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SMALL PLAQUE MUTANTS OF THE SINDBIS VIRUS

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Cl. Hannoun, J. Asso
and P. Ardoin
(with the technical
assistance of
M. Bersot)

The plaque size of certain viruses is variable when the virus is grown according to the technique of Dulbecco or its variants. Since plaque size is a genetically controlled factor, it is a useful marker in the study of, for example, the poliomyelitis virus[10], Coxsackie[4], and vesiculary exanthema in swine[5]. Variability in this factor has also been observed in certain viruses transmitted by arthropods (Arbovirus).

Quersin-Thiry in 1961[7] described the existence of the two kinds of plaques produced by the WEE virus grown on chicken embryo cells. The two kinds of plaques are reflective of nutritional differences in variants of the virus: these differences include serum concentration, bicarbonate concentration, and certain growth factors. Ushijima et al.[9] in 1962 observed the appearance of plaques of varying size in the later-generation descendants of viruses from an ancestral large-plaque clone. They isolated two variants which produced, respectively, plaques of 2 mm and 8 mm diameter after 48 hours of incubation. These variants were identified by sero-neutralization as being of the WEE virus type. Similarly, Marshall et al.[6] in 1962 observed the production of two sizes of plaques in their stock of the WEE virus, the larger size being the less frequent. Their study showed that wild-type WEE virus will produce large plaques regularly on egg and less regularly on mouse. However, when the virus is grown on tissue culture cells, the small size plaques predominate. According to the authors of that study, this phenomenon is due not only to conditions of the media, but also to a process of natural selection on a variable population.

We observed a similar phenomenon with a strain of sindbis virus (another virus of Group A of Casals)[1] and we attempted to separate and identify these variants in order to ascertain if the properties of the virious that had produced these various types of plaques were transmitted to their descendants.

MATERIALS AND METHODS

1. VIRUS. The typical virus strain used (Sindbis Eg Ar 339) was obtained from the Rockefeller Institute, (Dr. Casals). It was isolated in 1952 by Taylor[8] from mosquitos from the Nile delta, and is the typical strain currently used by numerous laboratories for serologic examinations. The material on which we based our work, was the twelfth passage on mouse brain.

2. PLAQUE TECHNIQUE. The plaque technique used was as previously described by Hannoun and Panthier[3] with the following modifications: the embryonic extract was replaced one part per hundred with 100% concentrated vitamins for the Eagle's medium; 60ml pharmaceutical bottles were used; and the duration of incubation for the adsorption of the sindbis virus was one hour.

3. CLONES. Plaques from stock cultures were chosen from non-crowded areas in the stock culture. Samples from these plaques were taken with a Pasteur pipette curved at the end in such a manner that no contamination was encountered in the transfer of the sample to the culture media. Samples thus taken were suspended in 1 ml Hanks solution prior to re-inoculation on plaque media or transfer to normal culture media.

RESULTS

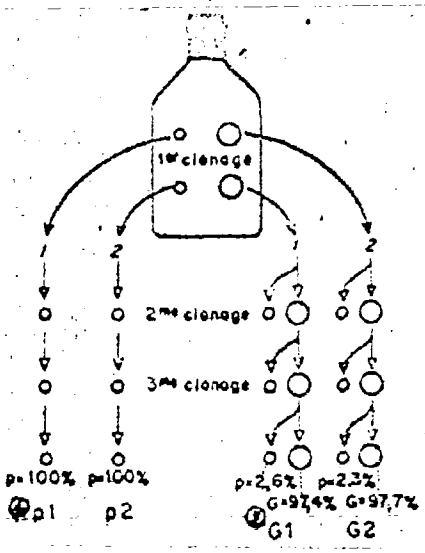
I. Appearance of Different Types of Plaques.

Upon the production of plaques with the Sindbis strain, the appearance of two kinds of plaques was observed. One type appeared after 24 hours and enlarged continuously until the diameter approached or exceeded 20 mm (figs. 3 and 4). The other type appeared more slowly (after 48 hours or more) and did not attain a diameter greater than 3mm (figs. 1 and 2).

II. Separation of Small and Large Plaques.

The re-inoculation of small plaques onto new plaque media, using the same method, gave rise only to small plaques (p), in which the virus has a descendance in which all plaques produced are small and very homogeneous in nature. To the contrary, the re-inoculation of the large

plaques onto new plaque media produced mainly large plaques, but also a small number of small plaques. Three successive re-inoculations were carried out for the descendants of each of two different plaques from the original stock culture, obtaining thus four sub-lines re-inoculated (cloned) three times: G_1 G_2, p_1 p_2 (fig. 1).



