses were inoculated into rabbit kidney cultures and examined from the point of view of cytopathic effect and of serotype. All the viruses remained unchanged, with the exception of 03. About 60% of the plaques of this strain were enlarged and syncytial; the remaining 40% of plaques were normal type 1 plaques. The syncytial variant was plaque-picked. The appearance of the plaque is shown in Fig. 2C. The plaque size of the variant is shown in Table 1, where it is also compared with the plaque size of the Roizman macroplaque virus (*see below*). Serologically the 03 variant was still type 1 (see Fig. 1G, H, and I). The density of the variant's DNA was the same (1.727 g/ml) as that of other type 1 viruses (Fig. 3).

Also examined were isolates from the eyes and spines of rabbits chronically infected with the 01 and Rodanus strains of type 1 and with the MS strain of type 2. The isolates were: six 01 isolates obtained from the eyes of 6 rabbits at 7, 8, 9, 10,

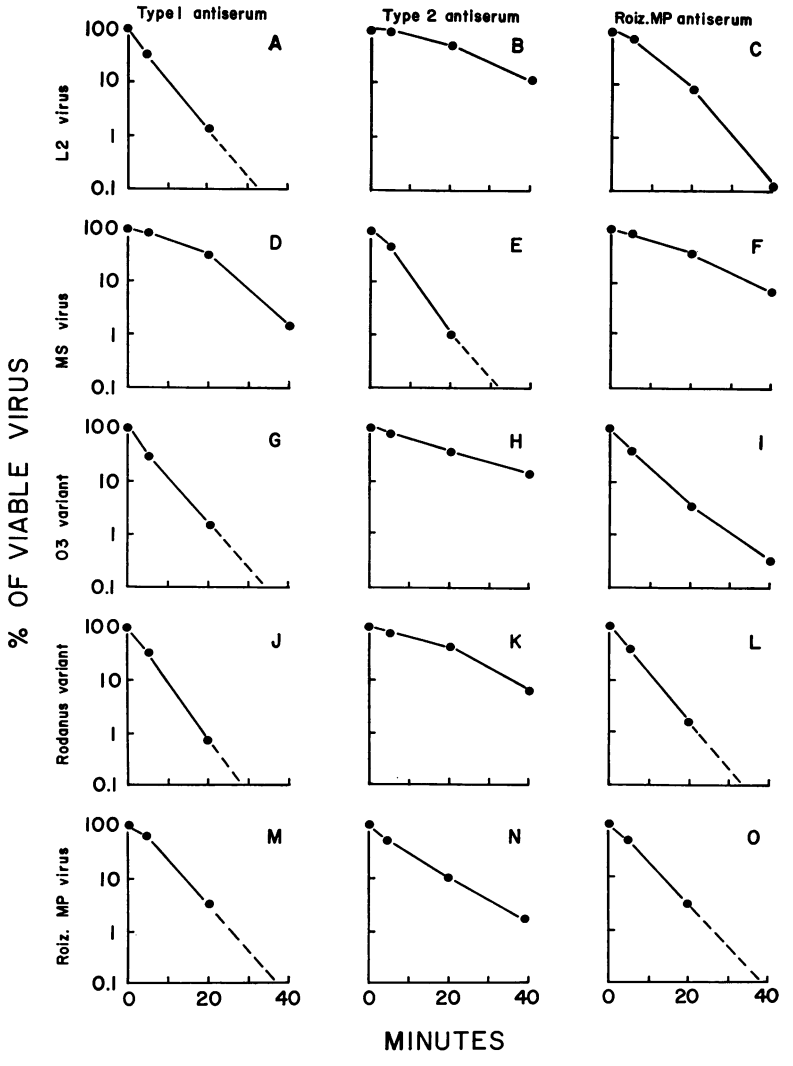

FIG. 1. *Neutralization by specific antisera of the prototype strain of type 1 virus (L2), the prototype strain of type 2 virus (MS), two variants of 03 and of Rodanus, and the Roizman macroplaque strain.*

13, and 16 weeks, respectively, after the original infection; six Rodanus isolates obtained from the eyes of 6 rabbits at 9, 14, 16, 18, 19, and 20 weeks, respectively, after the original infection; seven MS isolates, one obtained from the rabbit eye at 14 weeks after the original infection, and six isolated from rabbit spines at 30, 34, 36, 45, 46, and 47 weeks, respectively, after the original infection of the rabbits. All the isolates had retained their type-characteristics with the exception of the 19-week Rodanus isolate, about 70% of the plaques of which were enlarged and syncytial (the remaining 30% were normal type 1 plaques). The variant was plaque-picked. The appearance of the plaques of the variant is shown in Fig. 2D. The plaque size was similar to that of the 03 variant. The variant had, however, retained the serotypic characteristics and the DNA density of the parent strain (*see* Fig. 1J–L and Fig. 3, respectively).

