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Plaque Size Heterogeneity: a Genetic Trait of Lymphocytic Choriomeningitis Virus

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All of the ten strains of lymphocytic choriomeningitis virus assayed on BHK 21/13S cells showed various degrees of plaque size heterogeneity. The amount of virus released from these plaques was usually very small because of rapid photodynamic inactivation by neutral red. When virus from large and small plaques of a specific strain was plated, the same distribution of plaque size was obtained from each clone. Although it was shown that surface virus could possibly be randomly distributed at the time of addition of neutral red overlays, no virus could be isolated from nonplaque areas. Two different strains of virus (CA1371 and WE) with markedly different plaque size ranges were separated by plaque excision from plates infected with a mixture of both viruses.

Although several plaquing systems for lymphocytic choriomeningitis (LCM) virus have been reported (16), the recent BHK 21/13S agarose suspension assay employed by Sedwick and Wiktor (18) has been the only one found to be reproducible in our laboratory. With the standard use of a plaque assay, the need for cloned strains of virus in genetic and biochemical work is obvious. Although usually routine, the experiments demonstrating that cloning can be achieved with LCM virus are presented here for the following reasons: the virus is unusually sensitive to inactivation by neutral red in the presence or absence of light, and the degree of plaque size heterogeneity appears to be a heritable property of various strains of virus.

MATERIALS AND METHODS

Viruses. The origin and passage histories of the LCM strains are given in Table 1.

Cell culture. BHK 21/13S cells from T. J. Wiktor were received at the 18th-passage level. Cells were grown in monolayers (for no more than 15 passages) or in suspension. The BHK 21 monolayer medium was that used by Vahari et al. (22). Propagation techniques with Blake bottles were essentially as described by Sedwick and Wiktor (18). Cells were adapted for suspension by the following technique. Monolayers were dispersed with ethylenediaminetetraacetic acid (EDTA; 14), and these cells were used to form another monolayer which was again similarly dispersed. The cells treated in this way were then grown in suspension using minimal essential medium (5) with 2× vitamins and amino acids minus Ca and Mg, and supplemented with 0.1 mg of $FeNO_3 \cdot 9H_2O$ per liter, 5.5 g of D-glucose per liter, 20% tryptose phosphate broth, and 10% heat inactivated (56 C for 30 min) fetal calf serum. Cells were then taken from cultures at concentrations between 10^6 and 4.0×10^6/ml for use in the plaque assay. The log phase of growth contained between 0.2×10^6 and 10^6 cells/ml, having a division time of approximately 12 hr. Cultures were always split to between 10^5 and 8.0×10^5 cells/ml with no media supplement until a density of 4×10^6 cells/ml was reached. The heat-inactivated sera used in both types of growth media had to be screened before use since some lots completely inhibited plaque formation.

Measurement of infectious virus. The LD_{50} assay, with Twin Oak Farms Swiss mice, has been described (16). The plaque assay was that used by Sedwick and Wiktor (18), with the following modifications: plates were incubated in a 2.5% CO_2 atmosphere with 0.1% sodium bicarbonate the final concentration in the agarose-overlay medium. Instead of mixing virus dilutions with cell suspensions prior to the addition of agarose, the cell-agarose over-layer was poured and allowed to harden, and then 0.1 ml of the virus dilution was pipetted directly onto the surface (T. J. Wiktor, *unpublished data*). In addition to being equally as sensitive as the original, this modified technique provided excellent cell viability and enabled us to maintain the plates as long as 4 days before infection. Plaque counts on strains WE, CA1371, and G-45 were virtually identical whether plates were aged 1 or 4 days at the time of infection. All LCM stocks exhibited a linear relation between plaque number (the range examined was 0 to 100) and relative virus concentration. When plated in triplicate, dilutions of G-45, CA1371, or WE showed a standard error of no more than 5%. Falcon tissue culture (no. 3002) or bacteriology (no. 1007) grade dishes were used. The bacteriology grade dishes, known to be

