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Pathogenesis of a Lethal Mixed Infection in Mice with Two Nonneuroinvasive Herpes Simplex Virus Strains

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We previously reported that simultaneous inoculation of mice on abraded rear footpads with two nonneuroinvasive viruses (herpes simplex virus type 1 ANG and KOS) resulted in the deaths of 62% of the animals (R. T. Javier, F. Sedarati, and J. G. Stevens, Science 234:746–748, 1986). In the current study, to better understand the events responsible for the pathogenesis of this virus mixture, we investigated replicative capacity and spread of the virus mixture within specific tissues. We found that, compared with neuroinvasiveness of ANG or KOS alone, neuroinvasiveness of the virus mixture related to significantly increased amounts of the virus within spinal cords and brains of the mice. This finding indicates that ANG and KOS have defects in their capacities to spread and replicate within spinal cords. We also examined whether the increased neuroinvasiveness of the virus mixture related to complementation between viruses in tissues of the nervous system, generation and selection of neuroinvasive recombinants, or both. It was found that, although neuroinvasive recombinant viruses could be detected in the spinal cords of the infected animals, most of the viruses (both recombinants and nonrecombinants) isolated from all tissues tested were nonneuroinvasive (i.e., no mice died as a result of footpad infection with high doses of such plaque-purified isolates). As a result of these findings, we propose that the virulence of the virus mixture is a consequence of the complementation as well as the generation and selection of neuroinvasive recombinants in spinal cords of these mice.

Herpes simplex virus (HSV) is a neurotropic agent that spreads centripetally in the nervous system from the primary site of infection (2, 5, 12). In humans, invasion of the nervous system frequently leads to a latent infection in sensory ganglia, and in rare instances, a fatal encephalitis results (1, 4, 7). In this report, we describe additional studies of nonneuroinvasive HSV type 1 (HSV-1) ANG (8) and KOS (3, 11), which, unlike most HSV isolates, do not reach the brain and do not kill mice after rear footpad inoculation. They are, however, fully neurovirulent as defined by their ability to kill mice after intracranial inoculation at doses similar to those of prototype HSV strains (i.e., $<$100 PFU). Earlier, we reported that rear-footpad infection of mice with a 1:1 mixture of strains ANG and KOS resulted in a lethal infection in 62% of the infected animals (6). In a general sense, this mixed infection is unique, since to our knowledge it is the only system in which a lethal infection results from a simultaneous inoculation with two otherwise completely nonlethal viruses. In the current study, we investigated this mixed infection further to better understand how interactions between such agents lead to lethal infections.

Initially, to define tissues in which agents in the mixed infection gained an advantage over the parental strains, we examined the spread both of the virus mixture and of each parental virus from the rear feet through the nervous system into the brain. Mice were inoculated, as described elsewhere (10), on the abraded rear footpads with 10^7 PFU of ANG or KOS or a 1:1 mixture of the two. At several times postinfection, feet, lumbosacral spinal ganglia, spinal cords, and brains were removed (three mice per time point) and titrated for infectious virus (10). The ANG and KOS virus mixture did not replicate to substantially higher titers than KOS (the most efficient parental virus) in either the feet or the lumbosacral spinal ganglia of infected animals (Fig. 1). Reduced titers of ANG were observed in both types of tissues. Although viruses in all three virus groups reached spinal cords, the mixture replicated to significantly higher titers in this tissue, and agents in this group were the only ones to reach and replicate in brains.

Increased viral titers in the spinal cords could be the result of complementation between nonneuroinvasive agents, recombination resulting in generation and selection of neuroinvasive agents, or both. To investigate these possibilities, we established the genotypes and virulence phenotypes of agents recovered from feet, spinal ganglia, and spinal cords.

