Pathogenicity and Immunogenicity Herpes Simplex Virus Strains Propagated in Rabbit Kidney Tissue.* (26122)

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Cultivation of herpes simplex virus (HSV) in tissue culture of rabbit kidney (RK) has been reported(1,2,3). In this laboratory application of this technic has made possible a quantitative study of the pathogenicity and immunogenicity of strains of HSV. Results with strains of HSV with different passage histories will be compared.

Materials and methods. HSV strains: The HF strain used was first described by Flexner and Amoss(4) and has undergone numerous passages in rabbits, mice and chick embryos. The strain employed in this study (HF 378) was adapted to grow in the allantoic cavity of embryonated eggs. Infected allantoic fluid possessed 10^7 or more LD_{50} for 7-day-old chick embryos *via* yolk sac inoculation. Strain 1166,† a chick embryo adapted virus was originally isolated by Armstrong in mice from the spinal fluid of a case of aseptic meningitis (5). Strain 1266‡ was isolated in suckling mice from the oral lesion of a patient with aphthous stomatitis(6). Strain L4‡ was isolated from a labial vesicle and passed in chick embryo tissue culture. All strains were readily adapted to grow in RK cultures. For the present study, virus pools of the 8th RK passage of strain HF, 4th RK passage of strain 1166, 18th RK passage of strain 1266 and 5th RK passage of strain L4 were prepared. Virus titers in RK cultures were $10^{9.0}$, $10^{8.5}$, $10^{9.2}$ and $10^{9.0}$ $TCID_{50}$/ml respectively.

Cultivation and titration. Kidneys of young rabbits weighing 2 to 3 lb were trypsinized according to the technic of Youngner (7). The growth medium consisted of 0.5% lactalbumin hydrolyzate in Earle's balanced salt solution with addition of 0.01 M Tris (hydroxymethyl aminomethane), 5 - 10% horse serum and 100 μg of dihydrostreptomycin and 100 units of penicillin G or polymyxin B per ml. After complete sheets of epithelial cells had formed, the virus was propagated in Parker medium 199 without serum and cell degeneration was complete in approximately 40 hours. Fifty percent end point was calculated according to method of Reed and Muench(8). Virus titers were expressed as number (logs) of infectious or lethal doses per ml. By calculating the ratio between tissue culture titer and egg or mouse titer of the same virus fluid, the number of $TCID_{50}$ required to produce 1 LD_{50} was estimated.

Immunization of animals. Virus was inactivated at 37°C for 24 hours by adding formalin to the tissue culture fluid to a final concentration of 1:4000. For immunization of guinea pigs, 3 doses of 1 ml each were given i.p. at weekly intervals. In mouse protection experiments, mice were immunized intraperitoneally at weekly intervals with 0.5 ml of vaccine for 3 injections. Guinea pigs were bled one week post-immunization, the sera inactivated at 56°C for 30 minutes, pooled and stored at –20°C until used.

Results. Pathogenicity of HSV. A. *RK Cells.* Three types of cytopathogenic effect were distinguishable. With strain HF, the chief feature consisted of giant cell formation, whereas strain 1166 produced a clumping or fusing together of cells. The characteristic CPE of strains 1266 and L4 was rounding and "ballooning" of the cells and aggregates generally formed. The CPE characteristic for each strain remained constant through repeated passages in RK culture. B. *Chick embryos.* All 4 strains produced typical pocks on the chorioallantoic membrane. Irrespective of their previous passage history, all were lethal for the 7-day chick embryo upon yolk sac inoculation. However, strains differed in

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TABLE I. Pathogenicity of HSV for Chick Embryos and Mice.

Strain	TC passage	$TCID_{50}$/ml	Chick embryo (7 day) LD_{50}/ml	Chick embryo (7 day) $TCID_{50}/LD_{50}$	Mouse (4 wk) LD_{50}/ml	Mouse (4 wk) $TCID_{50}/LD_{50}$
HF	8	$10^{9.0}$	$10^{7.6}$	25	$10^{6.0}$	1,000
1166	4	$10^{8.5}$	$10^{5.9}$	500	$<10^{1.5}$	>10,000,000
1266	18	$10^{9.2}$	$10^{5.6}$	4,000	$10^{6.5}$	500
L4	5	$10^{9.0}$	$10^{5.1}$	7,900	$10^{6.1}$	790

virulence as indicated by the number of $TCID_{50}$ required to kill the embryo (Table I). C. *Mice.* With the exception of strain 1166, the other 3 strains were highly pathogenic for the mouse *via* the intracerebral route (Table I). Several hundred to a thousand $TCID_{50}$ were required to infect a 4-week-old mouse by intracerebral inoculation. This dosage remained at this level through repeated passages in RK culture (Table II).

