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Effect of Particle Aggregation on the Survival of Irradiated Vaccinia Virus

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ABSTRACT

GALASSO, G. J. (University of North Carolina School of Medicine, Chapel Hill), AND D. G. SHARP. Effect of particle aggregation on the survival of irradiated vaccinia virus. J. Bacteriol. 90:1138–1142. 1965.—The survival curve (plaque titer versus time) of vaccinia virus irradiated with ultraviolet light was found to be strongly dependent, in both shape and general slope, on the degree of aggregation among the virus particles. When special care was taken to produce completely dispersed virus, a straight steep line resulted. Some severely aggregated preparations produced a straight line also, but of much less slope. Thus, the straight line, often taken as evidence of complete dispersion, is not a reliable measure for vaccinia virus. None of the experimental curves resembled the classical multihit curve predicted by the Poisson function for the behavior of uniformly aggregated virus. The aggregation was observed, by electron microscopy, to be nonuniform, and of such a distribution that the survival curves can be qualitatively accounted for in terms of multiplicity reactivation of irradiated virus within cells that receive groups of two or more particles. The greatly increased survival value of aggregates of virus, which indicates multiplicity reactivation, is worthy of note.

When some of the original claims for a valuable procedure are shown to be less than universal in their area of application, there is sometimes a tendency to discredit the procedure as a whole. Often, however, the result of such findings is a wider usefulness of the procedure which comes from a better understanding of its meaning. This paper describes results with the plaque titration of vaccinia virus which we hope will have the latter effect.

Dulbecco and Vogt (1955) cited two experiments as proof that the number of plaques obtained is equal to the number of infectious virus particles present. The first of these must show that the number of plaques is proportional to the quantity of virus suspension put upon the cell monolayer. The second is a demonstration of linearity between the logarithm of the plaque survival ratio and the dosage of ultraviolet rays required to produce it, particularly for small doses. Thus, it is shown, first, that all plaques must arise from either single virus particles or groups (which do not break up on dilution) and, second, that no groups are present; therefore, plaques arise only from single particles.

Although it is probable that plaque titrations have been made and single plaques have been cultured for purifying purposes from virus preparations that do not meet the requirements of Dulbecco and Vogt, these do not concern us here. We will describe preparations of vaccinia virus that do meet these requirements while still containing a degree of particle aggregation such that a large fraction of the plaques must arise from clumps of virus particles. A plausible explanation of this behavior is presented in terms of the contribution of two factors: (i) the peculiar nature of the aggregation among the particles, and (ii) the influence of multiplicity reactivation, with aggregated virus, even when titrated at very low average multiplicities.

MATERIALS AND METHODS

Vaccinia virus from the American Type Culture Collection (WR, mouse neurotropic strain) was grown in Earle's L cells. The media for these cells was Hanks' balanced salt solution to which 16% horse serum, Yeastolate (Difco), and antibiotics were added as previously described (Galasso and Sharp, 1962). Sealed slant tube cultures of 10^6

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cells were inoculated with 10^7 virus particles (VP) and incubated for 2 to 3 days at 37 C, at which time the virus particles were released from the cells and dispersed by 9-kc sonic waves (Galasso and Sharp, 1962). The yield from such tube cultures was 5×10^9 to 8×10^9 virus particles. Suitable quantities of such lysates were pooled and clarified of some cellular debris by low-speed centrifugation. The virus particles were washed twice by sedimentation at $10{,}000 \times g$ in phosphate-buffered saline and diluted for ultraviolet irradiation and analysis of aggregation.

Irradiation of the virus was done by exposing it, in an open petri dish, to the unfiltered output of a small spherical germicidal lamp (Westinghouse Odorout), the output of which, in the effective range, is over 95% in the resonance line, 2,537 A, of mercury. The intensity of the rays incident upon the dish was 33 μw per cm^2. The depth of the virus suspension was about 1 mm. Most of the exposures were less than 3 min, and room lighting conditions were such that no photoreactivation of the virus was observed.

Aggregation analysis was made by the agar sedimentation procedure previously described for the counting of virus particles via electron microscopy (Galasso and Sharp, 1962). Briefly, it consisted of sedimenting the virus upon agar from which it was transferred by pseudoreplica to the specimen grid for photography. Correction for the coincidence aggregation, separate particles sedimenting upon each other, was made by methods already described (Sharp and Buckingham, 1956). Two kinds of data are presented, total particle count and the fraction of free particles.

