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Two Avirulent Herpes Simplex Viruses Generate Lethal Recombinants in Vivo

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While it is widely appreciated that infection with a virulent virus can produce disease in an animal, the ability of a mixture of avirulent viruses to produce disease by means of complementation or recombination in vivo has not been established. In this study, two weakly neuroinvasive herpes simplex virus type 1 (HSV-1) strains were simultaneously inoculated onto the footpads of mice. Many (62%) of the animals that received a 1:1 mixture of the viruses died, whereas the animals that received a similar or 100-fold higher dose of each agent alone survived. Of fourteen viruses isolated from the brains of ten mice that died after receiving the mixture of the two weakly neuroinvasive viruses, eleven were recombinants; three of these recombinants were lethal when reapplied to the footpads of mice. These results show that two avirulent HSV-1 variants may interact in vivo to produce virulent recombinants and a lethal infection. They also suggest that different genetic lesions account for the weakly neuroinvasive character of the HSV-1 strains ANG and KOS after footpad inoculation.

MOST STRAINS OF HERPES SIMPLEX virus are neuroinvasive and neurovirulent (*1–3*). When mice are infected on the footpad with such strains, the virus characteristically enters a peripheral nerve and travels centripetally through the nervous system (neuroinvasiveness) where it generally leads to a fatal encephalitis (neurovirulence). However, HSV-1 strains ANG (*4*) and KOS (*5, 6*) are atypical. Although they are fully neurovirulent as measured by intracranial inoculation [median lethal dose (LD_{50}) < 10 plaque-forming units (PFU)], they do not kill mice when inoculated onto mouse footpads, even when susceptibility to the virus is enhanced by treating the inoculation area with hypertonic saline (*7*). These agents may be classified as weakly neuroinvasive, because after rear footpad infection, only small amounts of virus (1 to 10 PFU) may be recovered from the mouse's brain; in contrast, about a million PFU may be recovered from the brain of a mouse infected on the rear footpads with a fully neuroinvasive HSV. The weak neuroinvasiveness of ANG and KOS does not represent a general restriction in mouse cells since both virus strains replicate as well as their virulent counterparts in mouse embryo fibroblasts (at the temperature of a mouse) in vitro and in mouse brains in vivo (*6, 8*).

To determine whether ANG and KOS have different genetic lesions responsible for this behavior in mice and to explore the possibility that two avirulent viruses might interact in vivo to produce disease, these two virus strains were co-inoculated onto murine footpads. If ANG and KOS had different genetic lesions, it was predicted that these viruses might complement each other or recombine in vivo to produce a lethal infection in mice. In two experiments with a total of 26 mice, we found that when ANG and KOS were mixed in a 1:1 ratio and inoculated onto both rear footpads of each mouse (1×10^6 PFU per foot), 62% of the mice died. Yet when the ANG and KOS strains were inoculated alone or on separate feet simultaneously at the same dose, no mice died (Table 1). If mice were given 100 times more than this dose ($\sim 10^8$ PFU) of either ANG or KOS alone, no mice were killed. Therefore, inoculating a mixture of these two avirulent viruses resulted in at least a 100-fold increase in lethality.

We then plaque-purified virus directly from the brains of killed mice to ensure that recombination of the two strains did not occur in vitro. The virulence of these purified viruses was then compared to both ANG and KOS in mice by footpad infection. Four of fourteen viruses isolated from the brains of ten mice produced lethal infections in mice inoculated on the footpad, indicating that recombination may have occurred in vivo in the mice from which the viruses were plaque purified. These viruses were designated AK13, AK23, AK26, and AK27; each was isolated from a different mouse brain (Table 2).

