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Replication at Body Temperature Selects a Neurovirulent Herpes Simplex Virus Type 2

RICHARD L. THOMPSON AND JACK G. STEVENS*

Department of Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

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A prototype strain of herpes simplex virus type 2 (HG-52) replicated at 31°C was avirulent when inoculated intracranially into mice. This property was not altered after serial passage of the agent at 31°C, but the virus became virulent after passage at 37.5°C. The selection was not merely for an agent which replicated more efficiently at the higher temperature, but for viruses with enhanced capacity to replicate in the brains of mice. Virulent descendants of plaque-purified avirulent stocks were obtained in each instance attempted.

Plaque purifications and basic stock preparations of strain HG-52, a prototype herpes simplex virus type 2 characterized at the Institute of Virology, University of Glasgow, Scotland, were carried out at 31°C (3; M. C. Timbury, personal communication). We obtained a seed pool (termed "elite") from the Glasgow group and initiated studies of neurovirulence after intracranial inoculation of mice. Contrary to our expectation, it was found that the agent was avirulent. During the course of subsequent studies, stocks prepared by serial passage at 37.5°C demonstrated a more than 1,000-fold increase in virulence, and the level attained was that usually demonstrated by herpes simplex viruses. The present communication documents this finding and, most importantly, shows that (i) the increased temperature of incubation does not merely select a virus capable of effectively replicating at higher temperatures, but selects for agents which have enhanced capacity to replicate in the central nervous system and (ii) neurovirulent descendants of all avirulent clones derived from a virus pool can be readily selected.

Virus stocks were prepared and titrated in a rabbit skin cell line or, less often, in BHK cells by methods which we have published earlier (4), and results were the same regardless of the cell type employed. Neurovirulence was assessed by scoring deaths after intracranial inoculation of outbred 4- to 6-week-old Swiss-Webster mice (Hilltop Farms, Chatsworth, Calif.). Four to six mice were employed per virus dilution, inoculated mice were observed for 21 days, and PFU to 50% lethal dose (PFU/LD_{50}) ratios were calculated by application of the Reed-Muench formula (1).

We initially found that two separate pools of virus prepared at 37.5°C (one passage from the elite virus) possessed PFU/LD_{50} ratios of 1.2×10^4 and 6.2×10^3, respectively. As is shown in Table 1, this ratio is dramatically influenced by serial passage at 37.5°C. Thus, although passage of the original stock at 31°C (seven serial passages) yielded high ratios, passage at the higher temperature resulted in at least a 1,000-fold increase in neurovirulence. Table 1 also indicates that the latter ratios are not greatly influenced by passage of the "virulent" agents at 31°C. There is, however, a consistent, approximately 10-fold increase in ratios after this manipulation; we have not studied this aspect further. The minimum number of serial passages necessary to effect the change to neurovirulence has not been established, but as will be seen later, four is sufficient.

An obvious and trivial explanation for these results is that passage of virus in this fashion resulted in selection of mutants with an enhanced capacity to replicate at 38.5°C (mouse temperature) in any murine cell or tissue. To investigate this possibility, one-step growth experiments were performed in mouse embryo fibroblasts with virus stocks prepared at 31°C (avirulent) or 37.5°C (virulent). As shown in Fig. 1, the kinetics of replication and the final virus yields obtained with the two virus pools were the same. The results were quite different, however, when a viral growth experiment was performed in mouse brains. There, as Fig. 2 indicates, the virus grown at 37.5°C replicated more rapidly, and the maximum yield demonstrated by this virus was 100-fold more than that of the virus grown at 31°C. Clearly then, selection was for an agent which replicates more efficiently in brain tissue, and not for a virus which merely replicates more efficiently at 38.5°C.

In the final group of experiments, we deter-

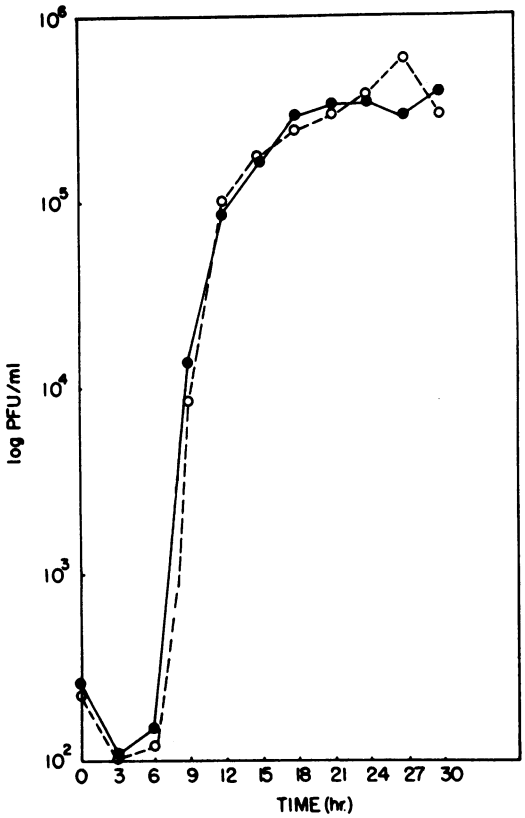

FIG. 1. One-step growth cycles of virulent and avirulent HG-52 strains in mouse embryo fibroblasts at 38.5°C. Avirulent (2.7×10^4 PFU/LD_{50}) and virulent (0.9×10^0 PFU/LD_{50}) viruses derived by passage of the avirulent stock at 37.5°C were employed. The mouse embryo fibroblasts were purified and propagated as described previously (2) in multiwell Linbro plates (Flow Laboratories). The cells were infected at multiplicities of 12 PFU/cell (virulent) and 20 PFU/cell (avirulent). After a 1-h adsorption period, cells were washed three times with 2 ml of medium, and the plates were placed at 38.5°C. At the times indicated, the cells were scraped from wells (three wells per time period), pooled with supernatants, and frozen at −70C. When all samples had been gathered, the samples were frozen and thawed twice and titrated on rabbit skin cell monolayers at 37.5°C. Symbols: ●, virulent virus; ○, avirulent virus.