Roizman's macroplaque strain of herpes simplex was compared with our syncytial strains. It formed somewhat larger plaques and more extensive syncytia than our variants (Fig. 2E and Table 1). Serologically it was essentially a type 1 strain, although it was neutralized a little better by type 2 antiserum than were other type 1 strains (see Fig. 1M, N, and O). The density of its DNA was the same as that of type 1 strains, i.e., 1.727 g/ml (Fig. 3).

Growth in different cell systems. Growth curves

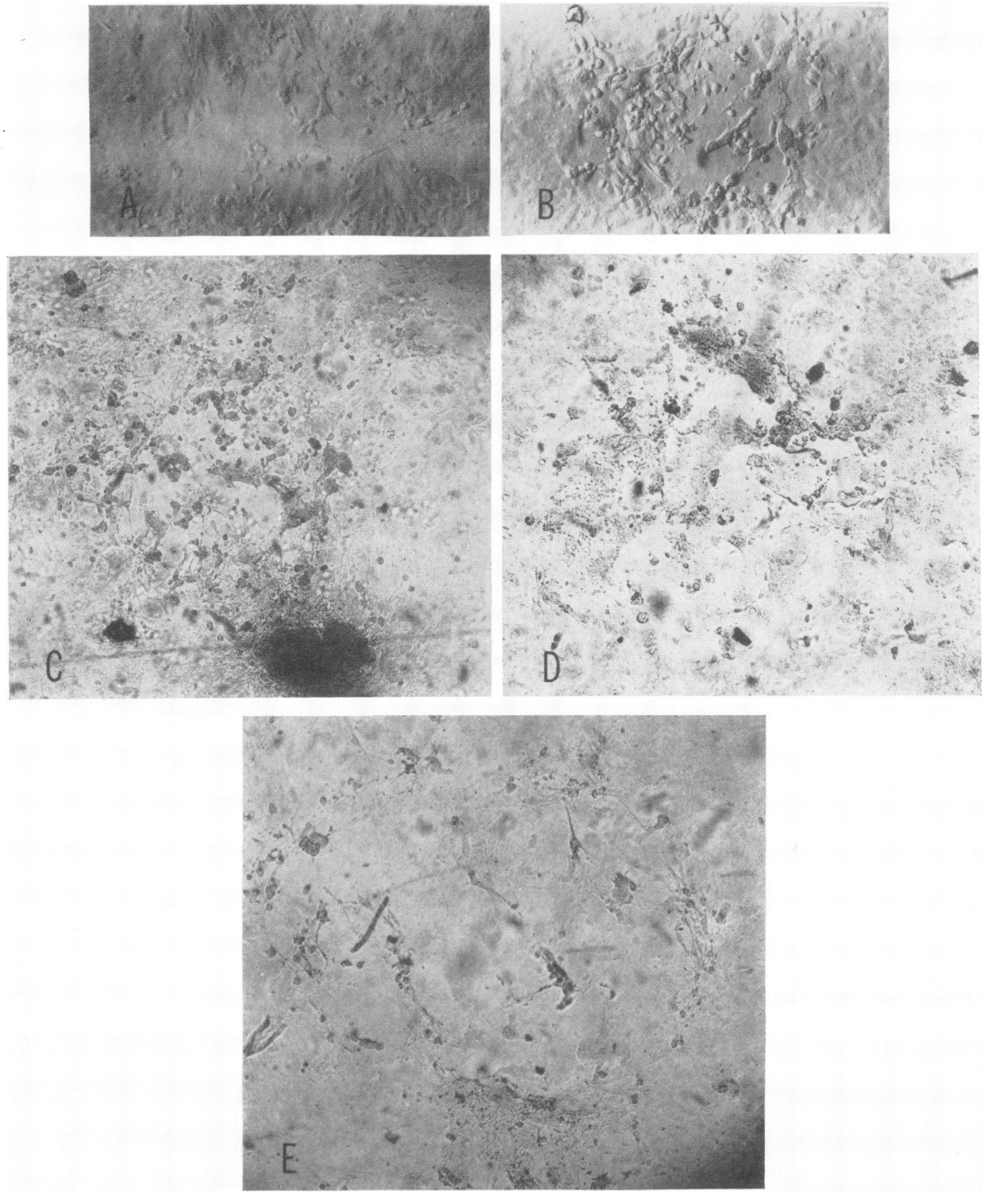

FIG. 2. *A and B are normal plaques of strains L2 and MS, respectively. C, D and E are plaques of the 03, Rodanus and Roizman MP variants respectively. All are about* × *40.*