Mouse rear footpads were infected with 10^7 PFU of a 1:1 mixture of ANG and KOS as described above. Since under these conditions mice died between 5 and 9 days postinfection, we tested rear feet, lumbosacral spinal ganglia, and spinal cords at 2 and 4 days postinfection for the presence of recombinants. These particular tissues were chosen because they represent tissues of the primary site of infection, the peripheral nervous system, and the central nervous system, respectively. Each sample was then homogenized individually, and a single virus was plaque purified from the homogenate by limiting dilution under an agarose overlay. The neuroinvasiveness of each plaque-purified isolate was then screened by infecting three mice on abraded rear footpads with approximately 10^8 PFU. When a virus isolate killed any mice, it was designated neuroinvasive and a ratio for PFU/50% lethal dose (LD_{50}) was established (9). To identify recombinant viruses, we examined the genomic structures of the isolates with the restriction enzymes *Asp* 718 and *Bam* HI. The recombinant nature of neuroinvasive viruses was confirmed by Southern blot hybridization experiments using the appropriate cloned viral DNA as a probe. It should be noted, however, that the frequency at which recombinant viruses were detected represents a minimum estimate since, despite restriction enzyme profile differences, strains ANG and KOS share extensive DNA homology.

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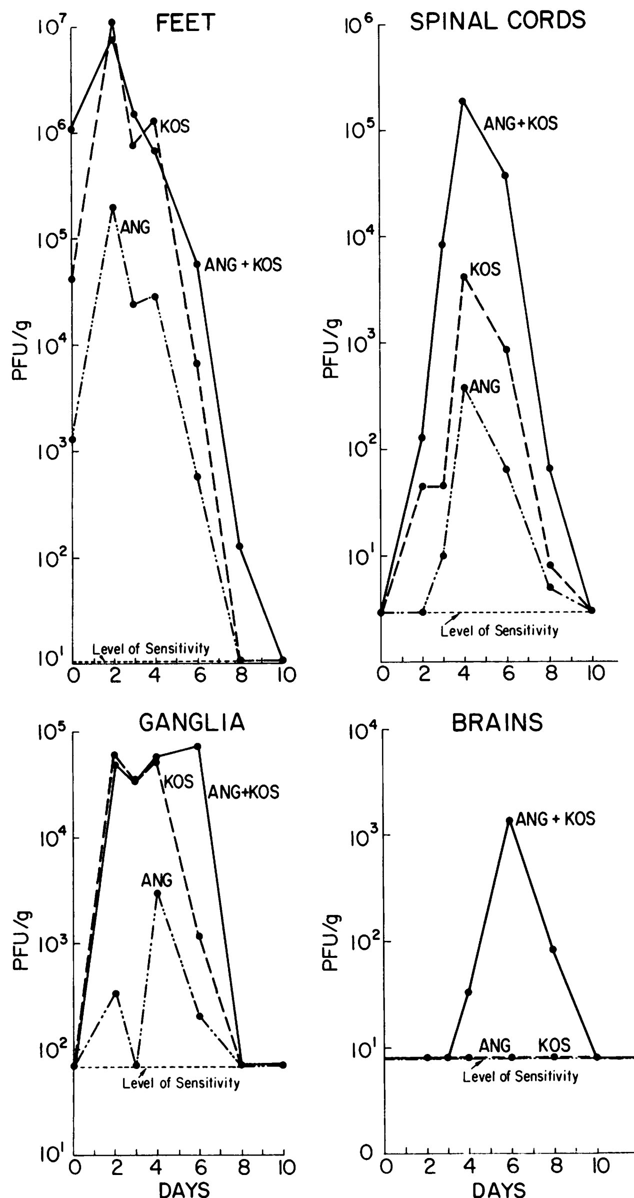

FIG. 1. Appearance of HSV in feet, lumbosacral spinal ganglia, spinal cords, and brains following footpad inoculation of mice with ANG, KOS, or the ANG and KOS virus mixture. Mice were inoculated on both rear footpads with 10^7 PFU of each virus group. At each time point indicated, three animals were sacrificed, and their feet, lumbosacral spinal ganglia, spinal cords, and brains were removed and stored at −70°C for later titrations. Values are presented as PFU per gram of tissues based on titers obtained from three animals. The level of sensitivity in each case was determined as the minimum number of plaques (1 PFU) detected in 0.8 ml (quadruple of 0.2-ml inoculums) of each tissue homogenate at the lowest dilution tested in the assay system used and is expressed as PFU per gram of tissue.

