incorporated into the overlay. Furthermore, if Agarose was substituted for Ionagar, large plaques also could be obtained. Other workers (4–9) have shown that DEAE dextran is essential for plaque production with certain rhinoviruses when ordinary agar is used in the overlay. Thus, present knowledge indicates that the conditions required for rhinovirus plaque formation include a sensitive cell system such as the HeLa cells used in this study, and an inhibitor free overlay medium. Additives, such as $MgCl_2$, DEAE dextran, or protamine sulfate can be used to facilitate plaque formation.

Summary. A reliable plaque assay is described for 60 rhinovirus strains representing 55 distinct serotypes and one subtype. Using HeLa cell bottle cultures, plaques were produced under starch gel and Ionagar overlay media. Employing the methodology described in this report, certain basic aspects of rhinovirus plaque formation were investigated.

1. Collaborative Report on Rhinoviruses, Nature **213**, 761 (1967).
2. Parsons, R. and Tyrrell, D. A. J., Nature **189**, 640 (1961).
3. Porterfield, J. S., Nature **194**, 1044 (1962).
4. Webb, P. A., Johnson, K. M., and Mufson, M. A., Proc. Soc. Exptl. Biol. Med. **116**, 845 (1964).
5. Behbehani, A. M. and Lee, L. H., J. Bacteriol. **88**, 1608 (1964).
6. Dans, P. E., Forsyth, B. R., and Chanock, R. M., J. Bacteriol. **91**, 1605 (1966).
7. Haff, R. F., Wohlsen, B., Force, E. E., and Stewart, R. C., J. Bacteriol. **91**, 2339 (1966).
8. Fiala, M. and Kenny, G. E., J. Bacteriol. **92**, 1710 (1966).
9. Abo-Ahmed, H. S., Behbehani, A. M., and Wenner, H. A., Proc. Soc. Exptl. Biol. Med. **126**, 463 (1967).
10. Conant, R. M. and Hamparian, V. V., J. Immunol. **100**, 107 (1968).
11. De Maeyer, E. and Schonne, E., Virology **24**, 13 (1964).
12. Lehane, D. E., Jr., Clark, H. F., and Karzon, D. T., Proc. Soc. Exptl. Biol. Med. **125**, 50 (1967).

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Unusually Large-Plaque Mutant of Sindbis Virus (32942)

SADAKO TANIGUCHI,[1] HISAYO TAKEUCHI, AND KAMESABURO YOSHINO[1]
Department of Bacteriology, Yokohama City University School of Medicine, Minamiku, Yokohama, Japan

In the course of a study on the plaque formation of various viruses at elevated temperatures, we noticed that Sindbis virus plaques formed at 40°C in primary chick embryo fibroblasts (CEF) revealed a pleomorphism, small plaques of various sizes mixing with the parent-type plaques. Then, it was attempted to separate the small-plaque clones by plaque purification. Thereby, a few of the small-plaque clones yielded progenies producing unusually large plaques. Analyses of some properties of this mutant have been done in comparison with the parent type and small-plaque type clones.

Materials and Methods. Virus. Egypt Ar339 strain of Sindbis virus was supplied by Dr. T. Okuno, the National Institute of Health, Tokyo, as a lyophilized suspension of infected baby mouse brains. It was passaged through CEF bottle cultures 61 times before starting the present experiments. The diluent for virus was 0.1% yolk-saline (1).

Plaque technique. The technique of plaque titration was essentially the same as described in a previous report (1). The CEF monolayer sheets formed were washed once with phosphate buffered saline (PBS) (2) and inoculated with serial decimal dilutions of Sindbis virus in the amount of 0.05 ml per dish. After an incubation at 37°C for 1 hour, the dishes were overlaid with an agar medium which contained 2% calf serum and 0.0025 M Tris buffer of pH 7.2. The dishes were then

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[1] Present address: Department of Virology, the Institute of Medical Science, the University of Tokyo, Minato-ku, Tokyo, Japan.

incubated either at 37°C or at 40°C, and 3 days later a second overlay with neutral red agar was made. After a further incubation at 20°C for 1 day plaques were counted.

CEF bottle culture. This was also essentially the same as described earlier (1), except that bottles measuring 4 × 7 × 2.5 cm were sown each with 5 ml of growth medium containing 5×10^6 cells. After 1 day's incubation at 37°C, the cell sheet was washed with PBS and received 0.5 ml of virus. After adsorption at 37°C for 1 hour, the inoculum was washed off with PBS, and 5 ml of maintenance medium was added which contained 2% calf serum and 0.0025 M Tris buffer of pH 7.2. Subsequent incubation was made at 37°C. In growth curve experiments, residual unadsorbed virus was removed by antiserum which was subsequently washed off with PBS before the addition of maintenance medium. For routine passage of virus, the inoculum was an appropriate dilution of the previous passage fluid which contained more than 1000 pfu (plaque forming units) of virus. Calculation of MOI (multiplicity of infection) was done as stated previously (1). At each passage, fluids were harvested after 1–2 days' incubation when the cytopathic effect was distinct, and stored at –20°C. Little degradation of virus occurred at this temperature within a month.

