Biological Characteristics of Cloned Populations of Herpes Simplex Virus Types 1 and 2

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Received for publication 30 September 1970

By using cloned types 1 and 2 herpes simplex virus, obtained by selecting large and small plaques produced by material from human lesions, studies were performed to compare properties between preparations of each type. Regarding the rate of inactivation by ultraviolet light, no differences were found between the two antigenic types and none between the preparations obtained from either type. In contrast, type 1 preparations were found to be more readily inactivated at 45 C than type 2. Plaque size of cloned preparations changed by passage in cell culture. A broader range of plaque sizes was obtained, and average plaque size was larger. After 20 passages, preparations obtained from different types gave rise to one of three kinds of cytopathic effect. The cytopathic effect produced by type 1 preparations remained as before 20 passages and consisted of round cells in a compact central mass. For type 2, two kinds of cytopathic effect were seen in cloned preparations. This consisted of aggregates of round cells (seen in preparations before 20 passages) or of large, loose aggregates of round cells of various sizes. Results from neutralization studies using virus before and after 20 passages in cell culture versus antisera prepared against live or ultraviolet-inactivated virus showed no differences between cloned preparations obtained from a given type.

During the past several years, antigenic type 1 and 2 of herpes simplex virus (HSV) have been found to differ in respect to a number of properties (2, 6, 8, 13, 15–17, 19–21).

Conclusions drawn from reports comparing properties of the two types should be viewed in light of the fact that most studies have been performed using uncloned-virus strains. This observation is especially interesting in view of the many cell-culture variants of HSV which have been isolated (9–12, 14, 18, 21, 22, 24).

It was the purpose of this investigation to analyze cloned preparations (selected on the basis of plaque size) of fresh isolates of both type 1 and type 2 HSV as to specific properties and the stability thereof. In so doing, it was hoped that the following questions might be answered: (i) Do plaque size variants exhibit those properties associated with the parent population? (ii) Does passage of cloned HSV in cell culture lead to populations which differ in one or more properties from the original cloned virus?

MATeRIALS AND METHODS

Cell cultures. Rabbit cornea cells (SIRC) were obtained from the American Type Culture Collection,

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Rockville, Md. Cells were grown in bottles at 37C in a 5% CO2 incubator and dispersed with trypsin before using.

Growth medium (GM) consisted of Eagle’s minimal essential medium in Earle’s balanced salt solution with 5% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and sodium bicarbonate (0.0012 g/ml). Maintenance medium contained 3% FCS.

Virus strains. Two virus strains isolated in this laboratory were employed in this study: "Del," a type 1 HSV, and "Arc," a type 2. Typing was done by multiplicity neutralization using type 1 antiserum.

Virus assay. Infectivity titers were determined by plaquing on SIRC. Monolayers were prepared by inoculating 60-mm plastic plates (Falcon Plastics, Div. of B-D Laboratories, Los Angeles, Calif.) with 5 x 10⁶ cells in 8 ml of GM and incubating at 37 C for 2 days. Monolayers were inoculated with 0.1 ml of virus in GM. After a 2-hr absorption period at 37 C, monolayers were overlaid with 5 ml of GM containing 0.7% agarose (Seakem, Bausch & Lomb, Inc., Rochester, N.Y.). Five days later a second agarose overlay (3 ml) containing a 1:10,000 dilution of neutral red was added. Plaques were counted 8 hr after the second overlay.

Cloning procedure. One-tenth milliliter of material obtained from a human lesion and referred to either as Del or Arc strain virus was inoculated onto monolayers. Five days after infection, virus from the largest and the smallest plaques was isolated and suspended in 1 ml of GM. After three cycles of freezing and thaw-
ing. 0.1 ml of the material in GM was inoculated onto fresh monolayers to repeat the procedure, which was then repeated a third time, still using plaque size as the basis for selection. Next, monolayers in bottles were inoculated with material obtained from the third plaque purification. After three days of incubation at 37°C, cells were frozen and thawed three times and the virus passed two more times in bottles. When cytopathic effect (CPE) reached approximately 100% on the final passage, preparations were frozen and thawed three times and centrifuged at 650 × g for 10 min at 4°C, then at 2,250 × g for 10 min. Supernatant fluids were filtered through a 0.45-μm filter (Nalgene filter unit, Nalge Co., Rochester, N.Y.). The filtrate was labeled 1L (or 15) if it originated from the Del strain, or 2L (or 25) if it originated from the Arc strain. In each case, L symbolizes large-plaque variant and S symbolizes small-plaque variant. Preparations were distributed into 1-ml volumes and stored at −70°C.