TABLE 1. *Characteristics of ten LCM strains*

Virus	Origin and primary literature citation	Passage history[a]	PFU/LD_{50}[b]
Armstrong	American Type Culture Collection, Rockville, Md. (1)	Mk_6, M/B_{206}–L_2	1:6
CA1371	W. P. Rowe, Bethesda, Md. (17)	M/B_4–L_{12}	1:12
Fortner	W. P. Rowe, Bethesda, Md. (10)	M/B_7–None	1:2
G-45	T. J. Wiktor, Philadelphia, Pa. (25)	$HDCS_{26}$, $BHK21/13S_1$, $HDCS_2$, $BHK21_1$–None	1:1
M-7	R. W. Sidwell, Birmingham, Ala. (19)	M/B_3–L_4	1:8
Traub	M. Volkert, Copenhagen, Denmark (23)	M/S_7–L_8	1:19
UBC	J. Hotchin, Albany, N.Y. (7)	M/B_6L_{11}–L_2	1:6
WCP	W. P. Rowe, Bethesda, Md. (17)	M/B_1KLS_4–L_{30}	1:23
WE	B. E. Kirk, Morgantown, W.Va. (12)	G/B_1–L_4	1:6
WE_3	F. Lehmann-Grube, Giessen, BRD (8)	M/B_3–L_1	1:6

[a] Passage history notations are those used by Hotchin and Benson (6). Passage to the left of the dash is that in the laboratory furnishing the strain; to the right is that in our laboratory. Abbreviations are B, brain; G, guinea pig; HDCS, human diploid cell strain; K, kidney; L, L cells; Li, liver; M, mouse; Mk, monkey; and S, spleen.

[b] PFU/LD_{50} ratios were determined by simultaneous use of a dilution series for injection into animals (6 mice/dilution) and infection of duplicate assay plates.

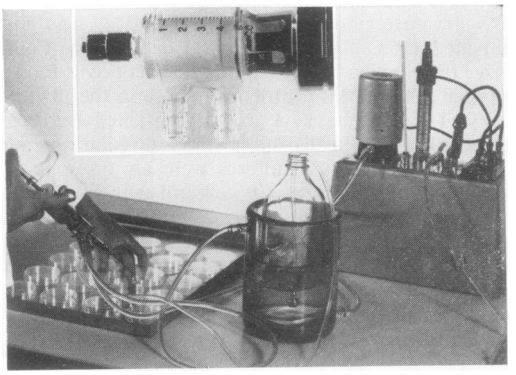

FIG. 1. *Apparatus for pouring BHK 21/13S agarose suspension plates. A constant temperature circulating pump (Haake) was connected in series to a double-walled glass beaker and a jacketed continuous pipetting syringe (Cornwall). Water temperature was maintained at 42 C. The double arm sleeve for the syringe (insert) was closed at both ends by a heat-resistant, fast-drying resin (5 min Epoxy, Devcon Corp., Danvers, Mass.).*

acceptable for cell-suspension assays (4), were found to lower the efficiency of plating of some LCM strains by about 30%. The efficiency of pouring plates and their uniformity were greatly increased by use of the apparatus shown in Fig. 1. In this way, 200 plates could be poured in 20 min. Further procedural aspects of the plaque assay may be found elsewhere (A. J. Pulkkinen, M.S. Thesis, Univ. of Massachusetts, Amherst, 1969).

Neutral red inactivation of virus. Liquid medium: virus-containing tissue culture fluid, with or without neutral red (0.1 g/liter), was incubated at 37 C for 2 hr. The glass tubes containing the virus were exposed to fluorescent light or covered by aluminum foil. Neutral red addition, dilution of virus samples, and plating were carried out in a dark room, the only source of illumination being overhead indirect ultraviolet lights. Semi-solid medium: duplicate BHK 21/13S agar plates were infected with a countable dilution of virus. After 4 days, one of the plates was stained with neutral red in the usual manner (18) At 4 hr, the cell layer from both the stained and unstained plates was removed and placed in polycarbonate tubes containing 1 ml of BHK monolayer medium. Samples were sonic treated for 30 sec under previously described conditions (15).

Release of virus from plaque areas. Agarose cylinders (1.0 mm inner diameter) were exised from plaque areas with sterile Pasteur pipettes. This was followed by vigorous pipetting in 1 ml of BHK monolayer medium, prior to sonic treatment for 30 sec (15).