Plaque purification. Limiting dilutions of a seed virus were inoculated to a number of CEF dishes, and dishes forming one plaque were selected. Each plaque was rinsed with 2 ml of diluent, and the rinse was put in CEF bottle cultures. Subsequent steps were the same as just described.

Results. Separation of small-plaque (sp) and unusually large-plaque (u) mutants from the parent large-plaque (lp) virus. The CEF monolayers inoculated with Sindbis virus were overlaid and incubated at 37°C and 40°C. Plaques formed at 37°C were mostly uniform in size (av diameter approximately 4 mm) while those formed at 40°C showed varying sizes from 1 to 3 mm. It was arbitrarily decided to call plaques larger than 2 mm as lp and smaller ones as sp. In 4 experiments, a total of 51 sp plaques were subjected to cloning. Of these, 24 produced progenies which formed small plaques at 40° C, while 24 others were of the parent lp type. The remaining three clones demonstrated an unexpected result that after one passage in CEF bottle culture a majority of the progeny virus formed plaques as large at 6–10 mm even at 40°C. This unusually large-plaque (u) mutant as well as the parent lp and sp viruses were clone-purified.

In the case of u mutant, a small portion of virus population was occupied by sp type virus even after repeated clonings. In contrast, purification of the lp and sp clones was successful after one or two clonings. After 3 consecutive clonings, they were subjected to passages in CEF bottle cultures in the routine passage manner. Plaques formed by these viruses at 37°C and 40°C are illustrated in Fig. 1. Identification of these viruses was done by a neutralization test with an antiserum which was produced by immunizing a rabbit with baby mouse brains infected with the lp clone. All the three viruses were neutralized to a similar extent.

Mutual exclusion between the three clone viruses. After serial passages of the above clones, only the lp virus could be maintained well, showing maximum fluid virus titers of about 10^8 pfu/ml at each passage. The sp virus showed much lower yields, which became gradually lower after repeated passages. An interesting fact was that the u mutant was replaced completely by lp type virus when examined after several passages. When passage was repeated from the stock fluid of the earlier passage using the technique of plaque purification at each passage, the u mutant could now be maintained. An explanation to account for this phenomenon was that an incidental appearance of the lp type virus by back mutation might result in exclusion of the u mutant. To test this possibility, experiments were performed in which CEF bottle cultures were infected with various combinations of the clone viruses and subjected to serial passages. As indicated in Table I, when given together with the u mutant the lp virus tended to exclude it. To the contrary, when a large amount of the sp virus was given to cells with a small amount of the u mutant, the sp virus was replaced by the u virus after a few

