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Efficiency of Plating on Chick Embryo Cells and Kinetic Neutralization of *Herpesvirus hominis* Strains

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Since it appeared that plaque formation in monolayers of primary chick embryo cells might provide a simple technique for the typing of *Herpesvirus hominis* strains, 100 isolates were tested for their efficiency of plating (EOP) on chick embryo cells versus plating on human embryonic fibroblasts. EOP values varied from 10^0 to 10^{-6}: 88% of the strains of genital origin had an EOP equal to or greater than 10^{-2}, and 82% of the oral isolates had an EOP equal to or less than 10^{-3}. Kinetic neutralizations were done with 53 strains, including those 12 with an EOP of 10^{-2} or 10^{-3}. An estimate of antigenic relatedness (R_a) between strains was calculated from the neutralization results. Although the site of recovery, EOP, and R_a generally correlated, the EOP of some oral strains did not agree with the neutralization results, and some genital strains showed type 1 EOP and R_a values. Selection of variants with increased EOP values did not result in accompanying changes in R_a. Thus, the two markers appeared to vary independently. These data support other findings which suggest that there may be no absolute correlation between biological and antigenic markers in herpesviruses and that a larger number with more diversity of strains should be examined for more markers before a typing system is established.

With the accumulation of evidence that *Herpesvirus hominis* strains from genital sources may differ from oral strains in antigenic and biological properties (2, 5, 9-11), there has been a search for markers which would help to divide isolates into two types. It appeared that plaque production in monolayers of primary chick embryo cells might provide a simple technique for such typing since it has been reported that genital strains plaque in these cells when oral strains do not (4, 8). To compare this technique with the results of an antigenic analysis, we tested 100 isolates for their efficiency of plating (EOP) on chick embryo cells versus that on human embryonic fibroblasts and classified 52 of these strains by kinetic neutralization.

There was not complete agreement between the EOP and neutralization, nor between either marker and the source of the strain. It appeared that the two markers vary independently. Selection of variants with increased EOP values did not result in an accompanying change in neutralization.

MATERIALS AND METHODS

Viruses. The 100 strains of *Herpesvirus hominis* were isolated between 1967 and 1971; 61 came from diagnostic specimens, 9 of which were isolated in Taiwan and the rest in Seattle, and 39 strains were isolated during a long-term family study, the Seattle Virus Watch (M. K. Cooney, K. S. W. Kim, and J. P. Fox, Bacteriol. Proc., p. 199, 1971). The 9 Taiwan strains were kindly supplied by James L. Gale (NAMRU-2, Taipei, Taiwan) and 24 Seattle strains by C. George Ray (Children's Orthopedic Hospital, Seattle, Wash.). Strains were isolated in either diploid or heteroploid human cell lines. They were propagated in human fibroblast cells and were in passages 4 to 9 when tested.

The age and sex distribution of the patients from whom these strains were isolated are shown in Table 1. All but three of the isolates presumed to have come from genital sources were obtained from lesions of cases diagnosed as herpes progenitalis. An isolate from the lesion of a neonatal infection and two from lesions of the lower back have also been classified as from genital sources (2). The isolates considered to be from oral sources were obtained from cultures of throats, brain tissue, mouth, or facial lesions. Among

TABLE 1. *Age and sex of patients from whom strains of Herpesvirus hominis were isolated*

Source of culture	Sex	No. isolated from patients of age (year)						Total
		<15	15-19	20-29	30-39	>40	Unknown (adult)	
Oral	Male	11	3	6	2	5	2	29
	Female	8	1	15	5	2	0	41
	Total	19	4	21	7	7	2	60
Genital	Male	0	1	4	2	1	0	8
	Female	1	6	16	3	0	6	32
	Total	1	7	20	5	1	6	40

these patients, 2 had encephalitis, 5 had pneumonia, 2 had leukemia, 1 had otitis media, 6 had a diagnosis of pharyngitis, and 11 had lower respiratory disease.