Since ANG and KOS viruses are distinguishable by restriction enzyme analysis of their DNA, the virulent and the avirulent viruses were examined to determine whether they were recombinants. Most of the viruses (11 of 14) isolated from the brains of mice killed by the mixed infection were recombinants. Three of these recombinants, AK13, AK23, and AK27, were virulent. When the DNA's from these three viruses were cleaved with Asp 718 (Kpn I isoschizomer), fragments were detected that comigrated with fragments derived from both ANG and KOS (Fig. 1A). These fragments were assigned to the appropriate parental virus by means of Southern blot analysis (Fig. 1B). Using four different restriction enzymes, we were unable to show that virulent virus AK26, which resembles KOS, was a recombinant since fragments comigrating with ANG-specific fragments were not found (see

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Table 1. Mortality of mice infected with ANG, KOS, or a 1:1 mixture of ANG and KOS on both rear footpads, or ANG on the left footpad and KOS on the right footpad at the same time. The procedures for saline treatment and virus infection were as described previously (*6*). Briefly, approximately 0.1 ml of a 10% (w/v) saline solution was injected subcutaneously into both rear footpads of mice (*16*). Six hours later, mice were anesthetized by an intraperitoneal injection of 0.2 ml of sodium pentobarbital (7.5 mg/ml) in water. The edematous footpad was then lightly abraded with a fine emery board. With the use of an 18-gauge needle, a drop of virus suspension containing 10^6 PFU (approximately 50 μl per foot) was applied and gently rubbed on the abraded area of each footpad with the shaft of the needle. Mice were observed for death resulting from viral encephalitis for 21 days after infection and then killed.

Virus	Total number of mice	Number of deaths (%)
ANG and KOS mixture	26	16* (62)
ANG	30	0 (0)
KOS	30	0 (0)
ANG, left foot and KOS, right foot	15	0 (0)

*This figure represents the sum of two experiments: 9 deaths out of 11 mice tested and 7 deaths out of 15 tested.

Table 2. Values for LD_{50} after footpad infection and genotypes of ANG, KOS, and virulent ANG-KOS recombinant viruses. Mice that had been treated with a hypertonic saline solution were infected with serial tenfold dilutions of the indicated virus containing 10^8 to 10^4 PFU/0.1 ml. For procedures used for saline treatment and virus inoculation see Table 1. Five mice were inoculated per dilution, and LD_{50} values were determined for each virus by the method of Reed and Muench (*17*).

Virus	Source*	Genotype	LD_{50} (PFU)
ANG		ANG	$>1 \times 10^8$
KOS		KOS	$>1 \times 10^8$
AK13	Mouse 1	Recombinant	7×10^6
AK23	Mouse 5	Recombinant	2×10^5
AK26	Mouse 7	KOS†	2×10^7
AK27	Mouse 8	Recombinant	3×10^5

*AK13, AK23, AK26, and AK27 were isolated from the brains of mice killed from the ANG and KOS mixture. Deaths ranged from 5 to 9 days after infection. At the time of death, brains were removed and frozen at −70°C until used for plaque purification of virus. †Restriction enzyme profile differences between AK26 and KOS were detected with Sal I. Since a Sal I restriction enzyme map for KOS is not available, we have not determined the locations of these differences, but the results suggest that AK26 is also a recombinant.

Table 2). However, since there are limited restriction endonuclease polymorphisms between ANG and KOS, some recombinational events may not be detected.

Although these results indicate that at least three of the four virulent viruses were products of ANG and KOS recombination, they do not prove that recombination is responsible for their enhanced neuroinvasiveness. Thus, variants with enhanced neuroinvasiveness may have been selected by passage in the appropriate tissues in vivo (*9*). To exclude this possibility, we attempted to select variants of ANG and KOS with enhanced neuroinvasiveness by inoculating the footpads of 20 mice and recovering viruses from the central nervous system. In these experiments, one virus was plaque-purified from each mouse and then inoculated (>10^8 PFU) onto both rear, saline-treated footpads of mice (five mice per virus). All 20 independent isolates (ten derived from ANG and ten from KOS) demonstrated LD_{50} values greater than 10^8 PFU. Similar results were obtained with 20 isolates derived from the brains of mice killed by intraperitoneal infection. Taken together, these experiments indicate that the lethal neuroinvasiveness of the recombinant viruses resulted directly from recombination in vivo rather than from selection of neuroinvasive variants by passage in the animal.

