Enhanced Detection of Western Equine Encephalitis Virus Plaque Variants in Crowded Cultures and Plaque Progeny—Potential Use in Genetic Studies

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Enhanced detection of large-plaque variants of Western equine encephalitis virus in the presence of large numbers of small-plaque Western encephalitis virus was achieved by using higher bicarbonate concentrations in the agar-overlay medium and increasing concentrations of CO₂ in an enclosed environment during the time plaques were developed with neutral red. By this technique we were able to detect large-plaque mutants in cloned small-plaque suspensions for the first time and to show an increase in detectable plaque progeny in plaques developed by neutral red. Further increase in the number of progeny per plaque was determined by assaying extracts of agar overlaying cultures containing known numbers of plaques. The significance of these findings for potential use in mutational studies is discussed.

Estimation of mutation rates from formulas developed by Luria and Delbrück (3) and applied to animal viruses by Dulbecco and Vogt (2) and Breeze and Subak-Sharpe (1) require accurate determinations of the mean numbers of virus in clones and either the number of virus mutants in a clone or the proportion of clones not containing the mutant. Mean clone sizes for viruses that form plaques can be accurately determined in plaque-forming units (PFU). The number of plaque mutants in a clone can be determined most readily by plaque assay, especially when culture methods are available that inhibit plaquing or replication of the parental type which otherwise would interfere with the detectability of the mutants.

In contrast, when methods are not available for inhibiting plaquing or replication of the parent virus, mutational studies with plaque mutants are usually confined to (i) viruses having high enough mutation rates to allow accurate counting of mutants in a reasonable number of cultures in the presence of parental type plaques, and/or (ii) the use of techniques that allow detection of clones not containing the mutant.

In this laboratory, numerous routine plaque assays performed over a number of years with cloned small-plaque (SP) suspensions of Western encephalitis (WEE) virus failed to reveal the presence of any large-plaque (LP) variants. We therefore believed that either SP → LP mutations of this virus did not occur or that the SP → LP mutation rate was extremely low. The studies described here relate to our enhanced ability to: (i) detect LP variants in cloned suspensions of SP by enhancing the resolution of plaques in cultures inoculated with large numbers of virus particles (preliminary findings had shown that, whenever large numbers of various group A togaviruses were plaqued on individual cultures, normal plaque development was often inhibited or obscured by interference and/or development of cytotoxicity); and (ii) obtain more accurate measurements of progeny present in plaques. Accurate determinations of the numbers of progeny in plaques for use in mutational studies are presently hampered by inherent difficulties in sampling techniques and by the inactivation of virus by neutral red (NR); therefore, any means of showing the presence of more virus in plaques would result in a more accurate determination of mutation rates in studies where plaque progeny are treated the same as subclones. We intend to use the information from these studies to investigate an apparently infrequent SP → LP mutation of WEE virus.

MATERIALS AND METHODS

Cell culture and virus plaque assay. Primary chicken embryo cell monolayer cultures for virus plaque assays were prepared in 60-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.)
according to standard technique (7). All dishes were seeded with 5.0 ml of a cell suspension containing 2 x 10^6 cells per ml and incubated at 37 C until cells became confluent, and cultures were maintained in plastic tanks (Lab-Line "Clear-Vue" Anaerobic Chambers, Lab-Line Instruments, Inc., Melrose Park, Ill.) containing optimal concentrations of CO₂. After incubation at 37 C, cultures were washed once with phosphate-buffered saline and infected with 0.2 ml of appropriate virus dilutions. The infected cultures were incubated for 1 h in a closed CO₂ environment at pH 7.4 before agar-overlay medium was added; after an additional 30- to 36-h incubation period, the virus plaques were developed with NR (2.0 ml per plate). In studies where plaques were picked to determine the number of progeny virus per plaque, a 48-h incubation period was used.

**Viruses.** The WEE virus plaque variants used were first described by Ushijima et al. (6). Plaque characteristics of the progeny of these viruses have remained stable in our laboratory since 1967. The isolated SP virus produces clear, slightly irregular plaques 2 to 3 mm in diameter after a 48-h incubation period at 37 C, whereas the LP variant produces round plaques 7 to 8 mm in diameter. The variants were subcloned in chicken embryo cells three times before use in this study, and the third clone was used to prepare seed virus. Before this study no LP had ever been observed in numerous assays performed with cloned SP suspensions.