Cells and media. Primary chick embryo (CE) cells were obtained from 9- to 11-day-old embryonated eggs. Monolayers were prepared by seeding 60-mm plastic dishes with 2.5×10^6 cells per plate and incubating the plates for 3 to 4 days in 2.5% CO_2 in air at 37 C. The FT line of human embryonic fibroblasts was derived in our laboratory from fetal tonsillar tissue (17). Both FT and CE cells were grown in Eagle's minimum essential medium (MEM) with 10% fetal calf serum, 6.6 mM sodium bicarbonate, 100 μg of streptomycin per ml, 100 units of penicillin per ml, and 10 units of mycostatin per ml. Viral inocula were diluted in MEM with 2% "GG-free" calf serum and antibiotics (MEM2).

Plaque assays. The use of a human fibroblast cell line for plaque assay of herpesvirus strains has been described (17). Inoculation, incubation, and staining were the same for either FT or CE cells. In brief, plates were inoculated with 0.1 ml of 10-fold dilutions of virus, which were allowed to adsorb for 30 min at room temperature. The plates were overlaid with 5 ml of overlay medium consisting of MEM2 with 8.0 mM sodium bicarbonate and 0.3% agarose. After 4 days of incubation in 2.5% CO_2, the monolayers were fixed with Formalin and stained with crystal violet. Plaques were counted with the aid of an automatic colony counter ("Markounter", Aztec Instruments, Inc., Westport, Conn.).

Immune sera. Experimental keratitis was produced in rabbits by inoculation of the scarified cornea with either prototype 1 or 2 viruses. The strains used to produce these infections had been typed with sera kindly supplied by A. J. Nahmias (Emory University) and W. E. Rawls (Baylor University). Viral inocula were propagated in the SIRC line of rabbit cornea in MEM without serum. The sera used in this study came from animals who exhibited signs of encephalitis and were bled 12 to 17 days postinfection. Sera from hyperimmune rabbits boostered with intramuscular injections after recovery from keratitis either had lower K values or did not show type-specific results. Animals given type 2 virus responded with antibody to both types and occasionally developed higher K values for type 1 than for type 2 virus.

Guinea pigs were given three intramuscular injections of virus at 9- to 10-day intervals and were bled 9 days after the last injection. The first injection contained 10^3 to 10^4 plaque-forming units (PFU) in Freund's incomplete adjuvant. The second injection contained 10^5 to 10^6 PFU and the third, 10^6 to 10^7 PFU; neither was mixed with adjuvant. Viral inocula were grown in FT cells by using MEM without serum. The supernatant fluids from cultures showing an extensive cytopathic effect were centrifuged and filtered to remove cells.

Neutralization. The sera were tested for K values at 37 C for 10 min, according to the method previously described (17). The type 1 rabbit antiserum had a homologous K of 10.2 with a standard deviation (SD) of 1.70 and a heterologous K of 3.0 (SD = 1.75) for type 2 strains in the neutralizations required for testing all the viruses. The type 2 rabbit antiserum had a homologous K of 4.7 (SD = 1.62) and a heterologous K of less than one with known type 1 strains ($K = 0.7$; SD = 0.4).

The type 1 guinea pig antiserum had a homologous K of 39.4 (SD = 7.65) and a heterologous K of 8.9 (SD = 2.77). The type 2 guinea pig antiserum had a homologous K of 10.2 (SD = 3.86), with the type 2 strain, and a heterologous K of 2.4 (SD = 1.29).

The test viruses were compared with the homologous strains in kinetic neutralization tests with these sera. An estimate of antigenic relatedness was calculated from the equation: $R_a = (K_2/K_{c2})/(K_1/K_{c1})$.

The K value for the type 2 serum with a test virus was divided by the K obtained with the homologous type 2 strain, and the same relationship was determined for the type 1 serum with test and homologous strains. The type 2 ratio was divided by the type 1 ratio to yield an R_a value. If the unknown virus is more closely related to the type 2 than to the type 1 strain, the R_a will be greater than one; and if the unknown is more closely related to the type 1 virus, the R_a will be less than one. An R_a approaching one indicates a strain which appears to be equally related to both types.

RESULTS

Efficiency of plating varied from 10^0 to 10^{-6} and is compared with the sources of the strains in Table 2. Among the oral isolates, 73% pro-

duced plaques on CE cells only at very low efficiencies, having EOP values equal to or less than 10^{-4}. About 78% of the genital strains plaqued with nearly equal efficiency on both kinds of cells, with EOP values of 10^{0} to 10^{-1}. Seven oral strains behaved like the majority of the genital strains, and five genital isolates behaved like oral ones. In addition, nine oral and four genital isolates fell into an intermediate range with EOP values of 10^{-2} or 10^{-3}; it was not clear which type, if either, these might represent. These 13 intermediate strains and 40 others which would be representative of other plating efficiencies (including all those whose EOP values were not consistent with source) were tested for neutralization indexes.