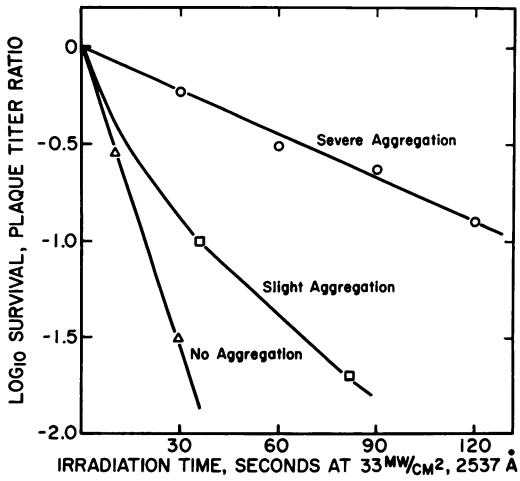

FIG. 1. *Inactivation of vaccinia virus by small dosages of ultraviolet rays. The three preparations of virus differ in degree of particle aggregation as determined by electron microscopy. None of the lines has the multihit form.*

TABLE 1. *State of aggregation of the vaccinia virus particles**

Group size	No. of groups	Total particles
1	149	149
2	24	48
3	17	51
4	11	44
5	5	25
6	1	6
7	2	14
8	0	0
9	1	9
10	2	20
11	1	11
12	1	12
13	1	13
14	1	14
16	1	16
18	1	18
20	1	20
26	1	26
34	1	34
36	1	36
70	1	70
81	1	81
Totals	224	717

* Per cent singles observed, 21; per cent singles in the suspension (calculated), 22.

Plaque titration of the irradiated virus was made on samples taken from the petri dish at appropriate times and immediately diluted with growth medium. The procedure for preparation of the monolayers of L cells was previously described (Galasso and Sharp, 1962). Plaques were counted on the 4th and 6th days of incubation.

RESULTS

Aggregated virus was obtained by resuspending the pellet, from the second saline wash sedimentation, with vigorous pipetting action but no sonic waves. The decline in titer of this virus with irradiation is shown in the top curve of Fig. 1, in which it may be seen to be quite linear. No suspicious curvature is evident, but the electron micrographs showed only 21% of the particles as singles (Table 1 and Fig. 2). Of these particles, 79% lay in aggregates varying in size, the largest of which contained 81 particles. These aggregates seem to be of structure loose enough to be deformed, upon sedimentation to the agar, into a plane. Seldom, even in the large aggregates, are particles piled upon one another. Probably the aggregates, which are moving toward the agar very slowly in the centrifuge, have ample opportunity to spread out and adjust themselves in such a manner that most of the particles actually

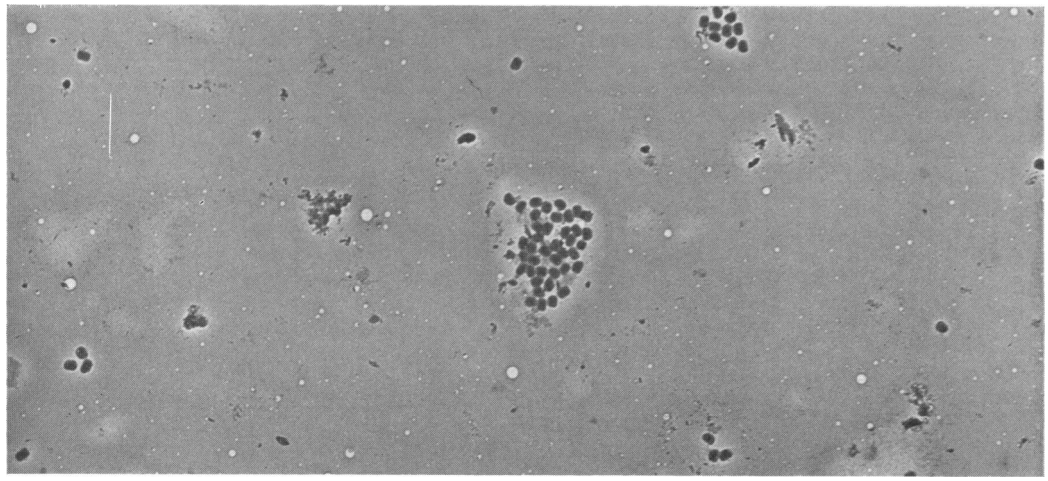

FIG. 2. *Vaccinia virus deposited by centrifugation from a very dilute suspension, showing the nature of the aggregates in the preparation producing the top curve of Fig. 1. This is a very small part of the total area used for particle count and aggregation analysis.*

touch the agar. Electron micrographs of aggregated virus sedimented directly upon aluminum-coated collodion films reveal the same appearance, so it is not an artifact of the replication process.