RESULTS

Dependence of plaque size on virus strain and method of cell propagation. When plates were prepared with 0.1% bicarbonate, all tested LCM strains (Table 1) plaqued. The size of the plaques depended not only on the cultivation history of the cells, but also on the strain of virus. The most striking examples of the former factor are shown in Fig. 2. With strains G-45 and WCP, both large and small plaques were apparent when suspension-grown (S) cells were used for the assay, whereas monolayer-grown (M) cells produced much smaller and less distinct plaques. The virus strains shown in Fig. 3 exhibited the same differences but to a lesser degree. The size of Traub plaques was nearly the same on both types of

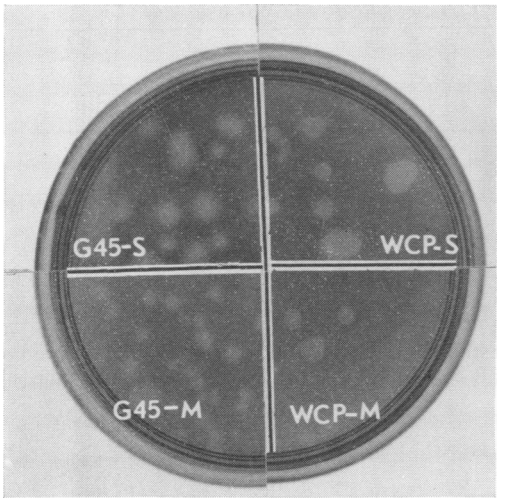

FIG. 2. *G-45 (right side) and WCP (left side) strains of LCM 96 hr postinfection. The cells used for the assays in the upper half of the picture were grown in suspension(S), whereas those in the lower half were grown in monolayers (M).*

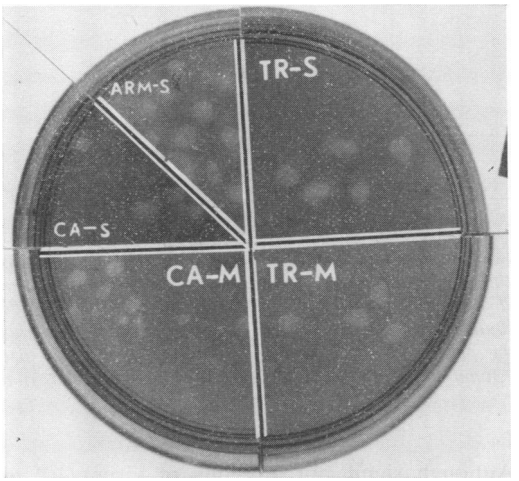

FIG. 3. *Armstrong (Arm.), CA1371 (CA), and Traub (Tr) strains of LCM 96 hr postinfection. The cells used for the assay in the upper half of the picture were grown in suspension (S), whereas those in the lower half were grown in monolayers (M).*

cells, but the plaques on M cells had more sharply defined edges. With strain CA1371, the plaque size gradient was larger on S than on M cells. The strains shown in Fig. 4 (FO, M-7, WE, WE_3, and UBC) were not strikingly dependent on the method of culture and exhibited a plaque size gradient from 1 to 7 mm.

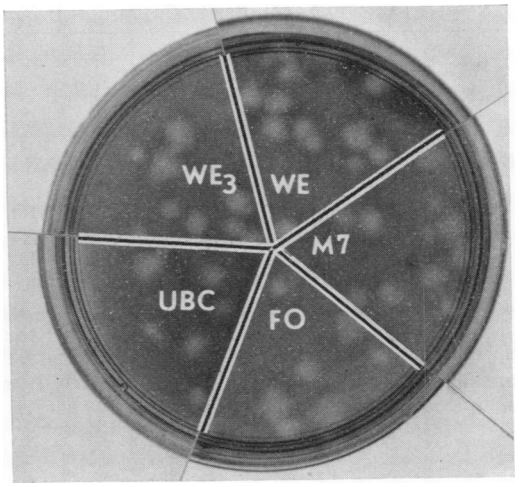

FIG. 4. *WE_3, WE, M-7, Fortner (Fo), and UBC strains of LCM 96 hr postinfection. All cells used for the assays were grown in suspension.*

Neutral red sensitivity of LCM. Since the amount of virus recoverable from excised plaques seemed unususally low, 6×10^2 to 60×10^2 plaque-forming units (PFU) depending on the strain of virus used, the effect of neutral red on the system was examined. Plates were exposed to a dilution of WE virus that would yield about 40 plaques. After the usual 4-day incubation period, one plate was stained with neutral red. All plates were incubated for an additional 4 hr at 37 C. The cell layer (plus neutral red layer) was stripped from the plates, sonic treated in liquid medium, and assayed. A typical result was that sonic-treated material of cells never exposed to neutral red yielded 3×10^6 PFU whereas those overlayed with the dye contained 1.2×10^4 PFU. No attempt was made to control the amount of illumination after neutral red addition. This neutral red sensitivity was then examined by using supernatants from L cell monolayers infected with either CA1371 or WE strain of virus. The results (Table 2) show that both strains of virus were inactivated in the presence of neutral red after incubation in the light and in the dark. In the dark with neutral red, WE-LCM lost 80% of its infectivity, whereas the CA1371 strain lost 99%. This sensitivity to neutral red was magnified greatly in the light; no infectivity was detectable in either strain at the end of the incubation period. It could be argued that inactivation of LCM by neutral red minus light was due to photodynamic inactivation during dilution or incubation of the assay plates. To rule this out, the kinetics of inactivation of the WE strain were followed. A