Although the rabbit and the guinea pig sera gave essentially the same results with these strains, the guinea pig sera proved more useful for typing since they yielded fewer intermediate results where the R_a values approached one. The $\log_{10}$ of the EOP and $\log_{10}$ of the R_a values are shown in Fig. 1. Both rabbit and guinea pig sera divided the strains into two main clusters where EOP and R_a correlated well, but about one-third of the strains did not appear to belong to these clusters. The correlation coefficient for $\log_{10}$ EOP and $\log_{10} R_a$ of the guinea pig sera is -0.652 and for the rabbit sera is -0.580, both of which are significant at $P < 0.01$. Table 3 shows that the 18 strains with R_a values greater than one all had EOP values of 10^{-2} or greater, but 10 of the strains with R_a values less than one also had EOP values in this range. If strains with an EOP equal to or greater than 10^{-2} are considered type 2 and those with smaller EOP values as type 1, the overall agreement between EOP and R_a is 81%.

TABLE 2. *Efficiency of plating (EOP) on chick cells of strains from oral and genital sources*

EOP[a]	Isolated from oral sources		Isolated from genital sources	
	No.	Per cent	No.	Per cent
0	1	1.7	5	12.5
−1	6	10.0	26	65.0
−2	4	6.7	4	10.0
−3	5	8.3	0	0
≦−4	44	73.3	5	12.5
Total	60	100	40	100

[a] Log_{10} virus titer on chick cells per virus titer on fibroblasts.

To determine whether there would be concurrent changes in EOP and R_a, 12 strains representing a range of EOP values from 10^{-2} to less than 10^{-4} were plaque-purified twice through chick cells for selection of variants with increased EOP values. High-titer stocks were then made in human fibroblasts to eliminate any possible host-induced modification. The same strains were also plaque-purified twice in fibroblasts, and all of the high-titer stocks were examined for changes in EOP and neutralization. Selection from chick cells increased EOP for these cells by 1 to 2

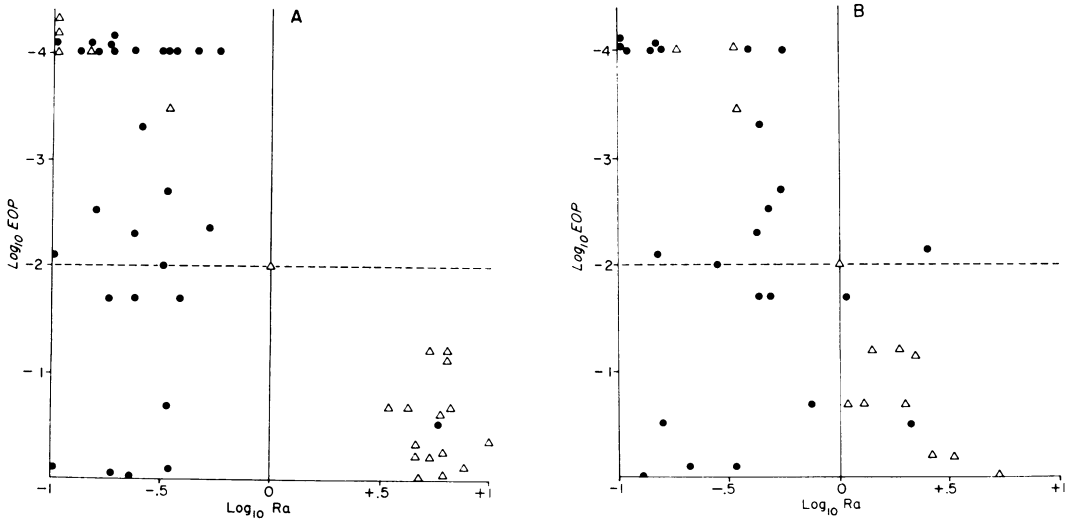

FIG. 1. *Log_{10} of the efficiency of plating on chick cells and $\log_{10}$ of the R_a values for virus strains from oral and genital sources. (A) R_a values obtained with guinea pig antisera; (B) R_a values obtained with rabbit antisera. Symbols: ●, strains from oral sources; △, strains obtained from genital sources.*

TABLE 3. *Comparison of efficiency of plating (EOP) on chick cells with R_a values from kinetic neutralization studies*

EOP[a]	$R_a < 1$	$R_a > 1$
0	1	2
−1	5	12
−2	4	4
−3	5	0
≦−4	20	0

[a] Log_{10} virus titer on chick cells per virus titer on fibroblasts.