The distribution of the particle population into groups of various sizes is shown in Table 1, which reveals two particularly significant facts which will be discussed later. Although the *fraction* of singles is small, the *number* of singles is far greater than the number of pairs; pairs are more frequent than triplets, etc. Thus, the frequency of the groups decreases rapidly with increasing group size. The second item of significance is the rather large fraction of the total population that is represented by the few large groups.

During the above experiment, part of the virus was removed from the treatment dish after 2 min of irradiation, the point at which its plaque survival ratio was $10^{-0.91}$, or about 20% (Fig. 1 and Table 2, preparation 2). It was diluted with growth medium to a concentration of 5×10^7 VP per milliliter and given sonic vibration (SV) treatment which increased the percentage of single particles in the pictures from 21 to 90 (Table 2). Titration of this virus showed the survival ratio to have been greatly reduced to $10^{-4.20}$, or by a factor of 1,950 times. Other experiments (preparations 1 and 3 of Table 2) show similar results obtained with aggregated virus irradiated to about the same survival level. The first of these was sonic-treated at a survival level of $10^{-0.61}$. Its titer after SV treatment fell by the somewhat smaller, but still relatively large, factor of 500 times. The sample of preparation 3, which had 16% singles, was sonic-treated at a higher VP concentration (10^9 per milliliter) than the others. Although it is not possible to achieve such high dispersion by this means (74% singles), the concentration is high enough to facilitate rather rapid reaggregation. This revealed the interesting result that the titer measured immediately after this treatment showed a drop of 417 times, but 3 hr later it had increased by a factor of 3.5 and the percentage of single particles had decreased during this 3-hr period, from 74 to 56 (preparations 3, Table 2).

Previous work showed that vaccinia virus SV-treated for 3 min in our apparatus at a concentration of less than 3×10^6 VP per milliliter is completely dispersed (Galasso and Sharp, 1964). Three different preparations of virus were so dispersed, then irradiated and titrated. After this, they were sonic-treated again with the same severity of treatment that was applied in the earlier experiments, and titration was repeated. The results at three different survival levels are shown in the last three lines of Table 2, in which it may be seen that little or no change occurs when irradiated virus is given SV when the particles are already dispersed.

Returning to the progress of inactivation (Fig. 1), we measured the rate of decline in survival of completely dispersed samples of virus and found it to be much more rapid than that of the heavily aggregated virus. As before, the line was straight but the slope was much greater, giving 90% inactivation in 19 sec rather than the 130 sec required with the highly aggregated preparations. VP, aggregated to an extent intermediate between the two extremes so far described, have

TABLE 2. *Effects of sonic vibrations on plaque titer of irradiated vaccinia virus*

Prepn	State of dispersion (per cent of free particles)				Log_{10} fraction of surviving PF	
	Before sonic treatment		After sonic treatment		Before sonic treatment	After sonic treatment
	In pictures (observed)	In suspension (calculated)	In pictures (observed)	In suspension (calculated)		
1	18	19	NA*	NA*	−0.61	−3.30
2	21	22	90	96	−0.91	−4.20
3	16	17	74	79	−0.83	−3.45
3 (3 hr later)	—	—	56	60	—	−2.91
4	CD†	CD†	CD†	CD†	−0.53	−0.57
5	CD†	CD†	CD†	CD†	−1.50	−1.64
6	CD†	CD†	CD†	CD†	−2.15	−1.90

* NA = no evidence of aggregation. These preparations were diluted to 2×10^6 virus particles per milliliter before sonic treatment.

† CD = completely dispersed before irradiation. Preparations 4, 5, and 6 were diluted to 2×10^6 virus particles per milliliter and sonic-treated for 3 min prior to irradiation. Extensive tests have shown that this procedure reduces all aggregates to singles (Galasso and Sharp, 1964).

given survival lines which are sometimes straight and sometimes curved like the one in Fig. 1. They have all been in the area between the two straight lines shown in the figures, but as yet we have not established a quantitative correlation between titer, dosage, and aggregation.