Legend:
1. 1st generation
2. 2nd generation
3. 3rd generation
4. p = small-plaque type
5. g = large plaque type

III. Characteristics of the sub-lines G and p.

The differentiating character between the two sub-lines is the evolution of the size of the plaques produced. This evolution is represented in fig. 2 which shows the growth of the two plaque types as a function of time: it was observed that the G (large) plaques grew rapidly over a long period of time, with a rather large variance in the final plaque size as seen after eight days. There appeared to be interference between closely-situated plaques, since these plaques generally gave rise to the smaller diameters in the range of diameters found for

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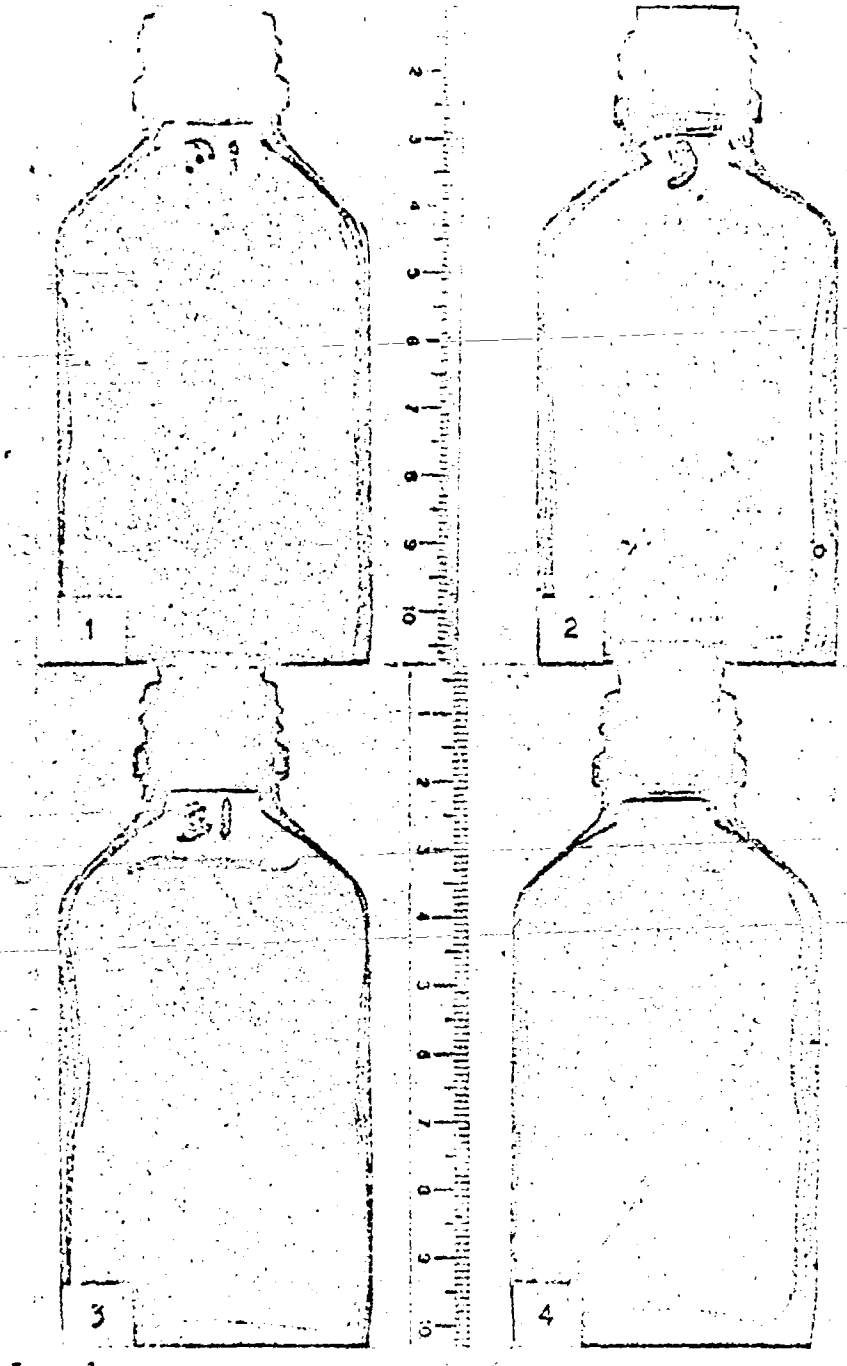
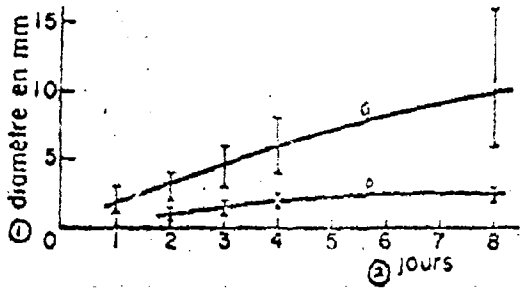