Results (Table 1) show that recombinant viruses were detected in all tissues tested as early as 2 days postinfection; at 4 days postinfection, a high proportion of viruses isolated from lumbosacral ganglia were recombinants. None of these recombinants was neuroinvasive when tested by the footpad reinoculation procedure (i.e., no mice died as a result of footpad infection with such plaque-purified isolates). Neuroinvasive agents were detected only in spinal cords and at a frequency that we previously reported for brains, following peripheral inoculation (6). Thus, at 4 days postinfection, 6 of 11 viruses isolated from spinal cords proved to be recombinants, 3 of which (S41, S42, and S43) were highly neuroinvasive (Table 2). The Southern blots (Fig. 2) show that these viruses are, in fact, recombinants. It should also be emphasized that all viruses (recombinants and nonrecombinants) isolated from the feet and ganglia and, more important, most of the viruses isolated from the spinal cords were not neuroinvasive when tested by footpad reinoculation.

TABLE 1. Recombinant and neuroinvasive HSV in the rear feet, lumbosacral ganglia, and spinal cords of 11 mice coinfected on both rear footpads with 10^7 PFU of strains ANG and KOS

Tissue	No. of mice from which recombinant or neuroinvasive viruses were isolated on[a]/11 mice tested			
	Day 2		Day 4	
	Recombinant	Neuroinvasive	Recombinant	Neuroinvasive
Foot	3	0	2	0
Ganglion	3	0	7	0
Spinal cord	2	0	6	3

[a] Recombinant and neuroinvasive viruses were identified as described in the text. Viruses (one virus per tissue homogenate per animal) were recovered from appropriate tissues of each mouse tested except for one sample of ganglia and eight samples of spinal cords at day 2 from which no virus could be isolated.

Although both parental viruses ANG and KOS are neurovirulent, it is possible that some agents generated as a consequence of recombination between the two were nonneurovirulent and that this factor was complicating the analysis. To exclude this possibility, we tested the neurovirulence phenotype (10) of the nonneuroinvasive recombinants (selected randomly) recovered from spinal cords. All proved to be as neurovirulent as the parents and demonstrated PFU/LD_{50} ratios of between 1 and 67 when tested by intracranial inoculations of mice.

On the basis of these data, we propose that both complementation between nonneuroinvasive agents or between neuroinvasive and nonneuroinvasive agents and generation and selection of neuroinvasive recombinants contributed to the pathogenesis of the virus mixture. Complementation is indicated since a majority of the viruses isolated from all tissues tested proved to be nonneuroinvasive when tested by footpad reinoculation. In this regard, it is important to emphasize that the majority of viruses (11 of 14) isolated from brains of mice inoculated with the virus mixture were also nonneuroinvasive (6) when tested by footpad reinoculation with plaque-purified isolates.

A contribution by neuroinvasive recombinants is also implied, since highly neuroinvasive recombinants were de-

TABLE 2. PFU/LD_{50} ratios of HSV-1 ANG and KOS and of three neuroinvasive recombinant viruses isolated from spinal cords of mice[a]

Virus	Genotype	PFU/LD_{50}
ANG	ANG	$>1.0 \times 10^8$
KOS	KOS	$>1.0 \times 10^8$
S41	Recombinant	4.5×10^5
S42	Recombinant	1.1×10^5
S43	Recombinant	5.6×10^5

[a] Mice were infected on rear footpads (10) with serial 10-fold dilutions of each virus containing 10^8 to 10^4 PFU/0.1 ml (five mice per dilution). PFU/LD_{50} ratios were determined by the method of Reed and Muench (9).