TABLE II. Effect of Passages in RK Culture on Pathogenicity of HSV for Mouse Brain.

Strain	Culture passage	LD_{50}/ml	$TCID_{50}$/ml	$TCID_{50}/LD_{50}$
1266	3	$10^{6.2}$	$10^{8.5}$	200
	8	$10^{5.9}$	$10^{8.3}$	250
	12	$10^{5.5}$	$10^{8.0}$	320
	18	$10^{6.5}$	$10^{9.2}$	500
1166	1	$10^{3.0}$	$10^{7.0}$	10,000
	2	$10^{2.0}$	$10^{8.3}$	2,000,000
	4	$<10^{1.5}$	$10^{8.5}$	>10,000,000

On the other hand, strain 1166, which had been shown to possess low pathogenicity for the mouse brain after passages in eggs(9), failed to produce infection following injection of 10^7 $TICD_{50}$ of virus. However, virulence for the mouse was not entirely lost, because the virus was still lethal for suckling mice or adult mice treated with cortisone (5 mg per mouse).

The mouse can also be infected through various routes. The threshold of susceptibility varied widely depending on route of inoculation. Contrary to expectation, intravenous inoculation was the least effective. In any case, CNS involvement appeared to be the principal cause of death (Table III). That susceptibility of the mouse to HSV was inversely related to age(10) was clearly confirmed by present results (Table IV). This was evident regardless of route of inoculation. The newborn mouse appeared to be equally or slightly more sensitive than RK cells for detection of HSV.

TABLE III. Susceptibility of 4-Wk-Old Mice to Strain 1266 via Various Routes of Inoculation.

Route of inoc.	LD_{50}/ml	$TCID_{50}$/ml	$TCID_{50}/LD_{50}$
I.C.	$10^{6.5}$	$10^{9.2}$	500
I.P.	$10^{4.6}$	$10^{9.2}$	40,000
I.M.	$10^{4.2}$	$10^{9.2}$	100,000
I.N.	$10^{3.5}$	$10^{9.2}$	500,000
I.V.	$10^{2.4}$	$10^{9.2}$	6,300,000

Pathogenicity of HSV for other Laboratory Animals. Table V shows differences in susceptibility of other laboratory animals to the 4 strains of HSV inoculated intracerebrally. Undiluted virus fluid was used and the inoculum contained 10^7 or more $TCID_{50}$. Adult hamsters and cotton rats were susceptible to all 4 strains. Although the strains were virulent for suckling rabbits and suckling white rats they failed to induce infection in adult animals. Guinea pigs appeared to be refractory to i.c. infection. However, all strains produced severe corneal and dermal lesions in the rabbit or foot-pad lesions in the guinea pig when inoculated accordingly. Pathogenicity of HSV for the CNS of monkeys would appear to depend on virus strain as well as species of monkey. For example, strain HF was non-pathogenic for both cynomolgus and rhesus monkeys; strain 1266 infected only cynomolgus but not rhesus; while strain 1166 was virulent for both species. The lesion produced was mainly a localized meningitis with variable amount of parenchymal involvement. The ganglion cells were only

TABLE IV. Influence of Age on Susceptibility of Mice to Strain 1266 via I.C. and I.N. Inoculations.

Route	Age of animal	LD_{50}/ml	$TCID_{50}$/ml	$TCID_{50}/LD_{50}$
I.C.	1 day	$10^{9.9}$	$10^{9.2}$	.2
	4 wk	$10^{6.5}$	$10^{9.2}$	500
	8 "	$10^{4.7}$	$10^{9.2}$	32,000
I.N.	3 days	$10^{5.0}$	$10^{9.2}$	16,000
	4 wk	$10^{3.5}$	$10^{9.2}$	500,000

TABLE V. Pathogenicity of Strains of HSV for Various Animal Species via I.C. Inoculation.

Strain	Rabbit		Guinea pig		Albino rat		Hamster	Cotton rat	Monkey
	Adult	Suckling	Adult	Suckling	Adult	Suckling			
HF	0/4*	2/3	0/3	0/3	0/3	1/3	2/3	1/2	0/2
1166	0/4	1/3	0/3	1/3	0/3	2/3	1/3	1/3	4/4
1266	0/4	3/3	0/3	0/3	0/3	2/3	3/3	3/3	1/4†
L4	0/4	3/3	0/3	0/2	0/3	1/3	2/3	2/3	ND

* No. died/No. inoculated. † One cynomolgus monkey was infected but did not die.
ND = Not done.

TABLE VI. Immunogenic Potency of Formalin-Inactivated HSV (Strain 1266) Grown in RK Culture.