**Hanks modified growth medium and agar-overlay medium.** Hanks balanced salt solution, modified to contain 1.0 g of sodium bicarbonate (HCO₃⁻) and 0.02 g of phenol red per liter and supplemented with 5% bovine serum, 0.5% lactalbumin hydrolysate (wt/vol), 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 50 U of mycostatin per ml, was used for cell culture medium and preparation of virus dilutions. Unless otherwise mentioned, nutrient agar-overlay medium was prepared by mixing twice-concentrated Hanks modified growth medium (further modified to contain 4 g of HCO₃⁻ per liter) with an equal volume of 1.8% (wt/vol) purified agar (Difco) in distilled water.

**Neutral red.** NR (1,5000 [wt/vol] in 0.85% NaCl) was prepared by dissolving the dye in several milliliters of 1.0 N HCl and adding it to the desired volume of 0.85% NaCl. The pH was then adjusted to 4.0 with 0.1 N NaOH or HCl, or both, before autoclaving. NR prepared in this manner was found to be less toxic to cells.

**CO₂ requirements for growing cultures and plaqueing virus in sealed tanks.** Since CO₂ was used to control the pH of cultures in sealed tanks, we had to establish initial guidelines for the volumes of CO₂ (100%) added to the tanks. For convenience, the flow rate of CO₂ was always adjusted to 1.6 liters/min, and the flow time varied until the optimal pH (7.2 to 7.4), as judged from the phenol red indicator present, could be produced. Once guidelines were established, minor adjustments were made as needed. For example, in a typical assay, CO₂ was added for 20 s to a 28-liter tank containing freshly seeded cultures. Twenty-four hours later, the cultures were infected, overlayed, and placed back into the tank along with a wet sponge to maintain 100% relative humidity. CO₂ was added for 40 s and the cultures were incubated for 30 to 36 h before NR was added. After the cultures were put back into the tank, CO₂ was added for 40 s, and the cultures were incubated for 30 min while the NR was diffusing down to the cells. At the end of this 30-min period, CO₂ was added to the tank for 40 more seconds and the cultures were incubated an additional 30 min while the NR was penetrating the cells. The cultures were then removed, and the plaques were photographed, counted, and/or picked for cloning.

**RESULTS**

**Detection of LP in cultures crowded with SP.** Preliminary studies had shown that the pH of cultures infected with large numbers of virus could be more effectively controlled in sealed tanks by increasing the HCO₃⁻ concentration in the agar-overlay medium and either increasing or decreasing the CO₂ concentration as needed. In addition, during the time NR was present on cultures, faster staining of plaques and less cytotoxicity was observed if the CO₂ concentration was markedly increased for a short period of time. Figure 1 is a photograph of 1 of 10 cultures infected with a 1,000 SP:1 LP virus suspension. After incubation of the cultures for 30 to 36 h, plaques were developed in the presence of high HCO₃⁻-CO₂ concentrations as described in Materials and Methods. Two LP were detected and cloned from this culture, and one was detected and cloned from another. The actual number of SP plaques present was uncountable; however, plaque counts from higher dilutions indicated that the plaques that were present represented a mean of approximately 2,200 SP per culture. Before the use of higher HCO₃⁻-CO₂ concentrations in the environment while virus and NR were present in the cultures, the most SP that could be present in single cultures without initiating areas of general cell lysis that obscured plaque types was from 200 to 300.

