VARIANTS OF HERPES SIMPLEX VIRUS: ISOLATION, CHARACTERIZATION, AND FACTORS INFLUENCING PLAQUE FORMATION[1,2]

FRED RAPP

Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

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ABSTRACT

RAPP, FRED (Baylor University College of Medicine, Houston, Tex.). Variants of herpes simplex virus: isolation, characterization, and factors influencing plaque formation. J. Bacteriol. **86**:985-991. 1963.—Variants of herpes simplex virus were isolated which differed in plaque size and in virulence for rabbits and mice. Keratitis on the rabbit cornea and generalized disease were associated with the large, but not the small, plaque variants. The large plaque variants were about as neurovirulent as was the parent strain for weanling mice, but the majority of the small plaque variants were only 1% as virulent for mice as was the parent virus. Agar inhibited the formation of plaques by the parent strain and all variants tested; development of plaques was retarded and the number of plaques was approximately 60% less than those developing under an overlay in which methyl cellulose had been substituted for the agar. Rabbit kidney cells and human embryonic lung fibroblasts were more sensitive than mouse embryo fibroblasts to infection with all variants tested.

Recent studies have revealed that strains of herpes simplex virus (HSV) may differ in a number of properties: in their cytopathic effects (Scott and McLeod, 1959; Hinze and Walker, 1961; Nii, 1961; Hoggan, Roizman, and Roane, 1961), in buoyant density (Roizman and Roane, 1961), in antigenicity (Florman and Trader, 1947; Jawetz, Coleman, and Merrill, 1955; Chu and Warren, 1960; Roizman and Roane, 1963), in virulence (Chu and Warren, 1960; Kohlhage and Siegert, 1962), and in resistance to 5-iodo-2-deoxyuridine (Smith, J. Immunol., *in press*). Many of these studies were conducted with fresh isolates of the virus and attempts to plaque-purify the virus were often not carried out.

During an evaluation of plaque formation by HSV under agar and methyl cellulose, variation in plaque size under methyl cellulose and inhibition of plaque formation by agar were noted. Variation of plaque size was then tested as a possible indicator for differences in virulence. During the course of this investigation, a number of other factors were encountered which affected the formation of plaques by HSV, and studies resulting from these observations are included in this report.

MATERIALS AND METHODS

Tissue cultures. Rabbit kidney cells were obtained by trypsinization of kidneys from 7- to 10-day-old rabbits, by use of a method described by Melnick (1956). The cells were grown in screw-cap bottles or in 60-mm plastic petri dishes and were generally used within 7 days after seeding. Mouse embryo fibroblasts were derived from mouse embryos taken after 14 to 18 days of gestation; they were trypsinized, and were grown in a similar fashion. The medium used to grow the cells consisted of 0.5% lactalbumin hydrolysate in Earle's saline and 10% bovine serum. The human embryonic lung cells were serially passaged a number of times prior to the addition of virus. Growth medium consisted of Eagle's medium with 20% bovine serum. All fluids contained 100 units of penicillin and 100 μg of streptomycin per ml, and the pH was adjusted to 7.1 with 7.5% $NaHCO_3$.

Virus. The strain of virus utilized was the JES strain of HSV originally isolated by G. Ruckle-Enders in primary human amnion cells. It had undergone 40 passages in primary rabbit kidney cells prior to onset of this study. (Virus used was kindly supplied by Albert Kaplan,

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[2] Early phases of this work were carried out at the Philip D. Wilson Research Foundation, The Hospital for Special Surgery, Cornell University College of Medicine, New York, N.Y.

Einstein Medical Center, Philadelphia, Pa.) The virus was passaged two additional times in primary rabbit kidney cells and will be referred to as the parent strain. Variants of this strain were plaque-purified three times under agar before characterization of their properties was attempted. Variants yielding large plaques (LPV) have been designated by Roman numerals; those yielding small plaques (SPV) have been given Arabic numbers.