Our data thus show that two avirulent HSV-1 strains may interact in vivo to generate virulent recombinant viruses and a lethal infection. RNA viruses are known to recombine (reassort) in vivo (*10–12*), but to our knowledge, this is the first evidence that, during a mixed infection, two avirulent viruses can interact in an animal to produce disease. The data also suggest that ANG and KOS have different genetic lesions responsible for their weak neuroinvasiveness in mice; however, further experiments are required to determine whether these lesions are located within the same or different genes (*6, 13–15*).

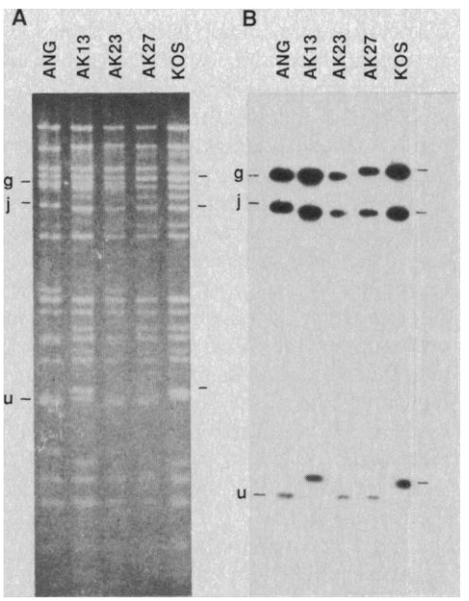

Fig. 1. (A) Agarose gel electropherogram of ANG, KOS, AK13, AK23, and AK27 viral DNA's cleaved with Asp 718 (Boehringer-Mannheim). All three of the virulent ANG-KOS recombinants possess an Asp 718 fragment j (0.73–0.78 map units) that comigrates with that derived from KOS. Also, AK23 and AK27 have an Asp 718 fragment u (0.03–0.05, 0.78–0.80 map units) and AK13 has an Asp 718 fragment g (0.05–0.11 map units), all three of which comigrate with specific ANG-derived fragments. The DNA was prepared by a modification of the procedure of Walboomers and Ter Schegget (*18, 19*). DNA fragments were separated in 0.8% agarose (Sigma) using TPE (0.08M tris-phosphate, 0.008*M* EDTA) electrophoresis buffer (*20*) at 40 V for 40 hours. The gel was stained with ethidium bromide. (B) Southern transfer of Asp 718-generated DNA fragments presented in (A) hybridized to ^{32}P-labeled Hpa I fragment n (0.036–0.063, 0.76–0.79 map units) of HSV-1 strain 17 syn$^+$. DNA fragments from each virulent virus that comigrated with Asp 718–derived fragments j from KOS and u or g from ANG are positively identified. The procedure for blot transfer and hybridization, under stringent conditions, was essentially as described by Southern (*21*) as modified by Wahl *et al.* (*22*) and Maniatis *et al.* (*20*). The procedure for nick translation of the DNA probe was as described by Rigby *et al.* (*23*). The Hpa I fragment n of HSV-1 strain 17 syn$^+$ was derived from Eco RI fragment j+k cloned into pUC12. Hpa I fragment n was purified from a Hpa I digest of the Eco RI j+k clone by electrophoresis in 0.8% agarose and electroelution (*20*).

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ANG and KOS without saline treatment of the rear footpads were as follows: 14 of 27 (52%) mice died at a dose of 10^7 PFU; 11 of 12 viruses isolated from different brains of dead mice were recombinants; and 2 of 12 recombinant viruses were virulent when reapplied to the footpads of mice.
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Intracellular Accumulation of T-Cell Receptor Complex Molecules in a Human T-Cell Line

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This work was aimed at understanding the mechanisms of T-lymphocyte function by studying the cellular distribution and traffic of molecules of the T-cell receptor complex. The accumulation of specific molecules in intracytoplasmic vesicles is related to the activation of T lymphocytes. Some of these molecules include acid hydrolases, the transferrin receptor, and class I antigens of the major histocompatibility complex. Molecules of the T-cell receptor complex have now also been found in intracytoplasmic vesicles in a human T-cell line derived from a lymphoblastic leukemia. Such vesicles were tightly associated with the cytoplasmic microtubule network. One functional aspect of this association is a cellular pathway by which vesicles traveling to and from the cell surface converge in an area of the cells that is rich in processing enzymes.