**Relative plating efficiency of LP in the presence of SP.** The maximum number of SP plaques that could be present without interfering with development or recognition of LP plaques was determined by infecting replicate cultures grown in 60-mm plates with suspensions of virus containing either 5 or 16 LP and 300 to 1,300 SP. Plaques were developed after a 30-h incubation period, and the results showed that no significant difference in plating efficiency of the LP was encountered until 1,000 PFU of SP were present per culture (Table 1). One thousand SP plaques per culture reduced
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FIG. 1. Representative photograph of 1 of 10 cultures infected with a 1,000 SP:1 LP virus suspension. Arrows point to two LP which were detected in a culture containing approximately 2,200 SP.

| Table 1. Relative plating efficiency of small numbers of LP in the presence of SP |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| SP added per culture (PFU)     | Mean plaque count and percent reduction of LP per culture |                  |                  |                  |
|                                 | LP (PFU/culture) | Percent reduction | LP (PFU/culture) | Percent reduction |
| 0                              | 16.5            | 0                | 5.0             | 0                |
| 601                            | 17.0            | 0                | 5.5             | 0                |
| 810                            | 16.5            | 0                | 7.0             | 0                |
| 997                            | 10.5            | 36.4             | 2.5             | 50.0             |
| 1,312                          | 7.5             | 54.5             | 1.5             | 70.0             |

The plaque count of the LP by an average of 43%, whereas no difference in the plaque count of the LP was detected when 800 or fewer SP plaques per culture were present. Thirteen hundred SP plaques per culture reduced the number of LP plaques an average of 62%.

Detection of LP in a cloned suspension of SP. In this study 100 cultures were infected with approximately 600 PFU of SP per culture. The suspension of SP used as the source of virus was prepared by infecting chicken embryo cell monolayers with 10 PFU per cell of thrice-cloned SP virus and harvesting virus at 12 h. The virus suspension titered approximately $5 \times 10^4$ PFU/ml. Only two LP were detected among the approximately 60,000 SP observed. It is noteworthy that this was the first time LP variants had been observed in cloned suspensions of the SP virus in spite of numerous plaque assays performed with this virus over a number of years.

Effect of high HCO$_3^-$-CO$_2$ concentration on detection of plaque progeny. Rapid inactivation of virus progeny in togavirus plaques by the photodynamic effects of NR has been discussed by Yukihiisa and Prince (8). Preliminary studies had shown that plaques could be seen and picked sooner in a high HCO$_3^-$-CO$_2$ environment, thereby reducing the time of exposure to NR. To determine whether this reduced exposure would result in an increase in measurable plaque progeny, the centers of 10 isolated SP plaques that had developed in a high HCO$_3^-$-CO$_2$ environment and 10 that had developed in a low HCO$_3^-$-CO$_2$ environment were picked with micropipettes (1 mm diameter) from cultures infected 48 h previously. The plugs and underlying cells and medium were suspended in 2.0 ml of growth medium. After freezing and thawing, viral progeny were assayed in triplicate (Table 2). All manipulations were performed with minimal exposure of virus-
infected cultures or virus suspensions containing NR to visible light. Plaques were sufficiently developed for picking after exposure to NR for 1 h in the presence of an overlay medium containing 2 g of HCO$_3^-$ per liter, whereas a 3-h incubation was required for clear visualization of plaques for picking when 1 g of HCO$_3^-$ per ml was present in the overlay medium. In repeated assays, plaques were always visible after 1 h of incubation when the high HCO$_3^-$--CO$_2$ conditions were used, whereas the time required to develop plaques varied considerably (range 1 to 6 h) when the low HCO$_3^-$--CO$_2$ conditions were used. There was an approximate 10-fold increase in the numbers of progeny virus detectable in the more rapidly developed plaques (2.3 x 10$^4$ PFU per plaque versus 3.0 x 10$^3$).

Enhanced detection of plaque progeny by assay of agar extracts. Because the previously determined estimate of the number of plaque progeny per plaque seemed low, we tried to determine the mean number of SP PFU per plaque by assaying liquid extracts harvested from agar-overlayed cultures containing known numbers of plaques (43 per culture). The liquid extracts were obtained after freezing and thawing the agar. Not only was there a major increase in SP PFU per plaque detected by this method, but there was also a major increase over a 24-h period in detectable LP (Table 3).