Stocks of virus were prepared by inoculation of rabbit kidney cells in 16-oz bottles. The bottles were incubated at 34 C and harvested when 50 to 75% of the cells exhibited cytopathic changes (CPE). Virus stocks used in these experiments contained at least 10^7 plaque-forming units (PFU) per ml.

Plaque assays. Cell monolayers grown in petri dishes were washed twice with warm 0.025 M tris(hydroxymethyl)aminomethane (tris) saline (pH 7.4) and 0.5 ml of tris saline was added to the cells after the final wash. The virus, at various dilutions in tris saline, was then added in 0.1-ml amounts into each of two to four cultures and allowed to adsorb for 2 hr at room temperature with intermittent swirling of the dishes. At the conclusion of the adsorption period, 5 ml of an overlay consisting of 10% bovine serum in Eagle's basal medium with either 1% Difco agar or 2% methyl cellulose were added to each petri dish. The overlay contained 100 units of penicillin and 100 μg of streptomycin per ml, and the pH was adjusted to approximately 7.4 with 7.5% $NaHCO_3$. The dishes were incubated at 37 C in an atmosphere of 5% CO_2. At the intervals noted below, 3 ml of a 1:7500 concentration of neutral red were added to the dishes; they were reincubated, and plaques were counted 3 to 4 hr after the addition of the dye.

Animal studies. Rabbit inoculations were carried out by introducing a single dose of each variant tested (800 PFU) onto the scarified cornea of young adult rabbits in 0.1-ml amounts.

The virulence of the variants for mice was investigated by injecting varying doses of virus in tris saline intracerebrally into 3-week-old animals; each mouse received 0.05 ml. Each titration involved 40 animals; 8 animals were inoculated with each dilution of virus. Simultaneous plaque assays were performed. Animals were examined daily for the first week and then at 2-day intervals during the succeeding 2 weeks. The dose required to kill 50% of the animals (LD_{50}) was calculated by the Reed-Muench formula.

RESULTS

Isolation of HSV variants. The parent strain regularly produced plaques under agar in rabbit kidney cells as described by Kaplan (1957). These plaques were from 1 to 2 mm in diameter in 4 to 6 days, although pinpoint plaques could be observed 2 days postinoculation. Plaques formed under methyl cellulose appeared earlier and were larger than those seen under agar; they were variable in size, ranging from 2 to 5 mm in diameter on the third day. Plaques were picked under agar and tested under both agar and methyl cellulose. A number of isolates yielded large plaques which could be counted 2 days after inoculation under agar or methyl cellulose. Others yielded small plaques which generally could not be enumerated until 4 days postinoculation. In both instances, plaques under agar were smaller than those under methyl cellulose. The variants isolated were plaque-purified two additional times under agar and continued to breed true.

Antisera prepared in rabbits against the variants neutralized the homologous variants as well as the parent strain in both tube neutralization and plaque-reduction tests. These sera, as well as pools of human sera known to contain antibodies against HSV, have also been successfully used to detect antigens of the variants in cultured cells by immunofluorescent techniques. These results confirm that the variants under investigation are HSV.

Quantitative comparison of plaque formation under agar and methyl cellulose. A series of experiments were performed simultaneously in rabbit kidney cells under agar and under methyl cellulose. Plaques were counted 4 to 5 days after inoculation of the virus. The results of a representative series of experiments are presented in Tables 1 to 3. In all instances, titers under methyl cellulose were higher than those obtained under agar. A series of replicate experiments with the parent strain of virus (Table 1) indicated an inhibition of plaque formation of about 65% under the agar. Virus derived from the LPV was inhibited by an average of 58% (Table 2). Titers of the SPV were reduced under agar by an average of 60%. Methyl cellulose was therefore

used in the overlay during the remainder of the experiments cited in this report.

Susceptibility of cells to HSV. A series of experiments were performed to determine the sensitivity of various cells to plaque formation by the variants of HSV. Differences in cell susceptibility were noted, but these differences remained constant regardless of the variants tested.