Uncloned preparations were obtained as follows. Virus-containing material from the corresponding human lesion was passed at low multiplicities [1 plaque-forming unit (PFU)/100 cells after the second passage] for a total of six passages. Final preparations were obtained as described above and labeled 1 P or 2P, representing, respectively, Del parental or Arc parental.

Ultraviolet inactivation. Virus preparations were diluted 1:10 in 0.005 M phosphate-buffered saline (PBS) and placed in 30- by 10-mm plates (Falcon Plastic). Virus was exposed, with constant shaking, to ultraviolet (UV) light. At predetermined intervals, virus was shielded from the UV light and assayed for infectivity.

Thermal inactivation. One-milliliter volumes of virus were placed into 2-ml glass ampoules. Sealed ampoules were then immersed in a water bath at 45°C. At intervals, ampoules were removed and stored at −70°C. Their contents were later assayed for infectivity.

Preparation of antiserum against live virus. For immunization, virus was selected and cloned as described in the procedure on cloning. For propagation of virus, SIRC was adapted to grow in GM in which 5% rabbit serum substituted for 5% FCS. The last two passages consisted of virus populations propagated in GM containing rabbit serum. This was done to minimize production of antibodies directed against materials other than the virus. Virus was harvested at time of maximum CPE, and final preparations were obtained as described.

Pre-bled New Zealand white rabbits were injected with 1 ml of virus intramuscularly and intraperitoneally as follows: (i) day 1, 10⁶ PFU/ml; (ii) day 10, 10⁶ PFU/ml; (iii) day 15, 10⁶ PFU/ml; (iv) day 20, 10⁶ PFU/ml. Animals were bled on day 30, at which time the serum was distributed in small volumes and stored at −20°C.

Preparation of antiserum against virus inactivated by UV light. The preparation of virus designed for immunization was modified from that described above. After centrifugation and filtration to remove cell debris, virus grown in GM with rabbit serum was sedimented at 38,000 × g for 30 min and resuspended in PBS. Suspensions of virus in PBS were placed in glass petri plates and exposed for 10 min, using constant agitation, to UV light as described previously. One-milliliter portions were frozen and stored at −70°C. No infectious virus was detectable in virus preparations used for immunization.

For immunization, virus preparations were thawed and combined with equal volumes of complete Freund's adjuvant (Difco). Two pre-bled rabbits were injected with 0.1 ml of emulsion into each footpad on days 1, 7, and 14 of the injection schedule. On day 45, 46, and 47 each animal was inoculated intravenously with 1 ml of the appropriate virus suspension in PBS. On day 55 each rabbit was bled. Immune serum was obtained and stored as described.

Multiplicity neutralization. For neutralization studies, the method of Wheeler et al. (25) was modified for use with the plaque assay developed for SIRC. Prior to use, antiserum was inactivated by heating at 56°C for 30 min. Approximately 2 × 10⁵ PFU of virus in 0.5 ml of GM was added to 0.5 ml of serial two-fold dilutions of antiserum in GM. Mixtures were first incubated for 1 h at 37°C then placed in an ice-water bath and, finally, assayed for infectivity.

Fifty per cent neutralization titers were expressed as the reciprocal of the serum dilution that would reduce by one-half approximately 100 PFU of test virus. Titers were determined by plotting the surviving fraction on the ordinate versus the log₂ of the serum concentration on the abscissa. At the point of 50% neutralization on the multiplicity neutralization curve (Fig. 4), a line was drawn to the abscissa and the serum concentration titer determined directly or by interpolation. Normalized titers were calculated by assigning a value of 100% to antiserum prepared against homologous virus or against homologous virus after 20 passages in cell culture. Normalized titer = (50% neutralization titer against heterologous virus-serum mixtures/50% neutralization titer against homologous virus-serum mixtures) × 100. In addition, neutralizing potency (pN) values of antiserum were determined using a formula devised by Fazekas de St. Groth (5): pN = log A + log log V₄/V₃; A = serum dilution expressed in terms of final volume. V₄ = initial virus concentration. V₃ = surviving virus concentration.

Multiplicity neutralization curves (Fig. 4) were constructed by connecting the points obtained from plotting the surviving fraction at serially doubling antiserum dilutions. Points were normally obtained in the region of complete neutralization to the region of no detectable neutralization. The pN value represents the mean of 3 to 5 separate pN determinations of those points.

In order to compare the neutralizing potency of an antiserum against various virus preparations, normalized pN values (25) were calculated according to the formula: Normalized pN = (test virus-serum mixture/homologous virus-serum mixture) × 30. 0.3
determined. Photographs of HSV plaques were taken so as to obtain a film image to subject ratio of 0.3.