TABLE 2. *Exposure of LCM strains WE and CA1371 to neutral red (NR) and light (L)*[a]

Determination	Exposure	PFU/ml	
		WE	CA1371
Before incubation	(+L−NR)	4.2×10^7	6.5×10^5
After incubation	(+L−NR)	3.0×10^7	1.3×10^5
	(−L+NR)	8.0×10^6	4.0×10^3
	(−L−NR)	6.2×10^7	6.9×10^5
	(+L+NR)	<10	<10

[a] Series of tubes received 1 ml each of either the CA1371 or WE strains of LCM and were held at 4C until neutral red addition. A 0.01 ml amount of a 1:100 stock of neutral red was added to foil-covered or unshielded tubes and immediately transferred (with all controls) to a 37 C incubator. After 2 hr, all samples were assayed.

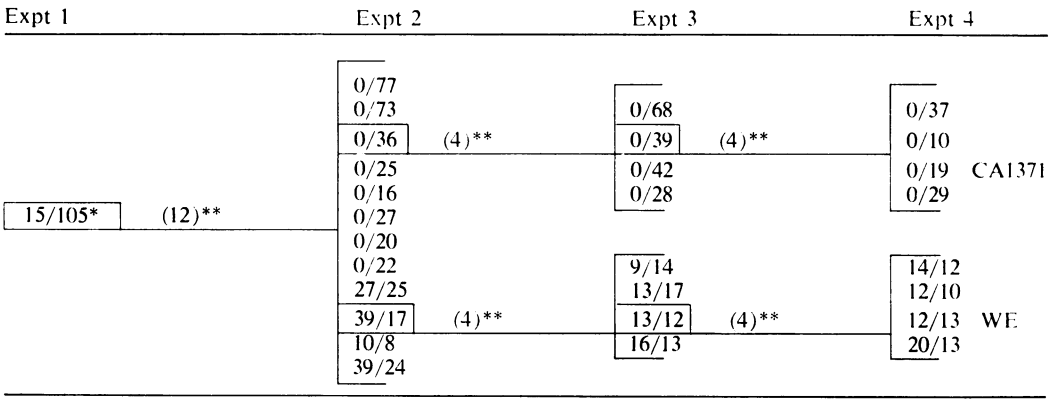

FIG. 5. *Flow diagram for strain identification in mixed infection. A plate was simultaneously infected with CA1371 and WE strains of LCM, the input ratio being approximately 2:1. Four days later, the plaque count was 15/105 (experiment 1). Cylinders were withdrawn from the centers of 12 small plaques on the above plate. Each cylinder, after being sonic treated and assayed, produced either small plaques or a wide gradient in plaque size (experiment 2). Agarose cylinders were again excised from both type of plates and repeated assays (experiments 3 and 4) showed that the plaque characteristics were stable. (*) Plaque size distribution on plate (no. >3 mm/no. <3 mm); (**) number of plaques <3 mm picked and plated at a countable dilution.*

gradual drop in infectivity was found over a 3-hr test period (unopened specimens of virus were sampled at 1, 2, and 3 hr intervals). Furthermore, the degree of plaque size heterogeneity was found to remain constant as the inactivation progressed.

Separation of the CA1371 and WE strains of LCM by cloning. Agarose cylinders from WE-infected plates were withdrawn from both large and small plaque areas, sonic treated, and re-plated. Virus liberated from either type of plaque did not breed true, i.e. heterogeneity in plaque size was still apparent. The same result was obtained whether an agarose cylinder was withdrawn from a plate containing only one plaque or from a plate containing as many as 50 plaques. Although significant amounts of virus (10^4 to 3×10^4 PFU) could be washed off the surface of a plate prior to neutral red addition (introducing the possibility of random distribution of virus on the surface of a plate after neutral red addition), no PFU could be detected in nonplaque areas. Thus, to prove that cloning was possible, two easily distinguishable strains of LCM were used to infect simultaneously a plate. Although the CA1371 strain produced predominantly small plaques (in the 1 to 2 mm size range), the WE strain produced a gradient of 1 to 7 mm in plaque size. Figure 5 shows that when small plaques were picked from the above type of plate, sonic treated, and assayed, the resulting plates contained either

predominantly small plaques (characteristic of CA1371) or a large gradient in plaque size (typical of WE). Two more cycles of picking showed that the plaque characteristics separated from the initial plate persisted through successive cycles of viral replication.