TABLE 1. *Effect of agar and methyl cellulose on plaque formation by the parent strain of HSV*

Parent strain expt no.	Titer (PFU per ml)		Per cent inhibition under agar
	Methyl cellulose	Agar	
1	3.6×10^4	2.7×10^4	25
2	2.6×10^5	7.7×10^4	70
3	1.2×10^7	4.3×10^6	64
4	4.5×10^6	6.0×10^5	87
5	2.0×10^6	4.5×10^5	77
Average			65

TABLE 2. *Effect of methyl cellulose and agar on plaque formation by large plaque variants*

Variant	Titer (PFU per ml)		Per cent inhibition under agar
	Methyl cellulose	Agar	
XIII	1.8×10^7	6.0×10^6	67
	1.9×10^7	9.0×10^6	53
XIV	7.6×10^7	3.5×10^7	54
XVII	1.2×10^8	2.9×10^7	76
XIX	1.2×10^8	7.2×10^7	40
Average			58

TABLE 3. *Effect of agar and methyl cellulose on plaque formation by small plaque variants*

Variant	Titer (PFU per ml)		Per cent inhibition under agar
	Methyl cellulose	Agar	
34	3.0×10^7	2.3×10^7	23
37	2.1×10^7	4.0×10^6	80
38	1.5×10^7	2.8×10^6	79
	1.6×10^7	7.0×10^6	56
Average			60

TABLE 4. *Representative results of the sensitivity of rabbit kidney and mouse embryo cells to HSV*

Virus	Titer (PFU per ml)		Efficiency of plating on mouse cells
	Rabbit kidney	Mouse embryo	
Parent	1.1×10^7	8.0×10^5	0.07
	1.3×10^7	7.0×10^5	0.05
	2.6×10^7	2.0×10^6	0.08
XIII	4.7×10^7	5.5×10^6	0.12
	9.0×10^6	1.9×10^6	0.21
	1.4×10^7	1.7×10^6	0.12
	3.8×10^7	2.2×10^6	0.06
38	2.6×10^6	3.2×10^5	0.12
	7.0×10^6	3.0×10^5	0.04
	2.6×10^7	6.4×10^5	0.02
	1.0×10^7	8.5×10^4	0.01

TABLE 5. *Sensitivity of rabbit kidney and mouse embryo cells to large plaque variant XIII replicating in mouse embryo fibroblasts*

Time post-inoculation	Titer (PFU per ml)		Efficiency of plating on mouse cells
	Rabbit kidney	Mouse embryo	
hr			
2	4.5×10^3	1.8×10^2	0.04
4	2.5×10^3	1.0×10^2	0.04
6	2.0×10^3	2.0×10^2	0.10
10	1.5×10^5	4.2×10^4	0.28
12	6.0×10^5	1.5×10^5	0.25

Table 4 represents a series of assays performed with the parent strain, LPV XIII, and SPV 38. Titers on the rabbit cells were always higher than on mouse embryo fibroblasts; differences were usually approximately 1 to 2 logs. All variants tested yielded similar results.

A number of the variants were then grown in mouse embryo fibroblasts. At various time intervals after inoculation, the cells were harvested and disrupted by freezing and thawing, and the cell-free virus was titered on rabbit kidney and on mouse embryo fibroblasts. The results of tests with LPV XIII are summarized in Table 5. Intracellular virus increased between the sixth and tenth hour after inoculation. Titers on the mouse cells were always lower than those in rabbit kidney cells, even though the virus tested had been grown in cells derived from mouse embryos.