TABLE 4. *Efficiency of plating (EOP) on chick cells and the R_a values of strains before and after plaque selection from chick and fibroblastic cells*

Strain no.	Plaque from	EOP[a]	R_a
276		−4	0.19
	CE[b]	−3	0.34
	FT[b]	−6	0.15
384		−3	0.02
	CE	−1	0.43
	FT	−3	0.09
405		−3	0.21
	CE	−1	0.64
	FT	−4	0.36
406		−2	0.32
	CE	−2	0.93
	FT	−2	0.37
682		−3	0.16
	CE	−1	0.53
	FT	−3	0.13

[a] Log_{10} virus titer on chick cells per virus titer on fibroblasts.

[b] CE = chick cells; FT = human embryonic fibroblasts.

log_{10} in eight strains with initial EOP values of 10^{-3} or less and in two of four strains with EOP values of 10^{-2}. The other two strains continued to have EOP values of 10^{-2} after plaque purification. Selection from fibroblasts decreased the EOP for chick cells in five strains and did not change the other seven. Although it was possible to select variants with type 2 EOP values from strains with type 1 EOP values, in no case was such a change accompanied by a corresponding change to an R_a greater than one. However, the minor changes seen in the R_a values were consistent in that selection from chick cells resulted in increased R_a values and selection from fibroblasts gave decreased R_a values. The results of five comparisons are shown in Table 4.

TABLE 5. *Comparison of the efficiency of plating (EOP) and R_a values of oral and genital strains*

EOP[a]	Oral strains		Genital strains	
	$R_a < 1$	$R_a > 1$	$R_a < 1$	$R_a > 1$
0	1	0	0	2
−1	5	1	0	11
−2	4	0	0	4
−3	5	0	0	0
≦−4	15	0	5	0
Total	30	1	5	17

[a] Log_{10} virus titer on chick cells per virus titer on fibroblasts.

When R_a values are considered in relation to source as well as EOP (Table 5), there are some major inconsistencies among the three. The overall agreement between source, EOP, and R_a is 70%. There are 25 type 1 and 18 type 2 strains with consistent R_a and EOP results. Five of the type 1 strains came from genital lesions and one of the type 2 strains from a throat culture. The 10 strains with inconsistent EOP and R_a values were all from oral sources. It has been reported that EOP on chick cells increased with laboratory passage on rabbit or human cells (8), which may account for the EOP of these strains, although they were in no higher passages when tested than those with a lesser EOP. The R_a values of these 10 strains varied from 0.08 to 0.39 and, as can be seen from Fig. 1, were not different from those strains with an EOP of less than 10^{-2}. R_a, therefore, appeared to be a better determinant for classifying oral isolates, but either EOP or R_a served equally well to differentiate genital isolates.

Since the source of a culture may not adequately indicate the clinical disease in the patient, history of illness was compared with the laboratory results on the isolates. Nothing distinguished the history of illness nor the clinical findings of the five patients with type 1 genital isolates from those of patients whose isolates had type 2 values. With the exception of the neonatal case, patients with genital isolates ranged between 16 and 35 years of age, regardless of the type of their isolate. There were six patients with genital isolates who also had some history of oral symptoms; all had type 2 isolates.

Patients from whom oral isolates were obtained were not concentrated into such a limited age span and ranged from infancy to 82 years of age. The age distribution was the same for those whose isolates showed inconsistent EOP – R_a patterns

as for the others. Three of the patients with oral isolates also had a history of genital lesions; one of these had an isolate with a type 2 EOP and a type 1 R_a, but the other two had isolates with type 1 EOP and R_a values. There was no indication in this series of "intermediate" strains isolated from cases with both oral and genital symptomatology.