were measured of two or more strains of both type 1 and type 2 viruses in rabbit kidney, human fibroblast, and mouse embryo cultures. The inoculum was similar in each case and was less than 1 PFU per cell; these curves do not represent one-step growth cycles. From these curves, shown in Fig. 4, it can be seen that in each cell system type 2 virus attains lower titers than does type 1 virus. The titers of extracellular type 2 virus decline much more rapidly after the maximum has been

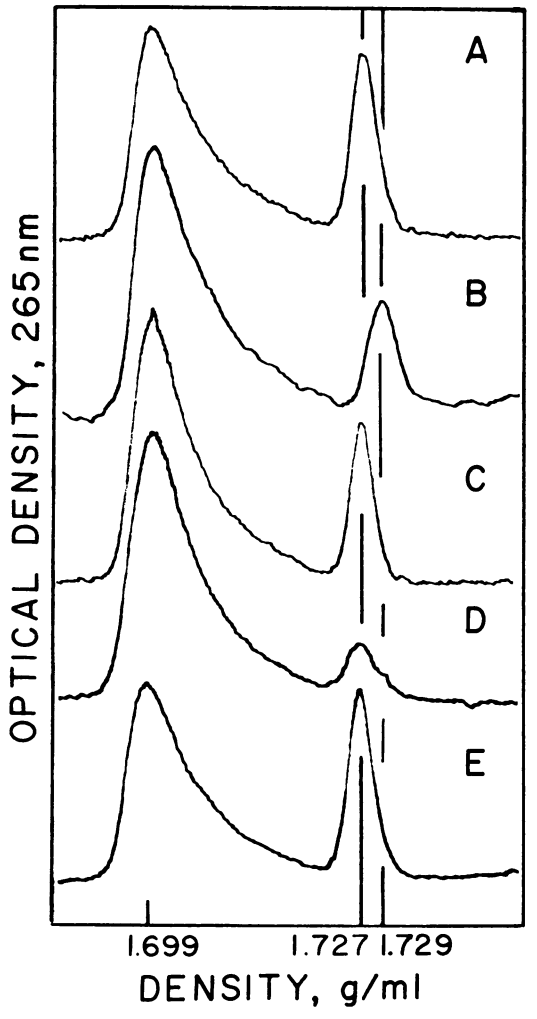

FIG. 3. *Scanner tracings showing the equilibrium distribution in CsCl density gradients of DNA extracted from rabbit kidney cells in primary culture infected with: A, the prototype strain (L2) of type 1 virus; B, the prototype strain (MS) of type 2 virus; C, the Rodanus variant; D, the Roizman macroplaque strain; E, the 03 variant. All runs were at 44,000 rev/min for a minimum of 24 hr at 25 C. Viral DNA densities were relative to cell DNA density (1.699 g/ml), which was calibrated in prior runs with bacterial DNA.*

attained than do the titers of type 1 virus; greater instability of the type 2 virus probably accounts for this. The inactivation rate of the type 1 and type 2 viruses, grown in the three cell systems and suspended in medium 199, is shown in Fig. 5.

Antigenic properties of the viruses when grown in different cell systems. Neutralization curves of the type 1 and type 2 viruses grown in rabbit kidney, mouse fibroblast, and human fibroblast cultures, and in the brains of rabbits and mice, are presented in Fig. 6 and 7. The antisera used in these experiments were prepared in rabbits by inoculation of strains L2 and MS. The curves shown involved strains L2 and MS as the challenge viruses, although similar results have been obtained by using other strains of type 1 and type 2 viruses. As can be seen, when the viruses were harvested from the brain tissue, the two serotypes were less easy to distinguish than when they were grown in cultures even though the virus extract had been filtered and diluted 10-fold. When virus grown in rabbit kidney cultures was mixed with healthy brain tissue and similarly homogenized and diluted, and then used as challenge virus, the two serotypes were readily distinguished. When viruses from the mouse brain, even after 10 passes therein, were subcultured back into rabbit kidney tissue cultures, they could, without any further passage in the tissue culture, be serologically distinguished from one another as readily as they could prior to passage in the mouse brain.