Comparison of rabbit kidney cells with cells

TABLE 6. *Representative results of the sensitivity of rabbit kidney and human lung cells to HSV*

Virus	Titer (PFU per ml)		Efficiency of plating on human lung cells
	Rabbit kidney	Human lung	
Parent	2.6×10^7	1.4×10^7	0.5
XIII	3.8×10^7	1.8×10^7	0.5
	4.7×10^7	3.2×10^7	0.7
35	2.0×10^7	1.3×10^7	0.7
	1.4×10^7	8.5×10^6	0.6
38	2.6×10^7	1.0×10^7	0.4
	1.0×10^7	2.6×10^6	0.3

TABLE 7. *Ability of variants of HSV to induce keratitis in rabbits**

Large plaque variants		Small plaque variants	
Variant	Keratitis	Variant	Keratitis
IX	0	29	±
XIII	+	33	0
XIV	+	34	0
XV	+	35	0
XVII	+	37	0
XVIII	+	38	0
XIX	+		

* A total of 800 PFU were introduced onto each cornea.

derived from human embryonic lung did not yield significant differences (Table 6). One titration with variant 38 did yield a higher titer on rabbit kidney cells, but this may represent variation among the different batches of human cells used.

In vivo studies. The ability of the different variants to induce keratitis, when inoculated onto the scarified cornea of the rabbit, was evaluated. Introduction of 800 PFU resulted in keratitis in all rabbits inoculated with LPV except variant IX (Table 7). Many of these rabbits subsequently died because of herpes encephalitis. The SPV did not cause keratitis under the same conditions (Table 7). In addition, intramuscular inoculation of approximately 10^6 PFU of LPV often resulted in disseminated disease and death of the rabbits. Large numbers of rabbits, inoculated with 10^6 PFU of the SPV for production of specific herpes antiserum, remained well throughout prolonged observation.

Results of inoculation of the isolates onto the chorioallantoic membrane of the embryonated hen's egg were variable. Both groups of variants included members capable of inducing the formation of pocks and others apparently unable to do so.

Intracerebral inoculation of 3-week-old mice revealed significant differences in the virulence of the plaque variants (Table 8). For the parent strain, approximately 50 PFU were required for 1 LD_{50}, when the results of two experiments were combined. The LPV were somewhat less virulent, requiring from 300 to 3400 PFU for 1 LD_{50}. This represents a 6- to 68-fold increase in number of PFU required to kill 50% of the mice. The SPV showed a greater variation. Variants 30 and 33 were similar to the parent strain. The five other variants tested required from 4600 to 18,000 PFU. This represents a 92- to 360-fold increase over the parent strain in PFU required for 1 LD_{50}. It is obvious that these variants are relatively attenuated for weanling mice. This is also reflected in the period required for death of the animals subsequent to inoculation of the viruses. Table 9 shows a comparison of data obtained with variants LPV XIII and SPV 35. Eight animals inoculated with $10^{5.9}$ PFU of variant XIII died within 3 days postinoculation. Introduction of $10^{5.6}$ PFU of variant 35 caused death of three mice in 3 days, four mice in the ensuing

TABLE 8. *Virulence of herpes virus variants in weanling mice*

Virus	No. of PFU in 1 LD_{50}	Fold increase*
Parent	50	—
LPV		
XIII	300	6
XIV	670	13
XVIII	960	19
XIX	2000	40
IX	3400	68
SPV		
33	50	0
30	100	2
38	4600	92
34	5100	102
37	6400	128
35	16,000	320
40	18,000	360

* $\frac{\text{No. of PFU in 1 } LD_{50} \text{ of variant}}{\text{No. of PFU in 1 } LD_{50} \text{ of parent}}$

TABLE 9. *Titration and latent period of variants XIII and 35 in weanling mice**

Virus	PFU inoculated (log)	Deaths per day postinoculation				Deaths/total inoculated
		3	5	6	9	
XIII	5.9	8				8/8
	4.9	7				7/8
	3.9	5	3			8/8
	2.9		4	3		7/8
	1.9			1		1/8
35	5.6	3	4	1		8/8
	4.6	1	1	1		3/8
	3.6		2		1	3/8
	2.6				1	1/8
	1.6				1	1/8

* For XIII, LD_{50} = 300; for 35, LD_{50} = 16,000.