Growth of virus stocks from excised plaques. Neutral red overlays were added in the dark to assay plates, and incubation was continued for no more than 2 hr. Virus was liberated from the plaque areas as described previously, except that all operations but the plaque excision were carried out in the dark. L cell monolayers in 25 cm^2 foil-covered plastic flasks were infected as previously described (16), and titers at 48 and 72 hr were comparable to routinely obtained virus stocks. Thus, any effect of neutral red in the inoculum was of minimal importance in the infection process.

DISCUSSION

Strains of LCM differ from one another in tissue culture with respect to growth curves (16), stability (16), sensitivity to various drugs (2, 16), character of persistent infections (9), and cell specificity (24). Further similarities and differences based on plaque size may now be considered. Three strains (WE, WE_3, and UBC), with similar plaque sizes and PFU/LD_{50} ratios, have a common origin (Table 1). Although the Fortner strain is similar in plaque size to the above three, it is an independent isolate and differs significantly with respect to the PFU/LD_{50} ratio. Parke, Davis & Co. obtained the Armstrong strain of LCM from the American Type Culture Collection in 1950 (F. A. Miller, *personal communication*), and this has recently been called the M-7 strain (Table 1). However, the plaque type of M-7 more closely resembles the WE-origin strains than our recently acquired Armstrong strain. Both the CA1371 and Armstrong strains originated in the laboratory of the late Charles Armstrong (Table 1; W. P. Rowe, *personal communication*). They are quite similar in plaque size but differ somewhat in the PFU/LD_{50} ratio. WCP and Traub plaques are most difficult to see, the difference in intensity of staining between background and plaque areas being very slight. Their inactivation kinetics are virtually identical (3), and their PFU/LD_{50} ratios (1:23 and 1:19) are quite apart from those of any other strains tested. Both the Traub (W) and WCP strains were originally obtained from the same source, yet Traub (21) and Camyre and Pfau (3) have found their pathogenicity in mice to be markedly different. Although it has been repeatedly shown that virus-passage history can affect plaque size (20), we have not observed a similar pattern in the LCM system. The method of propagating cells, however, has a marked effect on the plaque characteristics of certain LCM strains. We have not investigated whether this is due simply to the difference in media or to the general condition of the cell in monolayer versus suspension culture.

Neutral red-inactivation kinetics are greater with the CA1371 than with the WE strain of LCM. A difference in sensitivity to neutral red has been noted among strains of poliovirus (E. M. Opton and R. H. Green, Fed. Proc. **19:** 408). Stains other than neutral red were used in the present system to try to eliminate the strong photodynamic inactivation of the virus. These included 2,3,5-triphenyl-2H-tetrazolium chloride, tetrazolium blue (diformazan), alcian blue, and resazurin. Under the conditions we used, none of the dyes were concentrated by the cells. Decreasing the concentration of neutral red was also considered, but a dilution factor of two made plaques almost impossible to see.

The plaque characteristics of each LCM strain were found to be reproducible using either monolayer- or suspension-grown BHK 21/13S cells, respectively. The same characteristics were apparent after passage in carrier mice. The failure to separate genetically stable large and small plaque-forming virions from the WE strain did not seem to be due to cross contamination of the excised plaques. Had this been the case, it would have been impossible to separate the CA1371 from the WE strain in the mixed infection experiment (Fig. 5). Plaque size heterogeneity which cannot be altered by cloning has been noted with other viruses (11, 13). In the present system, it appeared that excised plaques contained only titratable virus originating in that area. Furthermore, the linear relation between plaque number and relative virus concentration indicated that virus particles in the plaque area were the progeny of virus originally released from a single cell. Sonic treatment, which breaks up aggregates of the WCP strain (15), did not alter the plaque characteristics of the WE strain. This seemed to rule out the possibility that clumping of virus particles ultimately led to plaque size heterogeneity. If the above assumptions are correct, the degree of plaque size heterogeneity is a genetic trait.

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