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RELATIVE PLAQUE-FORMING, CELL-INFECTING, AND INTERFERING QUALITIES OF VACCINIA VIRUS

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ABSTRACT

GALASSO, G. J. (University of North Carolina School of Medicine, Chapel Hill), AND D. G. SHARP. Relative plaque-forming, cell-infecting, and interfering qualities of vaccinia virus. J. Bacteriol. **88**:433-439. 1964.—The growth of vaccinia virus in slant cultures of L cells inoculated with different multiplicities of counted particles suggests a higher incidence of cell infection than can be accounted for by the number of plaque-forming units. From cultures containing antiserum or heated virus to limit the passage of progeny to uninfected cells, the data clearly indicate the ability of all the particles to infect cells even though the plaque titer is only one-tenth of this number. Analogous experiments show that an average of two heat-inactivated (56 C, 45 min) particles induce interference in L cells. There is nothing yet to show whether the few plaque-forming particles are different from the majority or whether they are just statistically fortunate in the complex process of plaque formation.

There is abundant evidence, from experiments in which animal virus particles have been counted by electron microscopy and titrated by the plaque method of Dulbecco and Vogt (1954), that the number of particles is substantially greater than the number of plaques (Isaacs, 1957; Sharp, 1963, Lab. Invest. *in press*). Among the many viruses tested in this manner, vaccinia is no exception, although it is one of those showing the highest ratios of plaques to particles, about 1:10 for well-adapted virus in L cells (Galasso and Sharp, 1963*a*). Some investigators regard this ratio as evidence that there is only one plaque-forming particle (PFP) in ten. Others think it denotes no such sharp difference among the particles but rather the probability that any one particle will make a plaque.

Through the use of sedimentation inoculation of cells, measurement of adsorption by particle counting, and determination of the degree of particle aggregation by electron microscopy, it is now possible to make more accurate estimates of the average number of virus particles which attach to the cells. Growth curves of virus produced in cultures of cells inoculated at multiplicites determined in this manner can be interpreted in terms of the multiplicity of infection of the cells. It is proposed that they can be used to determine the critical input multiplicity which is just sufficient to infect all the cells. Essentially the same techniques can be used to determine the minimal number of heated virus particles necessary to cause homologous interference. The following is an account of experiments which support this proposition and provide evidence that essentially all the vaccinia virus particles infect L cells and about one-half, when properly "inactivated" by heating, retain interfering power.

MATERIALS AND METHODS

Vaccinia virus. The vaccinia virus used in this work was the WR (mouse neurotropic) strain, obtained from the American Type Culture Collection and adapted to growth in Earle's L cells. It regularly yielded over 7,000 virus particles per cell in stationary cultures of 10^6 cells inoculated with 10^5 to 10^7 virus particles. The quality of this virus, when incubated to maturity in the cells, has been about 100 plaques per 1,000 particles, on monolayers of L cells (Galasso and Sharp, 1963*a*).

L cells. L cells were obtained from Wilton Earle. The media and techniques employed for growth experiments as well as for plaque titrations were described previously (Galasso and Sharp, 1962).

Counting of virus particles. Counting of virus particles and measurements of the degree of aggregation in preparations used for inoculation were done by electron microscopy as described by Galasso and Sharp (1962). All preparations were treated with 9-kc sonic waves prior to these experiments.

Antiserum. The antiserum used in experiment

D was prepared in rabbits. It is the same serum that was used in the work of Smith, Galasso, and Sharp (1961).

Inoculation of cells. Inoculation of cells used in all experiments involving virus growth curves was done by the sedimentation method of Sharp and Smith (1960) with the use of known numbers of cells and virus particles and sedimentation times of not more than 7 min.

Unadsorbed virus. After sedimentation inoculation, the cells were immediately resuspended in the same fluid and sedimented again at low speed (200 $\times$ *g*). Particles found and counted in this supernatant fluid are what we have called unadsorbed virus.

Multiplicity. Multiplicity is discussed at length, and the following abbreviations are used. M_a is the ratio of virus particles to cells when the inoculum is a preparation of fresh (fully active) virus. M_i denotes the same for heat-inactivated (56 C for 45 min) virus.

These symbols refer to numbers of virus particles and cells brought together in inoculation. They are the input multiplicities.

RESULTS

Adsorption of virus to L cells by the sedimentation process has been high and quite uniform. Data from seven different experiments (Table 1) indicate the degree of variation experienced as well as the average of 82% which was reached. There is no trend or systematic variation with multiplicity. Plaque titrations of the supernatant virus, which was counted to get the percentages, show it to be of the same quality as the input inoculum. There is no detectable selective action in this adsorption process.

TABLE 1. *Adsorption of vaccinia virus to L cells in sedimentation inoculation experiments with different input multiplicities**

Expt no.	Multiplicity (particles per cell)	Particles adsorbed (per cent by difference)
1	8	77
2	17	76
3	20	98
4	83	74
5	85	68
6	100	92
7	100	88
Avg		81.9

* Values were obtained by counting the particles in the supernatant fluid after suspending and resedimenting the cells. Plaque titrations have shown no difference in the quality of unadsorbed virus and inoculum; no selective action has been detected. SD, σ = 10.9.

TABLE 2. *Degree of aggregation observed in 11 different sonically treated suspensions of vaccinia virus particles just prior to inoculation of cells*

Prepn	No. of virus particles	No. of AU*	Per cent AU
1	101	96	95
2	165	137	83
3	128	118	92
4	74	68	92
5	140	112	80
6	164	116	71
7	114	97	85
8	59	56	95
9	198	137	69
10	180	125	75
11	169	153	91
Avg			84

* The active unit (AU) may be either a single particle or an aggregate. The sum of these is the number of AU. Results are the average from five pictures.