2 days, and one mouse died 6 days postinoculation. The deaths of animals receiving less virus followed a similar pattern. In all the above assays, plaque titrations were performed at the same time the virus was inoculated into the mice.

Discussion

A number of studies have been carried out with HSV in various cell systems. Plaque assays under agar have been developed for enumeration of this virus in rabbit kidney cells (Kaplan, 1957) and in human amnion cells (Osterhout and Tamm, 1959). In both instances, 3 to 4 days were required before the plaques could be visualized without additional magnification of the cultures. The results reported here indicate that plaque formation by HSV is facilitated when methyl cellulose is used as a substitute for agar. The use of methyl cellulose was originally suggested by Hotchin (1955) for fowl plague virus and adapted by Rapp and his colleagues (1959) for measles virus. Under methyl cellulose, plaques formed by HSV can be counted sooner, plaques become larger, and more plaques appear than under agar. The inhibition by agar of HSV may be similar to that reported for encephalomyocarditis virus by Takemoto and Liebhaber (1961) and for dengue virus by Schulze and Schlesinger (1963*a*), apparently owing to the sulfated polysaccharides in the agar (Liebhaber and Takemoto, 1961; Schulze and Schlesinger, 1963*b*). Even differences in the type of agar may be an important factor. Thus, several echoviruses which did not produce plaques under Noble agar readily did so under Difco agar (Wallis, Melnick, and Bianchi, 1962). It is of interest that another herpes virus, zoster, is not inhibited by Difco agar in the formation of plaques in human embryonic lung cells when compared with plaque formation under methyl cellulose. However, the zoster system involves plaques initiated by infected cells, and cell-free virus presumably plays no role in the development of the plaques (Rapp and Benyesh-Melnick, 1963).

Chu and Warren (1960) reported differences in virulence among four strains of HSV propagated in rabbit kidney cells. They made no attempt to plaque-purify these strains, but prolonged passage in culture under various conditions may have selected genetic variants present in the original populations. The present studies, performed on plaque-purified strains, support their observations and further suggest that a given population of wild HSV consists of a number of variants having different properties. It is likely that a number of our variants are identical to one another, since their properties coincide closely; preliminary attempts to demonstrate antigenic differences have failed. Nevertheless, they may differ in other properties not yet investigated. It appears that, although difference in plaque size is a useful marker for isolation of herpes variants, it is not a certain indication of neurovirulence. A number of the SPV were as virulent as the parent strain and even more virulent than some of the LPV. In general, however, the most attenuated variants were derived from small plaques. Preliminary experiments in suckling mice have confirmed the results obtained in the weanling mice. Experiments are also underway with selected variants to determine virulence and attenuation for other animals. It is hoped that the variants can be utilized in studies on latency and for studies concerned with viral genetics.

During the course of the present studies, Kohlhage and Siegert (1962) reported the isolation of two variants from the JES strain of HSV in HeLa cells. They purified the variants by terminal dilution and noted a difference in neurovirulence. They also failed to demonstrate antigenic differences. The method of plaque isolation used in our study with the same strain (JES) of HSV allows a cleaner selection of variants, and a virus population can be analyzed to a higher degree than when terminal dilutions are used to separate variants present in a parent population.

It is apparent that rabbit kidney cells and human embryonic lung cells are more sensitive to HSV than are mouse embryo fibroblasts. This difference is not related to differences in plaque size or in virulence and is possibly due to resistance of large numbers of mouse cells in the cultures. The mechanisms mediating this resistance were not investigated, although growth-curve analyses that have been performed with a few of the variants suggest that the time required for production of virus antigen and for virus maturation is similar in the mouse and rabbit cells. However, rabbit kidney cells are more susceptible to virus grown in mouse cells than are mouse embryo fibroblasts.

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ADDENDUM IN PROOF

Since submission of this report, Tytell and Neuman (1963) have also reported the inhibition by agar of herpes simplex plaques and have suggested use of methyl cellulose as a substitute for agar in the overlay.

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