Preparations used for CPE consisted of monolayers on 60-mm plastic plates 48 hr after infection.

RESULTS
Effect of cloning on plaque size. Plaques produced by cloned virus from the 2 HSV strains re-
Fig. 1. Plaques produced by cloned and uncloned preparations of type 1 and type 2 HSV at five days after infection.

...flected the criterion used as the basis for selection, i.e., plaques produced by 1L and 2L preparations were large and plaques produced by 1S and 2S preparations were small. Plaques produced by cloned and uncloned preparations of these prototypes are shown in Fig. 1. 1P refers to plaques obtained from uncloned preparations of the Del (type 1) strain. 1L refers to plaques obtained from the preparation resulting from selecting large plaques, and 1S refers to plaques obtained from selecting small plaques. Similar notation was used for the Arc (type 2) strain (see Materials and Methods).

CPE. The CPE produced in rabbit cornea cultures by the type 1 Del strain consisted of round cells in each focus of infection, with the infected cells observed also in the surrounding area. The CPE produced by the type 2 Arc strain was characterized by compact aggregates of uniformly round cells.
**Plaque size and CPE after 20 passages.** Plaques produced from both cloned and uncloned virus of the type 1 and type 2 prototypes after 20 passages in SIRC cells were similar in average diameter, showing that the cloned viruses did not breed true. In general, viruses after 20 passages produced plaque sizes of greater heterogeneity than those produced by the initial preparations. In addition, some unusually large plaques were seen with the type 2 preparations after 20 passages.

Although the plaque size produced by each type 1 preparation changed after 20 passages, the kind of CPE that characterized the initial virus preparations remained the same. This was not the case for type 2 virus, however; a new kind of CPE could be seen after 20 passages. Beside the kind seen originally, i.e., compact aggregates of uniformly round cells, CPE, consisting of large, loose aggregates of round cells of various sizes with occasional giant cells, appeared.

**Inactivation by UV light.** Since comparative studies concerning the effect of UV light on infectivity of type 1 versus type 2 HSV have not been reported, it was of interest to determine whether differences in the rate of inactivation could be found between (i) clones obtained from one type, (ii) clones obtained from different types, or (iii) clones and the corresponding parental population.

Preparations of type 1 (1P, 1L, 1S) and type 2 (2P, 2L, 2S) were exposed to UV light and assayed for surviving virus as described. Results obtained (Fig. 2) show a clear difference in the inactivation rates between the two types. The type 2 viruses were inactivated more readily, having a half-life of approximately 6 sec as compared with 10 sec for the type 1. No observable differences in inactivation rates were seen among the preparations of a given type, suggesting that they are homogeneous in regard to this criterion.

**Thermal inactivation.** For thermal inactivation, cloned and uncloned virus was incubated at 45 C for predetermined periods of time, then assayed as described.

Results obtained (Fig. 3) show a difference between type 2 and type 1 preparations when suspended in PBS but not when suspended in the medium of growth. Curves obtained with 1P and 1S were similar to the curve for 1L shown in Fig. 3. Likewise, curves obtained with the 2P and 2S viruses were similar to the curve for 2L.

**Neutralization studies.** Dowdle et al. (3) classified strains of HSV into two types using pN values determined from microneutralization tests. In a later study, Wheeler et al. (25) distinguished between two HSV strains with multiplicity neutralization tests using plaque assays. They reported that multiplicity neutralization tests, from which pN values can also be determined, were preferable in most instances for distinguishing between two strains of HSV to the often used kinetic neutralization test.

Neutralization of the six virus preparations by specific antiserum was studied using the multiplicity neutralization test (25), the object being to see if antigenic differences existed either among cloned virus or between cloned- and uncloned-
TABLE 1. Cross-neutralization scheme using antisera prepared (1) against live virus and (2) against ultraviolet (UV)-inactivated virus

| Virus population | Antiserum         |
|------------------|-------------------|
| (1)              |                   |
| 1P               | Anti-1L           |
| 1L               | Anti-1S           |
| 1S               | Anti-2L           |
| 2P               | Anti-2S           |
| 2L               |                   |
| 2S               |                   |
| 1P + 20          |                   |
| 1L + 20          |                   |
| 1S + 20          |                   |
| 2P + 20          |                   |
| 2L + 20          |                   |
| 2S + 20          |                   |
| (2)              |                   |
| 1P               | Anti-UV1S         |
| 1L               | Anti-UV2S         |
| 1S               |                   |
| 2P               |                   |
| 2L               |                   |
| 2S               |                   |

* Virus preparations after 20 passages.

virus preparations of the same strain. Cross-neutralization tests were performed by the scheme shown in Table 1.