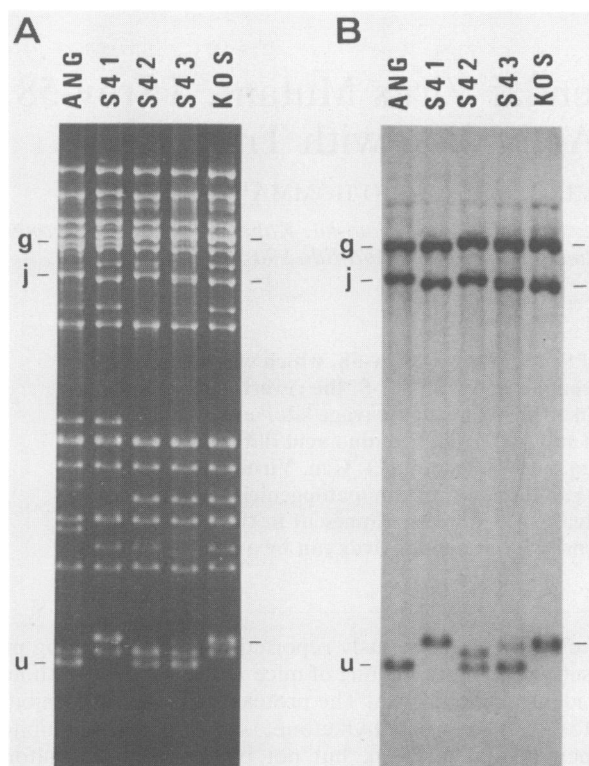

FIG. 2. (A) Agarose gel electropherogram of DNA fragments from HSV-1 ANG and KOS and the virulent viruses S41, S42, and S43 cleaved with *Asp* 718 and separated in a 0.8% agarose gel. Recombinant S41 has *Asp* 718 fragment g (0.05 to 0.11 map units), which comigrates with that of ANG, and fragments j (0.73 to 0.78 map units) and u (0.03 to 0.05 map units), which comigrate with those derived from KOS. Recombinant S42 has *Asp* 718 fragment g, comigrating with that of KOS, and fragments j and u, which comigrate with those of ANG. Recombinant S43 has *Asp* 718 fragments g and j, which comigrate with those derived from KOS, and fragment u, which comigrates with that derived from ANG. (B) Southern blot transfer of duplicate lanes presented in panel A, hybridized to a biotinylated *Hpa*I fragment n (0.036 to 0.063 map units) of HSV-1 17 syn$^+$ as probe. DNA fragments g, j, and u from each recombinant virus comigrating with those of ANG or KOS are identified. The ANG fragments are appropriately marked to the left of each panel, and the corresponding KOS fragments are marked to the right. Although some degree of variability was observed for *Asp* 718 fragment j, fragments g and u were highly stable upon passage of ANG or KOS through the nervous system (data not shown) and were reliably used in determining the recombinant genotype. No consistent association was found between a specific genotype detected here and the neuroinvasive phenotype. Procedures for blot transfer and hybridization, carried out under stringent conditions, were described earlier (6). Nick translation of the DNA probe in the presence of biotinylated dUTP was done according to the instructions of the manufacturer. *Hpa*I fragment n was purified from an *Hpa*I digest of the cloned *Eco*RI fragment j+k by electrophoresis in 0.8% agarose and subsequent electroelution.

tected in the spinal cords. At present, however, we cannot assess the relative importance of these two phenomena. It is also important to note that although neuroinvasive recombinants were generated and selected for in this system, they were not predominant in the spinal cords or in any other tissue tested.

Finally, the results presented here show that HSV-1 ANG and KOS are defective both in replication and in spread within the spinal cord. These defects are responsible for the lack of neuroinvasiveness of the agents, since neither, when inoculated alone in mouse footpads, can reach the brains and kill the animals. The restriction with ANG seems to be more severe and is compounded by possible defects for replication in other tissues, since reduced titers were observed for ANG in all tissues tested compared with titers for KOS or the ANG and KOS virus mixture. The genes involved in these restrictions should be identifiable through the use of marker rescue experiments in which clone d DNA fragments of one agent are employed with total genomic DNA of the other.

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