DISCUSSION

The examination of these herpesvirus strains suggests, as have the results of other studies (2, 9, 12), that neutralization divides strains into two rather distinct groups, which also differ in biological characteristics. Whether this antigenic difference is of sufficient magnitude and stability to warrant "type" distinctions is a matter of present controversy (6, 9). Although antigenic character does not appear to vary with EOP in this or another study (8), or with other markers (12, 13), there is great diversity in the degree of relatedness between strains within groups. Hampar et al. (6) have suggested that *H. hominis* strains should be classified into one serological group as a species by the use of 7*S* antibodies and that late 19*S* antibodies should be used for determining subtypes within that species. When our data for guinea pig sera are compared with data for rabbit sera, it can be seen that the degree of relatedness between strains was influenced by the sera used for neutralization. The efficacy of the guinea pig sera may be due to a relatively higher concentration of 19*S* antibody.

Be they types or subtypes, however, present evidence suggests the existence of at least two kinds of herpes simplex viruses with differing antigenic and biological characteristics, one of which is associated predominantly with oral infections and the other with genital ones. In this series, as in others (2, 9, 11), the majority of the isolates from genital sources had the properties now assigned to type 2 viruses. There were, however, five type 1 genital isolates to be added to those previously reported. Nahmias et al. (11) identified eight type 1 strains among 226 genital isolates, and three of Schneweis' nine genital isolates fit the type 2 designation (9). Other investigators have not reported type 1 strains from genital sources (8, 12). The weight of evidence at present indicates that type 2 virus is limited to the genital tract and has a venereal mode of transmission, but it appears that at least certain type 1 viruses may also behave like genital strains. The number of strains examined so far is relatively small, since data on only about 500 isolates have been reported (8, 9, 11, 12), and it is possible that type 1 strains may be more prevalent among genital isolates from other populations. The diversity of strains examined by investigators is limited by the epidemiology of infection in the population under study and the relationships between individuals from whom strains were isolated. The strains from the 61 diagnostic specimens in our study were chosen to represent patients with no known epidemiological relationships, but this does not insure that we, any more than other investigators, have not been examining repeated isolations of the same virus. Some family relationships are represented among the 39 strains from the Virus Watch study. Since 10 families had more than one infected family member, 25 families comprise the population for these strains. These families have no known contact with one another.

When the source of strains is known, EOP on chick cells may serve to screen isolates for types. EOP is a simpler procedure than those required for neutralization, and there appears to be excellent correlation between the EOP and R_a of genital isolates. Lowry et al. (8) tested 40 genital isolates which were type 2 by neutralization and found no discrepancies between the chick cell and neutralization results. Since we did not find the same agreement with oral strains noted by these investigators, neutralization may be needed to clarify the status of oral isolates with type 2 EOP values.

If *H. hominis* strains are to be classified with some amount of sophistication, markers which vary independently and are suitable for use with a large number of strains will be needed. A difference in deoxyribonucleic acid density between type 1 and type 2 viruses has been reported (5, 7), but this is not a procedure which could readily be used for typing. Other markers which have been investigated include pock production (8, 10), growth characteristics (4, 8, 14, 15), thermostability (4, 8, 12–14), ultraviolet irradiation (13), cytopathic effect (1, 12, 15), animal virulence (4, 12), and sensitivity to inhibitors (8, 14). Although these tend to show correlation with antigenic typing, in most cases they have been applied to only a few strains used as prototypes. Some of these markers are probably interrelated and could not be expected to vary independently, such as pock production and efficiency of plating on chicken cells, and there is conflicting evidence in regard to some others (1, 4, 8, 12–14). Roizman and co-workers (3, 15, 16) have shown that there is considerable variation in the "social behavior" of herpesvirus-infected cells and have suggested that there may be more than two types or that there is a continuum in the range of viral behavior which makes it difficult to identify two

distinct types. The lack of correlation between source, EOP, and R_a and the diversity in the EOP and R_a values seen here could be construed as support for this hypothesis. It seems particularly important that a typing scheme should not be based on the study of a limited number of strains by a few investigators. It will require the investigation of a much larger number of strains for several markers which vary independently to determine whether the differences now seen between strains are indicative of two types or whether they represent the extremes of a continuum of variation.

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