Neutralization using antisera prepared against live virus. To reduce the possibility of antibodies being formed against a heterogeneous virus population, antisera was prepared in rabbits against the cloned-virus preparations. Cross-neutralization tests were performed using combinations of cloned- or uncloned-virus preparations and antiserum prepared against cloned virus. Results obtained using anti-1L and anti-2L serum are shown in Fig. 4. Neutralization with anti-1L serum resulted in a relatively large degree of difference (between 8- and 16-fold) between viruses of the two types, but no observable difference was seen among preparations of the same type. Use of anti-2L serum provided no observable distinctions between the two types of HSV. It also did not allow for separation of preparations from the same type.

Results obtained using other combinations in cross-neutralization tests did not reveal any pertinent information not shown in Fig. 4. Neutralizing potency values and 50% neutralization titers determined from multiplicity neutralization curves are shown in Table 2.

When it became apparent that different preparations from either type of HSV did not differ antigenically, each virus was passaged 20 times in SIRC cells to see if detectable antigenic differences would then emerge. Virus preparations after 20 passages were centrifuged, filtered, and stored as described. Later, each virus was cross-neutralized with anti-1L or anti-2L serum. The multiplicity neutralization curves obtained were similar to those obtained before passaging. No new neutralization characteristics were detected.

Neutralization using antisera prepared against UV-inactivated virus. In the neutralization tests described in the preceding section, the possibility existed that virus replication in the rabbits might have led to the masking of antigenic differences, especially among cloned preparations of the same virus type.

In an attempt to exclude this possibility, cross-neutralization tests were performed using virus and antiserum prepared against UV-inactivated 1S or 2S virus preparations. Again, the multiplicity neutralization curves failed to show any differences among different preparations of the same virus type. Differences between the two strains were similar to those found in neutralization tests carried out with antiserum prepared against live virus. Neutralizing potency values and 50% neutralization titers obtained are shown in Table 3.

**DISCUSSION**

Results from the present study indicate that plaque size variants of HSV are present in human...
TABLE 2. Fifty per cent neutralization titers and pN determinations obtained from multiplicity neutralization curves using virus preparations before and after 20 passages versus anti-1L and anti-2L sera

| Anti-serum | Virus prepn | Titers | pN values | Anti-serum | Virus prepn | Titers | pN values |
|------------|-------------|--------|-----------|------------|-------------|--------|-----------|
|            |             | (50%) Titer | Normalized titer | pN⁵ | Normalized pN⁶ | (50%) Titer | Normalized titer | pN⁵ | Normalized pN⁶ |
| 1L         | 1P          | 362     | 138       | 3.35 | 105         | 2L         | 1P          | 206 | 59         | 3.08 | 94 |
| 1L         | 1L          | 256     | 100       | 3.19 | 100         | 2L         | 1L          | 269 | 76         | 3.20 | 97 |
| 1L         | 1S          | 239     | 93        | 3.12 | 98          | 2L         | 1S          | 298 | 85         | 3.01 | 92 |
| 1L         | 2P          | 19      | 7         | 1.90 | 60          | 2L         | 2P          | 256 | 73         | 3.14 | 95 |
| 1L         | 2L          | 19      | 7         | 1.97 | 62          | 2L         | 2L          | 352 | 100        | 3.19 | 100 |
| 1L         | 2S          | 24      | 9         | 2.11 | 66          | 2L         | 2S          | 213 | 61         | 3.11 | 95 |
| 1L         | 1P + 20     | 240     | 100       | 3.13 | 99          | 2L         | 1P + 20     | 270 | 120        | 3.18 | 102 |
| 1L         | 1L + 20     | 240     | 100       | 3.15 | 100         | 2L         | 1L + 20     | 256 | 113        | 3.19 | 102 |
| 1L         | 1S + 20     | 268     | 112       | 3.15 | 100         | 2L         | 1S + 20     | 306 | 136        | 3.20 | 102 |
| 1L         | 2P + 20     | 13      | 5         | 1.86 | 59          | 2L         | 2P + 20     | 189 | 84         | 3.04 | 97 |
| 1L         | 2L + 20     | 26      | 11        | 2.18 | 69          | 2L         | 2L + 20     | 225 | 100        | 3.13 | 100 |
| 1L         | 2S + 20     | 16      | 7         | 1.96 | 62          | 2L         | 2S + 20     | 241 | 107        | 3.08 | 98 |

⁵ Value of antisera against homologous virus or against homologous virus after 20 passages is taken as 100%.