A. Antibody production in guinea pigs		B. Immunization of mice against I.C. homologous challenge								
Immunization	Serum neutralization* titer†	Immunization	Virus dilutions 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer	Protection index
None	<10	None		5/5‡	4/5	5/5	3/5	0/5	0/5	$10^{-4.0}$	—
1 dose	80	1 dose	2/5	3/5	0/5	1/5	0/5	0/5		$10^{-0.7}$	3,000§
3 doses	640	2 doses	3/5	0/5	1/5	0/5	0/5	0/5		$10^{-0.3}$	5,000
		3 "	0/4	1/5	0/5	1/5	0/5	0/5		$<10^{0}$	>10,000

* *Vs* 100 $TCID_{50}$ in RK tubes. ‡ Mice died/mice inoculated.
† Titer expressed as reciprocal of serum dilution. § No. of LD_{50} resisted.

slightly affected or not at all and virus could be recovered from the infected nervous tissue.

Immunogenicity of HSV. Study of immunogenic potency was limited to production of neutralizing and protective antibodies in guinea pigs and mice. However, it should be pointed out that antiserum against strain 1166 failed to neutralize the other 3 strains and *vice versa,* whereas no antigenic difference was noted among the other strains. Neutralization tests were carried out in tube cultures of RK cells. Antiserum against B virus§ also did not neutralize strain 1166.

TABLE VII. Comparison of Immunogenicity of Formalin-Inactivated HSV Propagated in Egg, Mouse Brain, and RK Cultures.

Strain	Virus preparation*	Guinea pigs: Serum neutralization titer*	Mice: Protection index†
HF	Allantoic fluid	40	10
	RK culture "	320	>10,000
1266	Mouse brain suspension	640	>32,000
	RK culture fluid	320	25,000

* *Vs* 100 $TCID_{50}$ in RK tubes; titer as reciprocal of serum dilution.
† No. of LD_{50} resisted.

§ The authors are grateful to Dr. K. Hummeler, Childrens Hosp., Philadelphia, Pa. for this serum.

Results of an evaluation of immunogenic potency of formalin-inactivated RK grown virus (strain 1266) are presented in Table VI. Virus titer of the fluid before inactivation was $10^{8.5}$ $TCID_{50}$/ml. Immunogenic potency of preparations of HSV grown in RK culture, embryonated eggs and suckling mouse brain were compared by employing similar evaluation procedures. In this experiment, virus titers of the preparations were adjusted to $10^{8.0}$ $TCID_{50}$/ml before inactivation by formalin. Results indicated that RK culture virus and mouse brain virus were antigenically comparable, while the chick embryo propagated virus appeared to be much less so both in antibody production in guinea pigs as well as in mouse protection tests (Table VII).

Discussion. It is well recognized that strains of HSV vary with respect to pathogenicity for different hosts and tissues. Moreover, HSV manifests varying types of cytopathogenicity in tissue culture systems(11, 12). Difference in cytopathogenicity would appear to suggest heterogeneity of the virus population and by laboratory manipulation strains could be obtained which showed predominance of certain types of response(12). A similar explanation may be applied to dif-

ferences in pathogenicity for various animal species. It is conceivable that passage of the virus in certain host animal or tissue may well serve as a selective process for virus particles with the specific attribute.

It is generally held that very little antigenic variation exists among strains of herpes simplex virus. In this regard, it is of interest that strain 1166 which came to us after 31 egg passages showed little or no antigenic relationship to the other strains. This finding, however, agrees with the results of Florman and Trader(13) who found that the mouse-passage virus of the Armstrong strain was antigenically unrelated to other herpes strains in cross neutralization tests in mice. Since these observations were at variance with those in the original report(5) the matter merits further investigation.

Except for the results reported for strain 1166, the other strains grown in RK culture were highly pathogenic for the mouse and the chick embryo irrespective of their previous contact with these hosts. In contrast to their virulence for the mouse CNS, the same virus failed to induce disease in the rabbit brain, although a much larger dose of infectious virus was inoculated.

Our results indicate that HSV propagated in RK cultures is highly antigenic even after formalin inactivation. The finding that virus grown in eggs is much less potent in protecting mice against intracerebral challenge agrees with the observation of Jawetz and associates(14). As tissue culture grown virus can be prepared relatively free from extraneous proteins, such preparation may be more suitable for immunization studies in man.

Summary. Four strains of HSV have been propagated in high titer in RK monolayer culture. Marked strain differences exist with regard to cytopathogenic effect as well as virulence for chick embryos, mice and other animals. Tissue culture propagated HSV is much more highly antigenic than the same virus strain grown in the chick embryo.

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