THE SPECIFIC ACTIVATION AND function of regulatory and effector T cells is mediated through a receptor that recognizes foreign moieties in the context of self antigens of the major histocompatibility complex (MHC) (*1, 2*). This receptor is a multimolecular complex on the surface of human and murine lymphocytes (*3, 4*); it consists of a clonotypic molecule (Ti) that is a glycosylated heterodimer tightly associated with the oligomeric T cell–specific T3 antigens.

Two clonotypic monoclonal antibodies have been prepared by independent laboratories against HPB-ALL, a human T-leukemic cell line (*5, 6*). The use of these antibodies with indirect immunofluorescence microscopy revealed, in addition to the anticipated Ti reaction on the cell surface, the presence of Ti molecules within intracytoplasmic vesicles in HPB-ALL cells (Fig. 1A). Other membrane glycoproteins that were also located intracellularly in the HPB-ALL cells included T9 and class I MHC antigens as well as T3. In contrast, T4, T8, and T11 antigens were all expressed on the cell surface as determined by fluorescence-activated cell sorting (FACS) analysis and visualized by fluorescence microscopy (Fig. 1A); but T4 and T11 were present only at low levels in intracytoplasmic vesicles, and T8 was undetectable.

To confirm and quantitate intracellular Ti and other molecules, we treated viable HPB-ALL cells with protease until these membrane glycoproteins were no longer detectable on the cell surface by indirect immunofluorescence and FACS analysis. The cells were then lysed onto nitrocellulose, and the cell lysates were immunoblotted with the appropriate monoclonal antibodies and then with rabbit antiserum to mouse immunoglobulin (Ig) followed by radiolabeled protein A. Only those antigens that were shown by immunofluorescence microscopy to have an intracellular localization were protected from digestion by extracellular protease (Fig. 1B). Intracellular T3 and Ti were determined by densitometric scanning of the autoradiogram to be 60 to 70% of that expressed by the same cells mock-treated with bovine serum albumin (BSA). In contrast, only 10% of the T8 antigen expressed by these cells was insensitive to extracellular proteolysis.

The collection of Ti-containing vesicles in a central area, their juxtanuclear position, and the occasional appearance of cells in a log-phase culture with scattered vesicles suggested the association of these structures with the cytoskeleton (*7*). Costaining with monoclonal antibodies to tubulin by indirect immunofluorescence and isotype-specific antibodies to mouse IgG conjugated to different fluorochromes confirmed the association of Ti-containing vesicles with the cytoplasmic microtubule network (CMTN): vesicles containing Ti were clustered around the microtubule organizing center (MTOC) in interphase cells, but were scattered in cells undergoing mitosis (Fig. 2A). Treatment of HPB-ALL cells with colchicine led to dispersion of Ti-containing vesicles in the entire culture, further indicating their association with the CMTN.

The expression of acid hydrolases and lysosomelike organelles is a marker of T-cell activation and neoplasia (*8–10*). Cytochemical staining showed that HPB-ALL cells were positive for acid phosphatase, but negative for esterase and β-glucuronidase. Furthermore, acid phosphatase–positive vesicles collected in a central area of these cells, like Ti-containing vesicles, became dispersed upon treatment with colchicine (Fig. 2B), indicating their association with the CMTN. Several recent reports describe the association of Golgi elements with the CMTN in nonlymphoid cells (*11, 12*). Acid phosphatase is known to be localized not only in lysosomes and in the matrix of multivesicular bodies but also in *trans*-Golgi stacks and post-Golgi reticular elements (*11, 13, 14*). The post-Golgi region was found to be an acidic compartment (*15*) where uncoupling and sorting of recycling receptors may occur (*14, 16*), and there was evidence for the intracellular resialylation of transferrin receptors treated with extracellular neuraminidase (*17*).

When we costained for T9 [the transferrin receptor (*18*)] and Ti in HPB-ALL, these two molecules were found in the same vesicles, or at least in vesicles collected in the

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