Virulence in mice and in rabbits. In preliminary experiments, type 2 strains seemed more virulent for laboratory animals than did type 1 strains. To explore this possibility further, strains of type 1

TABLE 1. *Comparison of plaque diameters of normal type 1 and type 2 plaques, the 03 variant, and the Roizman macroplaque strain*

Expt	L2 Strain	03 Strain	MS Strain	03 Variant	Roizman large plaque
	mm	*mm*	*mm*	*mm*	*mm*
1	1.2		0.8	1.0	3.0
	0.8		1.2	1.8	2.8
	0.8		0.8	1.6	3.1
	0.8		0.6	1.4	3.8
	1.0		0.7	1.2	3.5
	0.7		1.0	2.0	3.8
	0.8		1.4	1.5	3.2
	0.8		0.8	2.0	3.6
Avg	0.9		0.9	1.6	3.4
2		0.8	0.9	1.4	2.1
		1.2	0.7	1.5	2.4
		1.1	1.1	2.4	1.9
		1.1	1.0	1.6	2.5
		1.0	0.8	2.2	2.4
		1.0	1.2	1.8	2.4
		1.1	1.0	1.8	2.2
		1.0	0.8	1.4	2.4
Avg		1.0	0.9	1.8	2.3

[a] Experiments 1 and 2 were done in two different batches of rabbit kidney cultures; plaques were measured two days after infection.

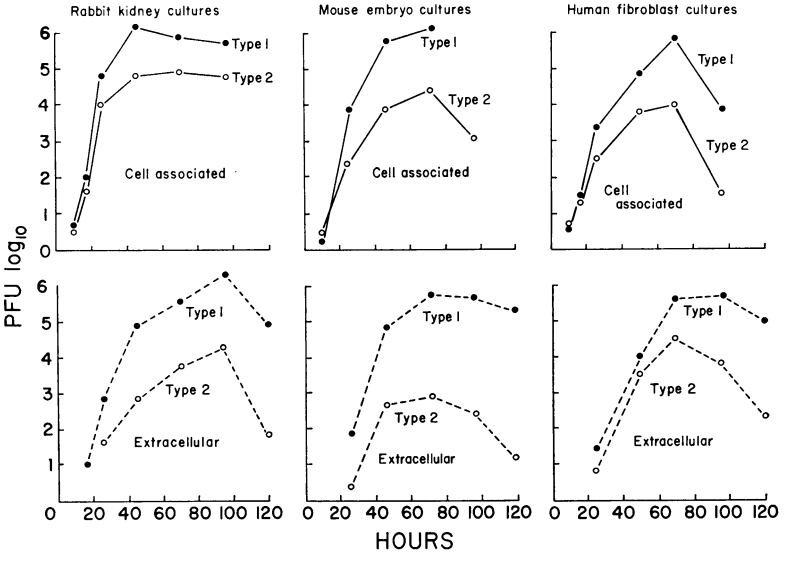

FIG. 4. *Growth curves in primary rabbit kidney, human fibroblast, and primary mouse embryo cultures. These curves were obtained with L2 and MS viruses.*

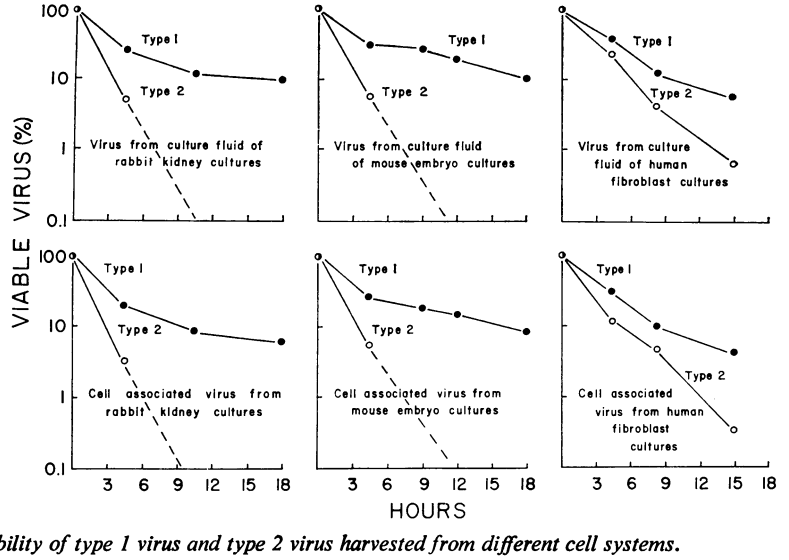

FIG. 5. *Stability of type 1 virus and type 2 virus harvested from different cell systems.*