Legend:

1. Sub-line p_1. Plaques after 48 hours
2. Sub-line p_1. Plagues after five days
3. Sub-line g_1. Plagues after 48 hours
4. Sub-line g_1. Plagues after five days

the large plaque type. At times, closely-situated plaques appeared to merge completely. On the other hand, the p (small) plaques grew slowly for two days at most, and did not appear to grow after this period. The p plaques stabilized between 2mm and 3mm.

The G plaques were perfectly round with very crisp borders, the margin sometimes being slightly more colored than the neighboring cells. To the contrary, the p plaques did not have well-defined borders and had no definite shape. Their color was generally clear, with the general appearance of a halo.



Legend:

1. diameter in millimeters
2. days

IV. Proportions of Small and Large Plaques.

The percentage of small plaques depended on the method of preparation. Table 1 shows the actual numbers of G and p plaques in the original stock and in the sub-lines.

These results were obtained with incubation for one hour at 37°C. It was observed that the proportion of p plaques in the original stock was considerable and that this proportion decreased as selection was made for the larger G plaques in the sub-lines. The proportion of p plaques in the re-inoculations from the G sub-lines was small but constant, even after three and four generations. Because of the unlikelihood that this small but constant reappearance of p plaques could be due to simple continuing intermixture of p virus particles in the G plaques, a spontaneous mutation was probably occurring, in the direction G p. According to Table 1, the mutation rate appeared to be on the order of 2-3%.

① TABLEAU I

②	o	p	% p
Souche originale	148	52	26
③			
G_1 (3 clonages).....	493	14	2,6
G_2 (4 clonages).....	593	15	2,5
G_3 (3 clonages).....	792	19	2,3
P_1 (3 clonages).....	0	462	100
P_2 (3 clonages).....	0	904	100

Legend:

1. TABLE I
2. original strain
3. g_1 (3 generations, etc.)

DISCUSSION

The present study, using the Sindbis virus, encountered a situation very comparable to the one described by Marshall et al.[6] which concerned the WEE virus. At least two types of plaques were found: one type corresponding to a mutant virus "fixed" on a character for smallness (p), and another type corresponding to the wild stock, capable of undergoing a mutation which reduces its size considerably and transforms it into the smaller type. The rate of mutation appears to be constant and is reproducible experimentally. With other viruses the "small plaque mutation" appears to be accompanied by an attenuation of pathologic potency (as in poliomyelitis and Coxsackie). No such attenuation was found in the p mutants, there being 100% mortality observed in mice inoculated with the mutant virus. Therefore, thus far, we have no factor in favor of an attenuation of the pathogenic power of these generations.

The wild-type virus is without doubt the sort which gives rise to large plaques. It appears that the environment found in embryonic chicken cells favors the mutant strain, and since the mutation rate is quite appreciable, the mutant small-plaque virus can quickly replace the large-plaque wild type. But small-plaque mutants multiply themselves much less rapidly, and thus several days must pass before the mutant strain produces noticable plaques.

For the WEE virus, however, Marshall et al.[6] found that this was not the case: when inoculation was made on chicken embryo or on chicken, the large plaques predominated. This predominance perhaps accounts for the conservation in nature of the large-plaque type as the wild strain of the WEE virus.

RECAPITULATION

In embryo cell cultures in gel medium infected with the Sindbis virus, two types of plaques appear: large (G) and small (p) plaques. The G plaques appear in 24 hours and grow until they attain a diameter of more than 20mm. The small plaques (p) appear after 48 hours but never attain a diameter exceeding 3mm. After three successive re-inoculations stable sub-lines of the p type are obtained, while the G sub-lines always give rise to a predominance of G but a small, constant percentage of p plaques. The interpretation of this phenomenon is that a mutation is spontaneously occurring, giving rise to small-plaque mutants from clones of wild-type, large-plaque viruses.

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