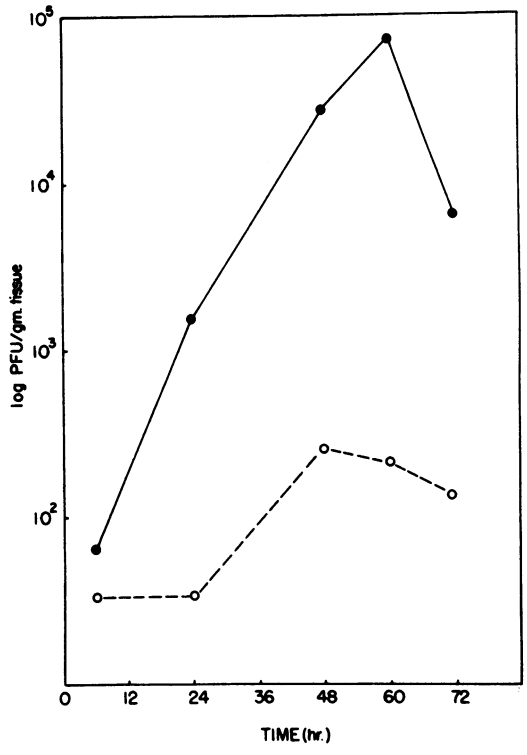

FIG. 2. Growth cycles of virulent and avirulent HG-52 strains in mouse brains. Avirulent (2.7×10^4 PFU/LD_{50}) and virulent (0.9×10^0 PFU/LD_{50}) viruses derived by passage of the avirulent stock at 37.5°C were employed. Four- to six-week-old Swiss-Webster mice were inoculated intracranially with 0.03-ml inocula (2.1×10^3 PFU of avirulent virus or 0.9×10^3 PFU of virulent virus). At the times noted, three mice in each group were killed, and their brains were removed and stored at −70°C. Brains were then thawed, and those from each time point were pooled, ground as 10% suspensions in Ten Broeck homogenizers, clarified by centrifugation at 5,000 × *g* for 5 min, and titrated on rabbit skin cell monolayers at 37.5°C. Symbols: ●, virulent virus; ○, avirulent virus.

mined whether descendants of each avirulent virus in the original stock were capable of mutation and selection for neurovirulence. Five isolates were plaque purified two times at 31°C, and the progeny were serially passaged four to five times at 37.5 or 31°C. As shown in Table 2, all five isolates passaged at 31°C retained the property of non-neurovirulence; those at 37.5°C became virulent.

We do not know the basis for this change in an important biological property which is irrevers-

TABLE 1. PFU/LD_{50} ratios for strain HG-52 after serial passage of virus at 31 or 37.5°C[a]

Passage history of virus	PFU/LD_{50} ratio	
	Expt 1	Expt 2
31°C, 7 passages	2.7×10^3	3.0×10^4
37.5°C, 7 passages	0.3	1.2
37.5°C, 7 passages; 31°C, 7 passages	9.6	18.6

[a] The Glasgow elite stock of HG-52 was serially passaged on rabbit skin cell monolayers as noted, and PFU/LD_{50} ratios were established by plaque titration in cell cultures and intracranial inoculation (0.03 ml; decimal dilutions of virus) of mice. Further details are given in the text.

TABLE 2. PFU/LD_{50} ratios for plaque-purified HG-52 passaged four times at 37.5 or 31°C[a]

Isolate	Passage temp (°C)	PFU/LD_{50}
A	31	$>1.4 \times 10^4$
	37.5	<2.1
B	31	1.7×10^3
	37.5	<7.5
C	31	$>1.1 \times 10^4$
	37.5	<2.7
D[b]	31	$>1.9 \times 10^4$
	37.5	$>1.04 \times 10^4$
E	31	$>1.2 \times 10^4$
	37.5	<9.0

[a] The Glasgow elite strain of HG-52 was plated at 31°C, and five isolates were plaque purified twice at this temperature. These agents were then serially passaged four times at 31 or 37.5°C, and PFU/LD_{50} ratios were established after intracranial inoculation of mice.

[b] On passage 5, the PFU/LD_{50} ratio of the 37.5°C-passaged virus dropped to 100, but that of the 31°C-passaged virus remained at $>10^4$.

ibly evoked by increasing the temperature at which the virus is propagated. However, it is not related to a capacity of the virus to replicate more efficiently at the elevated temperature and can be demonstrated to occur with regularity in individual plaque isolates of the avirulent agents. Finally, although the generality of the overall phenomenon has not been systematically and completely studied, we isolated (passaged once at 31°C) herpes simplex viruses from genital lesions on five different individuals and then determined PFU/LD_{50} ratios. In each instance, the ratio was less than 5. On the other hand, the phenomenon was demonstrated with another laboratory strain (Curtis, obtained from A. Nahmias, Emory University, Atlanta, Ga., in 1973) which had been passaged twice at 37°C in this laboratory before passage five times at either 37.5 or 31°C. From the results obtained here with HG-52 and Curtis strains, it is clear that influences of temperature must be taken into account when biological studies of herpes simplex virus type 2 are undertaken.

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