⁶ Each pN value is a mean of 3 to 5 determinations at different serum concentrations.

lesions, so it would seem that for some properties at least HSV populations in human lesions are quite heterogenous.

The findings presented regarding the effect of cloning on the size of plaques produced are in contrast to those reported by Germanov and Sokolov (7). They found that the distribution pattern of plaque size produced from selecting small and large plaques in either chick embryo fibroblasts or human embryo fibroblasts was similar to that of the original strain (type 1). Rapp, (22) on the other hand, reported that plaque size variants were obtained after selecting small and large plaques of HSV (type 1) produced in PRK cells. It should be mentioned that in the two cases just cited (i) plaques were selected under an agar overlay and (ii) fresh isolates of HSV were not used. Since in this study agarose was used in the overlay, the problem of what effect agar may have on plaque selection was not encountered.

The cloning procedure employed in this study
followed established techniques. It is probable that the emergence of larger plaques from virus preparations that originally produced small plaques only (1S and 2S) resulted from the selection of mutants that arose during the process of passaging virus preparations in cell culture. It is also possible that the cloning procedure produced progeny that were not 100% pure, perhaps because of recombination taking place among the deoxyribonucleic acid (DNA) of noninfectious virus particles leading to reactivation or between the DNA of noninfectious virus particles and live virus. Interpretation of the results would then be based on the emergence of large plaque-forming infectious viruses present in noninfectious form in the preparation, rather than to mutation.

The CPE produced by fresh isolates was found to correlate with the antigenic type of HSV. It is doubtful, however, that CPE in cell cultures can be used as a sole criterion for classifying strains of HSV. Too much variation exists in the descriptions of CPE given in the results obtained from different laboratories (4, 21). It is sometimes difficult even to characterize the CPE produced by a single strain. Our results, for instance, showed that in the case of preparations of the type 2 strain used at least two kinds of CPE could be seen after 20 passages in cell culture. Results were the same for both cloned- and uncloned-virus preparations.

A difference was detected between preparations of the two HSV types in their rate of inactivation by UV light. Since results of similar experiments have not been reported, there is no basis for comparison. The reasons for the differences between the two antigenic types are open to speculation. Before conclusions are drawn concerning the universality of the differences shown between the two types of HSV, a number of strains will have to be tested besides the two here.

There have been several reports comparing the rates of thermal inactivation of type 1 versus type 2 HSV strains (4, 6, 20, 21). In most cases workers found that type 2 strains of HSV, grown in a variety of cell lines and under a number of different conditions, were more thermolabile than type 1 strains of HSV (6, 20, 21).

The results obtained contrast with those reported, and reasons for these contrasting results are not clear. One difference in procedure from those reported was the use of SIRC cells for replication of HSV strains. This could be a major factor because the particular cell line used for virus replication has been shown to alter the difference in rates of inactivation obtained with the two types of HSV (21). Other possibilities for the difference in thermolability could be: (i) unrepresentative strains of HSV. Since only two strains of HSV were employed, the results may only apply to these two strains. (ii) Medium for virus suspension. Wallis and Melnick (23) have reported that the medium used for virus suspensions plays an important role in studies on thermal inactivation. They found that HSV would remain relatively stable at 50°C with certain changes in the isotonicity of medium used for suspending the virus. It is not known how PBS, which was used in this study, compares with media used in other studies on the thermolability of HSV. Moreover, in the medium of growth, thermal inactivation rates were the same, contrasting with results obtained by other researchers (6, 21). (iii) Cell-associated virus. Plummer et al. (21) have shown that there are differences in the rate of thermal inactivation between cell-associated and extracellular virus for both antigenic types of HSV. Since no attempt was made to separate the two in this study, it can be suggested that what is being shown in curves for preparations of the two viruses is the effect on different proportions of cell-associated and extracellular viruses.

Results obtained showed that cloned-virus preparations were of the same antigenic type as uncloned preparations. Passaging 20 times in cell culture did not alter these results. In addition, results obtained using antiserum prepared against both live and UV-inactivated virus were similar. These results indicate that plaque purification and subsequent subculturing did not select antigenic variants of HSV and are in agreement with most results obtained either from studies in which cloned virus (1, 22) or cell-culture variants (3, 11, 14, 18, 21) were tested for antigenicity against the parent strain.

ACKNOWLEDGMENT

We acknowledge the help of A. J. Nahmias of Emory University, Atlanta, Ga., for typing virus strains for us and for providing us with reference antisera.

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