and type 2 grown in rabbit kidney cultures were titrated in parallel in 3-week-old mice (inoculated intracerebrally) and in rabbit kidney, mouse embryo, and human fibroblast cell cultures. The results presented in Table 2 indicate that the type 2 strains are more virulent than type 1 strains. Virus stocks prepared in human fibroblast cultures showed the same difference in virulence.

To test further for a difference in neurovirulence between the two serotypes, 1 month-old rabbits were inoculated extraneurally (femoral muscle of left hind leg) with virus strains of both types prepared in rabbit kidney cultures. Table 3 presents the results. The viruses used as inocula had been harvested at a similar time in their growth cycles; the type 1 inocula therefore contained 10 to 100 (average 33) times as many PFU as the type 2 inocula. The animals were observed for

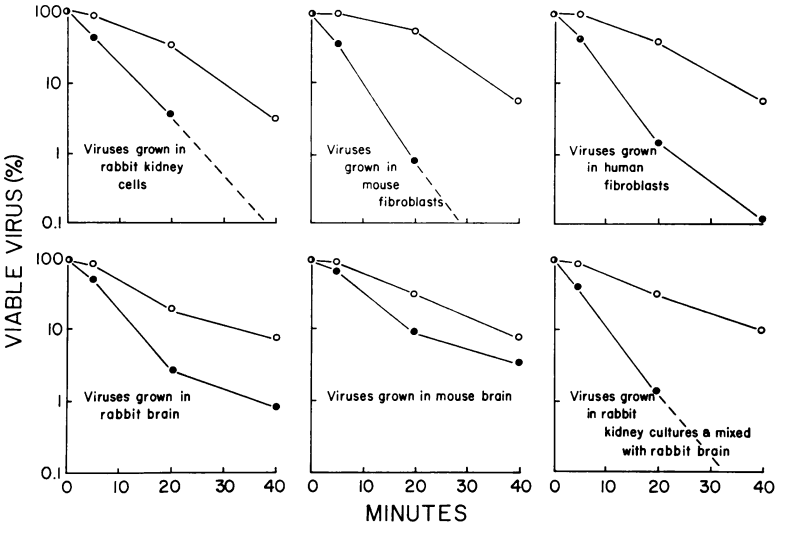

FIG. 6. *Neutralization curves between type 1 antiserum and type 1 and type 2 viruses grown in different cell systems. Type 1 virus, ●; type 2 virus, ○.*

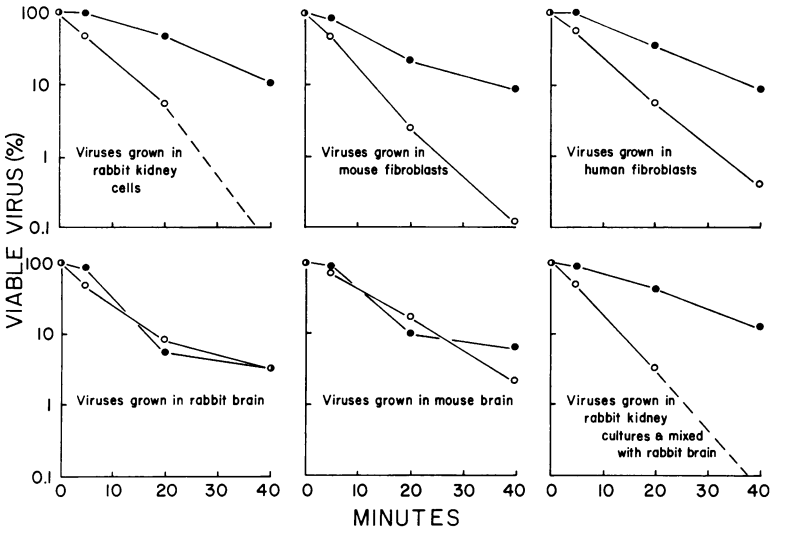

FIG. 7. *Neutralization curves between type 2 antiserum and type 1 and type 2 viruses grown in different cell systems. Type 1 virus, ●; type 2 virus, ○.*

paralysis, and, at the conclusion of the 18-day observation period, or sooner if an animal became severely ill, the spinal cords were removed and examined histologically. Both measures of neurovirulence, i.e., frequency of paralysis and signs of infection observed histologically, show that type 2 is much more neurovirulent than type 1, even though more type 1 PFU had been inoculated.