**DISCUSSION**

The study of the genetics of plaque type changes of viruses are most easily done when plaquing methods are available that inhibit replication of the parental type (2, 5). In contrast, when such methods are not available, mutational studies are usually confined to viruses having mutation rates high enough to allow accurate counting of mutants in a reason-

**Table 2. Effects of high and low HCO$_3^-$--CO$_2$ concentrations on time of development and detection of progeny in Western encephalitis virus small plaques**

| No. of plaques | HCO$_3^-$ concentration in agar-overlay medium (g/liter) | Total CO$_2$ flow* into tanks after addition of NR (s) | pH of overlay medium | Time to visualize plaques for picking after addition of NR (h) | Average PFU virus per plaque |
|----------------|--------------------------------------------------------|------------------------------------------------------|----------------------|----------------------------------------------------------------|-----------------------------|
| 10             | 2                                                      | 80                                                   | 6.6–6.8              | 1                                                                | 2.3 x 10$^4$                |
| 10             | 1                                                      | 20                                                   | 7.0–7.4              | 3                                                                | 3.0 x 10$^4$                |

*1.6 liters/min.

**Table 3. Detection of plaque progeny in liquid extracts harvested from agar following freezing and thawing**

| Incubation time (h) | Total detectable SP per culture (PFU) | PFU of SP per plaque$^a$ | Total detectable LP per culture (PFU) | PFU of LP per plaque |
|---------------------|--------------------------------------|--------------------------|---------------------------------------|---------------------|
| 24                  | 1.37 x 10$^4$                        | 3.1 x 10$^4$             | 0                                     | 0                   |
| 48                  | 3.33 x 10$^4$                        | 7.74 x 10$^4$            | 5.5 x 10$^4$                          | 1.28 x 10$^4$       |

*43 SP plaques per culture.

Available number of cultures in the presence of parent-type plaques and/or to the use of techniques that allow detection of clones not containing the mutant (1).

In preliminary studies to detect SP → LP mutations with WEE virus, we found that whenever LP variants were plated with NR in the presence of relatively large numbers of SP, normal LP plaque development was often inhibited or obscured by interference or development of cytotoxicity, or both. Furthermore, the NR used to develop plaques often appeared to enhance cytotoxicity in crowded cultures and increased the difficulty of distinguishing plaque types.

Since the development of cytotoxicity appeared to be related to the low pH of the cultures (especially cultures heavily infected with virus) at the time the plaques were developed, the bicarbonate concentration in the agar-overlay medium was raised until a more alkaline pH could be maintained. The plaques, however, developed more slowly under the more alkaline conditions, and rapid cell lysis often occurred when cultures were taken out of their CO$_2$ environment for observation. This toxicity appeared from microscopic examination to be related to the formation of crystals of NR in and around the surface of the cell monolayers. Therefore the concentration of CO$_2$ was doubled for a short time to lower the pH while NR was present on the cells. When this was done, NR became more soluble and diffused down and penetrated cells more rapidly. More importantly, with these conditions more of the NR was taken up by the cells and less was available for crystallization, and subsequent cell lysis did not occur, even when the cultures returned to an alkaline state. The enhanced resolution of plaques and absence of cytotoxicity in crowded cultures resulting from the use of this technique are shown in Fig. 1. Two LP were detected and cloned from this culture, which contained approximately 2,200 SP. Before the use of higher
HCO$_3^-$-CO$_2$ concentrations in the environment while NR was present in the cultures, no more than 200 to 300 could be present in single cultures without initiating areas of general cell lysis. The fact that only 3 out of an expected 20 LP were detected in this experiment indicates a significant loss of plating efficiency of the LP when plated in the presence of large numbers of SP.

To determine the relative plating efficiency of the LP in the presence of large numbers of SP, cultures were infected with small numbers of LP and varying numbers of SP. The results showed that 800 or fewer SP could be present without significantly decreasing the detectability of 15 or fewer LP (Table 1).

We then tried to detect LP variants in a cloned suspension of the SP by infecting 100 cultures with approximately 600 PFU per culture. Only 2 LP were detected among the 60,000 SP observed. This was, however, the first time that LP variants were observed in cloned suspensions of the SP. This finding was in marked contrast to a cloned SP variant of Sindbis virus, also used in this laboratory, that routinely gives rise to LP variants.

The fact that LP variants of WEE virus had never been previously observed and are apparently present in such low concentrations in cloned SP suspensions suggests that the SP → LP mutation rate of this virus is very low and that techniques requiring calculation of mutation rates by enumeration of mutants in clones would not be feasible for studying this particular mutational event.