DISCUSSION

Electron microscopy has contributed little in the past to the knowledge of VP aggregation, because most methods for preparation of suspended particles for observation involve their deposit from a shrinking, drying drop of saline diluent. Thus, their final resting places in relation to one another are largely influenced, particularly in the last moments of water removal, by rapidly increasing salt concentration with corresponding changes in electrical forces and surface tension. This complex situation has been further aggravated by subsequent washing operations which are necessary to remove the large amount of salt deposited with the virus before the particle can be photographed. By sedimenting the virus particles from dilute suspension directly upon an agar receiving surface, they are fixed in a two-dimensional presentation before the removal of the suspending fluid. No washing is necessary, when this is done on agar, so there is good reason to expect the observed arrangement of particles to truly represent the three-dimensional arrangement that existed in the suspension. Our previous work has shown the degree of aggregation of vaccinia VP observed in this way to be quantitatively related to the plaque titer on L cells (Galasso, Sharp, and Sharp, 1964; Sharp, 1965). Dispersal of aggregates by means of SV resulted in an increase in the titer of fresh virus preparations, the magnitude of which could be calculated from the observed aggregation data.

The present experiments are based upon an extension of the above work and upon our results (Galasso and Sharp, 1963) and those of Abel (1962), which have shown that multiplicity reactivation occurs with vaccinia virus in L cells and that it is present in aggregated virus preparations. The persistence of plaque-forming units (PFU) under irradiation (top curve, Fig. 1) can be explained in part by the need for multiple hits for inactivation of aggregates, but this is a very insignificant part of the effect observed. The large drops in PFU with subsequent dispersal of the irradiated particles shows that, as individuals, they are mostly incapable of plaque formation. Similarly treated dispersed virus shows that the trauma of SV does not destroy any PFU. It merely prevents most of the multiple infection of cells that would otherwise take place.

Three points of interest are notable. (i) Multiplicity reactivation is a major factor in determining the PFU titer of the irradiated, aggregated virus, even when the cells of the monolayer may receive an average of far less than one irradiated VP. (ii) It is possible to obtain a straight-line relationship between log survival and dosage even though there is a large amount of aggregation present. (iii) It should be possible, by analyzing considerably more data of this kind, to arrive at an estimate of the number of critical break-points in the deoxyribonucleic acid (DNA), an estimate quite independent of the one made by Luria and Dulbecco (1949), and discussed at length by Dulbecco (1952) and later by Barricelli (1955).

Whereas the first of these points would seem to require no further discussion, ii and iii doubtless do.

A qualitative account of the top curve of Fig. 1 can be made in terms of the frequency distribution Table 1. Traditionally, a straight line has been taken as evidence for single-hit inactivation with no multiple targets nor aggregation among infectious unit particles. How, then, can aggregated virus which exhibits multiplicity reactivation be capable of yielding a straight line, even for a 1 factor of 10 decline in titer? This is possible because of the combination of two effects which influence the survival curves oppositely. If *all* the particles were in groups of two, the curve would doubtless be given by the second term of the Poisson series, clearly convex upward. But the data of Table 1 show more singles than pairs, more pairs than triplets, etc. Rapid inactivation of the prevalent singles, then the next most frequent pairs, etc., tends to make the curve fall most rapidly at the start and less rapidly as successively larger groups of particles remain as survivors. Clearly, some particular distribution of group sizes can produce a relatively straight line, at least for a limited dosage of ultraviolet rays. Others might be expected to resemble, initially, the line for singles, diverging later as the few groups predominate in the surviving titer (middle curve, Fig. 1). Such curves must, of course, eventually fall again as dosage increases, since resistant forms have not been found among the survivors of the ultraviolet treatment of this virus.

In the calculation of N, the probable number of points at which the viral DNA can be readily broken by the photons, Luria and Dulbecco (1949) made use of the Poisson function to calculate the frequency distribution of bacteria infected by one, two, three, etc., irradiated phage particles. Because of aggregation, it would not be possible to perform the experiment in this way with vaccinia virus. It should, however, be possible to calculate N from data such as those of Fig. 1, obtained at very low average multiplicity. In this case, the observed aggregation distribution would be used in place of the calculation based on the Poisson function. Only the early, low-dosage parts of the inactivation curves from severely aggregated preparations would be useful because of the difficulty of determining the frequency of the few large aggregates. Preparations with less aggregation could be followed farther. Precise calculations will require more extensive data than those available now. These are being accumulated, and means are being sought to make reproducible degrees of aggregation for these studies.

The decrease observed in the titer of irradiated vaccinia virus after treatment with sonic waves affords a sensitive test for aggregation inasmuch as the virus does not suffer destruction by these waves. Either this test or direct observation of the sedimented virus by electron microscopy is necessary if one wishes to be sure of a high probability of getting plaques which arise from single particles. Of the plaques produced by the virus of Table 1, for example, almost half would probably have been from groups of two or more. This degree of aggregation is not unusual (Galasso and Sharp, 1962), and it may account for some of the difficulty encountered by workers with the pox viruses, in isolating pure strains by the plaque method.

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