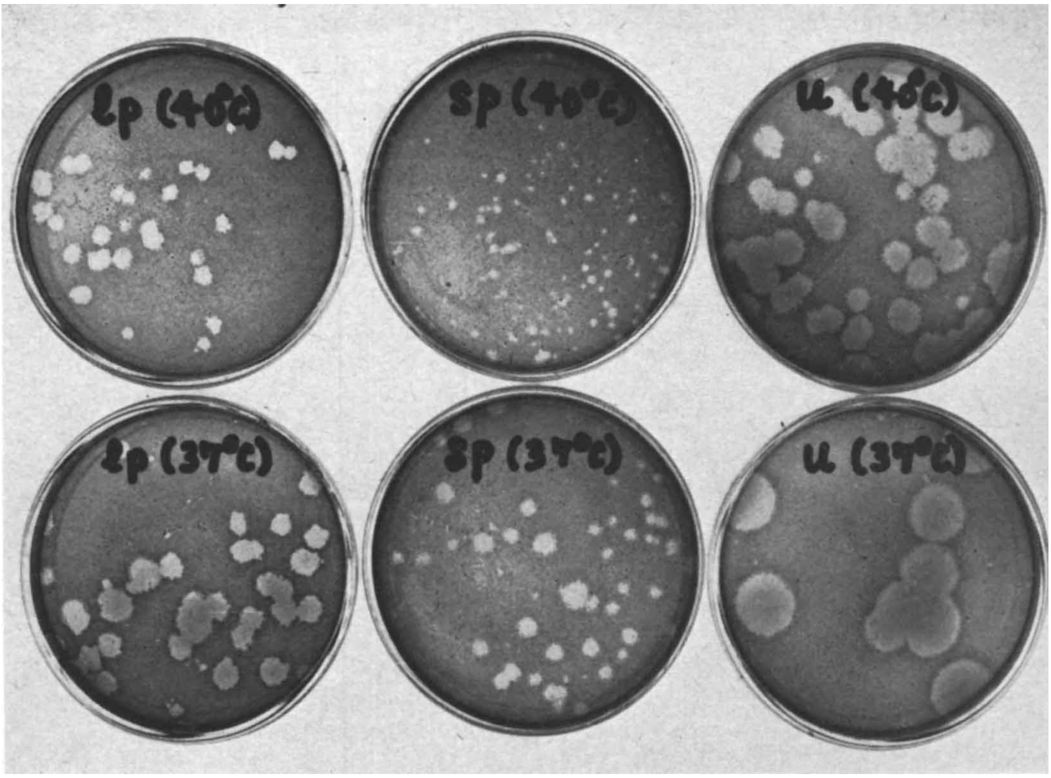

FIG. 1. Plaque formation by the lp, sp, and u clones of Sindbis virus in CEF monolayer dishes at 37 and 40°C. (The dishes were 60 mm in diameter.)

passages. These relations are expressed as

$$lp > u > sp,$$

where the left-hand virus can exclude the right-hand one.

The reason why the lp virus excluded the rapidly growing u mutant was then studied. The possibility that the per cell yield of the u mutant may have been lower was denied by the fact that growth curves of those viruses in CEF bottle cultures following infection at comparable MOI (ca. 5) testified a quicker rise and a higher peak of the u mutant.

Sensitivities to interferon of the three clones. The exclusion of the u mutant by the lp virus was then thought ascribable to difference in the sensitivity to interferon, and the

TABLE I. Analysis of Progenies after Mixed Infections of CEF with Two Different Types of Virus and Serial Passages Therefrom.

	Virus in starting inoculum (pfu/bottle)			Distribution of plaque types (%)					
				first pass.			third pass.		
Expt.	lp	sp	u	lp	sp	u	lp	sp	u
1	1.5×10^7	0	6.0×10^7	92	3	5	—[a]	—	—
	0	1.5×10^6	6.0×10^7	0	1	99	—	—	—
2	2.3×10^5	0	7.0×10^1	98	0	2	100	0	0
	0	2.8×10^6	7.0×10^1	0	97	3	0	0	100

[a] Not done.

TABLE II. Reduction of Plaque Sizes of the Three Clone Viruses under Overlay Containing Interferon.

	37°C			40°C		
Virus	Interferon (mm) (a)	Control (mm) (b)	Ratio (a:b)	Interferon (mm) (a)	Control (mm) (b)	Ratio (a:b)
lp	0.7[a]	3.7	0.2	0.7	2.2	0.3
sp	0	4.1	0	0	1.4	0
u	0.8	8.1	0.1	0	7.3	0

[a] Average of ten plaques randomly selected from 3 parallel dishes.

following experiment was carried out. An interferon preparation, produced from CEF bottle cultures infected with the parent Sindbis virus by means of dialysis against pH 2 buffer followed by dialysis against PBS, was diluted 1:4 with maintenance medium and given to CEF dishes. Control dishes received plain maintenance medium. After 24-hours' incubation at 37°C, the dishes were used for plaque titration of the three clone viruses. In the interferon-treated dishes, the plaque numbers of the lp and u mutant viruses were reduced to 8 and 2% of respective controls, while no visible plaques were formed in the interferon-treated dishes in the case of the sp virus. A clear difference between the lp and u viruses was observed in the plaque size rather than in the plaque number. For further confirmation of this point, the three viruses were inoculated to untreated CEF dishes and an overlay medium was added which contained the above interferon at 1:8. Control dishes were given interferon-free overlay medium. As can be seen in Table II, the sp and u viruses were proved to be more sensitive to interferon than the lp virus. The difference was especially marked when the dishes were incubated at 40°C

Discussion. The parent Sindbis virus maintained by our standard passage contained the sp and u mutants in addition to the parent lp type virus. The sp virus herein defined may be different from the conditional lethal mutants which cannot grow at 42°C (3). The u mutant has not been found before. The reason for this may be that, although the u mutant appears at a certain rate during the standard passage of the parent virus, it is excluded by the parent type virus due to its higher sensitivity to interferon. Here remains a question of why the sp virus, which is also sensitive to interferon, occupied a considerable portion of virus population of the standard passage seed. There may be the possibility that mutation from the parent type to the sp type occurs frequently. In fact, the lp clone, when examined at the sixteenth serial passage, included sp type progenies at 9.4%.