DISCUSSION

Our results confirm that there are two serotypes of herpes simplex virus, and that strains isolated from the mouth are type 1 and those from the anogenital region are type 2. In our experiments there seemed to be no serologically intermediate strains, although the exact shapes of the neutralization curves for a given virus did show

TABLE 2. *Parallel titrations of type 1 and type 2 isolates in three different tissue culture systems and in mice (inoculated intracerebrally)*[a]

Virus strain	LD_{50} log_{10} in mice	PFU[b] log_{10} in cultures of			Ratio of LD_{50} titers to rabbit cell PFU titer
		Rabbit kidney	Human fibroblasts	Mouse-embryo	
Type 1					
L2	5.8	6.0	5.9	5.8	1:2
01	4.7	5.3	5.1	4.7	1:4
02	4.1	4.1	3.9	3.6	1:1
03	2.8	4.7	4.5	4.4	1:79
04	4.7	5.1	4.7	4.5	1:3
05	4.3	4.7	4.6	4.4	1:3
06	5.0	5.3	5.2	5.1	1:2
Type 2					
MS	4.0	3.3	3.2	2.5	5:1
G1	4.4	4.0	3.9	3.4	3:1
G2	3.4	3.0	2.8	2.5	3:1
G4	4.5	3.2	2.8	2.8	20:1
G5	2.9	3.0	2.5	2.4	1:1
G6	4.0	3.0	2.8	2.4	10:1
G7	4.3	3.8	3.7	3.4	3:1

[a] The four titers in each horizontal column represent the amount of virus per 0.2 ml of a given stock of each strain as measured by the different assay systems.

[b] Plaque-forming units.

TABLE 3. *Neurotropism of strains of type 1 and type 2 viruses*

Virus strain	Pass level	Inoculum[a]	No. paralyzed	No. with inflammation of dorsal ganglia and horn
Type 1				
01	6	4.7	0/4	0/4
03	6	4.8	0/5	0/5
05	6	4.7	0/4	0/4
06	6	4.6	0/5	1/5
07	3	4.9	1/5	1/5
09	3	4.9	0/5	0/5
010	3	5.2	0[b]/5	1/5
L2	?>15	5.2	0[b]/4	2/4
03 Variant		4.8	0/5	0/5
Roizman MP		4.9	0/4	0/4
Type 2				
G1	3	2.9	1/5	2/5
G4	6	3.0	5/5	5/5
G5	6	3.0	1/5	2/5
G7	6	3.6	5/5	5/5
G9	3	3.4	3/4	4/4
G10	3	3.6	4/5	5/5
G11	3	3.8	2/5	2/5
G12	3	3.5	3/4	3/4

[a] Values expressed as plaque-forming units of log_{10}.

[b] Two of the 010 and two of the L2 rabbits showed changes which were not the typical limb paralyses of the type 2 rabbits, but which can be described as ataxia and marked behavioral changes.

some variation in replicate experiments. It is also interesting that the Roizman macroplaque strain was consistently neutralized by type 2 antiserum slightly better than were other type 1 strains. The variants we obtained by passage in animals took the form of syncytial variants, but they did retain the serological characteristics and DNA density of the parent strains. At no time did we find any evidence for conversion of one serotype into the other.

The type 2 strains seem to be more virulent, although the difference between the two serotypes in this respect was not entirely clear-cut inasmuch as some of the strains occupied an intermediate position in both the mouse and rabbit experiments. The greater virulence and greater neurovirulence could result from a number of factors. Perhaps, for instance, the type 2 strains grow faster and to higher titer in brain tissue than do type 1 strains. Or, perhaps type 1 strains cause a greater interferon response. It is also possible that the virions in type 2 preparations retain the ability to cause neural infection after losing the ability to initiate plaques. It is not possible to decide between these and other possibilities from the present data.

We have in this paper referred to the two viruses as "types," although a strong argument can be made for calling them "subtypes," particularly in view of the strong cross-neutralization between them. But if cross-neutralization between two viruses precludes their separation into distinct "species" or "types," then B-virus, which strongly cross-neutralizes with herpes simplex virus, must be regarded as a subtype of herpes simplex virus.

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