Based on these studies, another, possibly more fruitful approach for determining mutation rates of plaque size determinants of this virus could be to pick plaques (subclones) and assay for total progeny and the presence or absence of mutants. Slobodà et al. (4) showed that plaque progeny could be used as real subclones and that statistical analysis for inhomogeneity with the Chi-square test and variance with the F test gave significant results for a clonal distribution of mutants in plaques. The main problems associated with this technique, especially when used with labile lipid enveloped viruses, such as WEE virus, are accurate determinations of the total plaque progeny and the detection of mutants. Concerning the former, our studies (Table 2) have shown that the number of detectable plaque progeny could be increased approximately 10-fold (3.0 × 10$^3$ PFU per plaque to 2.3 × 10$^4$) by picking plaques developed (for 1 h) in a high HCO$_3^-$-CO$_2$ environment.

However, because this figure (2.3 × 10$^4$ PFU per plaque) still seemed unreasonably low, extractions and assays of virus present in agar-overlayed cultures containing a known number of plaques were performed. The results (Table 3) indicated that considerably more SP virus was present per plaque than could be detected by picking and assaying progeny (7.74 × 10$^4$ versus 2.3 × 10$^4$). In addition, many LP variants were detected that increased dramatically over a 24-h period. This finding, which is apparently due to the selective action of agar for the LP, suggests that SP plaques in which SP → LP mutations occur contain on the average much more LP than previously expected. Proper sampling of plaques should therefore enhance the chances of detecting mixed SP and LP plaques and thereby allow more accurate determinations of the numbers of clones that do not contain the LP mutant. Theoretically, therefore, the determination of mutation rates by the technique of picking plaques and determining the number of negative subclones in a system where the mutant population is enhanced would function opposite those where the parent type virus is inhibited. In this regard, Breeze and Subak-Sharpe (1) found little difference in the mutation rates of EMC/r$^*$ → EMC/r$^-$ determinants whether they used techniques that relied on accurate determinations of the clone size and number of mutants, or clone size and the number of negative clones.

In conclusion, the results of these studies suggest that mutational studies involving virus determinants that cannot be selectively inhibited may, even under optimal conditions, be impractical for study by classical methods; however, the opportunistic finding that certain LP plaque mutants may be selected during the growth of SP plaques under agar may eventually allow more accurate calculations of SP → LP mutation rates based on the enhanced capabilities of detecting the number of negative subclones (plaques that do not contain the mutant) and more accurate determinations of the number of progeny per plaque.

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**LITERATURE CITED**

1. Breeze, D. C., and H. Subak-Sharpe. 1967. The mutability of small-plaque-forming encephalomyocarditis virus. J. Gen. Virol. 1:81-88.
2. Dulbecco, R., and M. Vogt. 1968. Study of the mutability of d lines of polioviruses. Virology 32:220-235.
3. Luria, S. E., and M. Delbrück. 1943. Mutations of bacteria...
from virus sensitivity to virus resistance. Genetics 28:491–511.

4. Slobodă, E., E. Buimovici-Klein, and R. Klein. 1969. Value of the fluctuation test for establishing the origin of agar-resistant L particles emerging in S subclones of ECHO virus 19. Arch. Gesamte Virusforsch. 28:1–6.

5. Takemori, N., and S. Nomura. 1960. Mutation of polioviruses with respect to size of plaque. II. Reverse mutation of minute plaque mutant. Virology 12:171–184.

6. Ushijima, R. N., D. W. Hill, G. H. Dolana, and L. P. Gebhardt. 1962. Plaque mutants of WEE virus. Virology 17:356–357.

7. Welsh, H. H., B. J. Neff, and E. H. Lennette. 1958. Isolation and identification of Western equine encephalomyelitis virus from mosquitoes by tissue culture methods. Amer. J. Trop. Med. Hyg. 7:187–196.

8. Yukihisa, T., and A. M. Prince. 1963. Photodynamic inactivation of arbor viruses by neutral red and visible light. Proc. Soc. Exp. Biol. Med. 112:887–890.