It is of interest that the u mutants were all isolated from sp plaques. Attempts thus far made failed to obtain a u type mutant from any of 32 lp plaques tested. Then, we considered that the first-step mutation is represented by the sp virus, which is more interferon-sensitive than the parent virus, and the u mutant is a daughter variant which does not stimulate interferon production in infected cells to such an extent as the parent lp or sp virus. However, when interferon production in CEF bottle cultures infected with these viruses was examined in the manner described earlier (1), the results obtained were quite irregular, failing to substantiate the above explanation. This was so even when the influences of subtle changes of cultural conditions upon interferon production (4–6) were taken into consideration. It may also be possible that some helper virus is responsible for the u type plaques, since such an effect of mumps virus is known (7). The true mechanism underlying the present phenomenon is being studied.

Summary. When Sindbis virus plaques were formed in chick embryo fibroblasts (CEF) at 40°C, plaques varying in size were observed. Plaques measuring less than 2 mm

(sp) were subjected to cloning. Out of 51 sp plaques so examined, three yielded predominantly, on passage through CEF, unusually large-plaque (u) mutants. This mutant as well as the parent large-plaque (lp) and sp viruses were purified by consecutive clonings. Mixed infection of CEF with the sp and u viruses tended to exclude the sp virus. Contrarily, mixed infection with the lp virus and the u mutant tended to exclude the latter. Thus for maintenance of the u mutant, frequent clonings had to be done, because otherwise an incidental appearance of the parent type virus by back mutation seemed to result in exlusion of the u mutant virus. The u mutant as well as the sp virus was more sensitive to interferon than the lp virus. The reason why the interferon-sensitive u mutant can grow more rapidly and yield a higher amount of virus than the parent lp virus remains to be elucidated.

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1. Yoshino, K., Taniguchi, S., and Arai, K., Proc. Soc. Exptl. Biol. Med. **123**, 387 (1966).

2. Dulbecco, R. and Vogt, M., J. Exptl. Med. **99**, 167 (1954).

3. Burge, B. W. and Pfefferkorn, E. R., Science **148**, 959 (1965).

4. Friedman, R. M., J. Bacteriol. **91**, 1224 (1966).

5. Carver, D. H. and Marcus, P. I., Virology **32**, 247 (1967).

6. Vilcek, J. and Lowy, D. R., Arch. Ges. Virusforsch. **21**, 253 (1967).

7. Frothingham, T. E., J. Immunol. **94**, 521 (1965).

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The Effect of Dimethyl Sulfoxide (DMSO) on Cellular Systems (32943)

CHUNG-YI CHANG[1,2] AND EDWARD SIMON (Introduced by H. Koffler)
Department of Biological Sciences, Purdue University, Lafayette, Indiana

Dimethyl sulfoxide (DMSO), a dipolar aprotic solvent with a high dielectric constant, differs from protic solvents such as water and alcohols because of its tendency to accept rather than donate protons. It has been used as a chemical solvent and a biological preservative (1,2), and also provides protection from radiation damage (3). It enhances the percutaneous penetration of several steroids (4), and the permeability of the frog skin to mannitol, urea, sodium, potassium, and chloride (5,6). However, it is possible that the effects are mainly caused by changes in osmotic activity of the bathing solution (5,6).

In this report the effects of DMSO at the cellular level are investigated. We found that DMSO inhibited the growth of L-cells, HeLa cells, *Escherichia coli*, Mengo virus, and bacteriophage T4. We could find no evidence that DMSO altered the permeability characteristics of any of these systems.

Materials and Methods. DMSO: spectroquality reagent from Matheson, Coleman and Bell was used. Maximum evaporative residue was 5 ppm.

Mammalian cells and tissue culture. HeLa strain S_3 and L-cell strain 929 were grown in a modified Eagle's medium custom-made by General Biochemicals with 20% horse serum (for HeLa) and 10% new born calf (for L-cells). The cells were maintained as monolayers on 60 × 15-mm plastic petri dishes (Falcon) and incubated at 37°C in a 5% CO_2 atmosphere.

Bacteria. Escherichia coli strain FW3A, Sm^r T^- L^- B^- M^- lac^- (y^-) was obtained from Dr. F. Neidhardt. *E. coli* strain P4X6, M^- L^- lac^+ and strain PA106, M^- lac^- were obtained from Dr. P. Margolin.

Virus. Mengo virus was obtained from Dr. R. Franklin, and passaged in L-cell monolayers in growth medium. The Beaudette strain of NDV was maintained by passage through embryonated eggs. The *r*II mutants *r*147 and

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