Inasmuch as an accurate estimate of the number of cells receiving one or more virus particles at a given multiplicity is dependent on the state of dispersion of the particles, this was measured in several typical preparations. These were diluted sufficiently so that when they were sedimented on agar for particle count and aggregation analysis the average distance between particles would be at least 12 diameters. Under these conditions, the error from chance superposition of previously separated particles is very low (Sharp and Buckingham, 1956). Table 2 shows the total counts and the active unit (AU) or group counts for five pictures each of several preparations of virus just before they were mixed with cells in the following experiments. The AU count is the total of all free particles, pairs, triplets, etc. It is the number of units of virus that are free to act independently during the process of approach and contact with the cells during adsorption, and it is this number, rather than the total number of

particles, that determines the plaque titer of an aggregated preparation of this virus (Galasso and Sharp, 1962; Galasso, Sharp, and Sharp, J. Immunol. *in press*). These preparations were all sonically treated and the table shows them to be well dispersed, as indicated by an average of 84% AU.

Cultures inoculated with fresh virus at a multiplicity of $M_a = 0.1$ were tested at various times to determine the total number of virus particles produced. For this purpose, infected cells were broken and the virus particles were dispersed for count, by the use of 9-kc sonic waves. A plot of these counts on a scale of incubation time constitutes the growth curve. Data from seven such experiments, done on different days, were averaged to give the curve on Fig. 1. Plotted with it is a similar composite curve from 16 experiments done at $M_a = 10$, from previously published work (Galasso and Sharp, 1963*b*). The purpose here was to provide a comparison between the shapes of these two curves, so that individual differences in maximal yield, of the two sets, have been eliminated by making the ordinate the fraction of maximum rather than the actual yield. Whereas the curve of cultures inoculated at $M_a = 10$ denotes a maximal rate of virus production beginning immediately after eclipse, the curve for the cells which received only

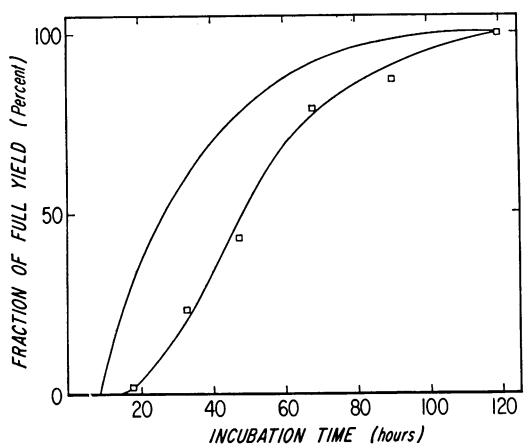

FIG. 1. *Increase of vaccinia virus particles as a function of time. L cells were inoculated by sedimentation at time 0 with an input multiplicity of 0.1. Each point is the average value from seven separate growth experiments. The curve without points is from cells inoculated at $M_a = 10$, a composite of 16 experiments (Galasso and Sharp, 1963).*

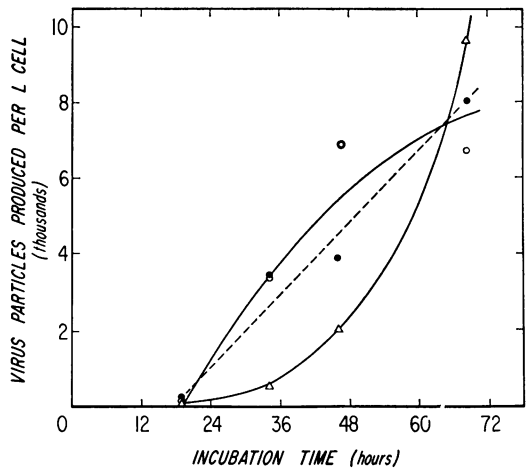

FIG. 2. *Increase of vaccinia virus particles in L cells incubated with antiserum. Cells were inoculated with input multiplicities of 10 (○), 3 (●), and 1 (△). Many cells do not receive virus at input multiplicity 1 until multiplication occurs in those that do.*

$M_a = 0.1$ shows a distinct lag, presumably owing to the waiting period before the majority of the cells could become infected with the progeny of the first few. If this is indeed the reason for the lag, then it should be possible to accentuate this lag in the curve of any culture inoculated with insufficient virus to reach all cells initially, by increasing the difficulty of passage of progeny virus to uninfected cells.

Cells were inoculated at $M_a = 10$, 3, and 1, and antiserum (1:50) was added to the cultures. This antiserum, at a dilution of 1:1,000, was sufficient to reduce the plaque titer of the virus by a factor of 2 (Smith et al., 1961). Virus particle growth curves are shown in Fig. 2. The growth of virus in cells inoculated at $M_a = 1.0$ shows the characteristic lag. At 34 hr, these cultures had produced only about one-fourth as much virus as those at $M = 3$. (It will be shown later that, through the influences of incomplete adsorption, particle aggregation, and statistical fluctuation, there will be a large fraction of initially uninfected cells even at an input multiplicity of $M_a = 1$.) At $M_a = 10$, the curve is convex as it was in Fig. 1, without serum. At $M_a = 3$, the curve seems to be at the point of transition between the convex and lag shapes. Probably the serum has not slowed the growth of virus in these cultures, because it was not dependent on transfer of infectious virus from one cell to the next.

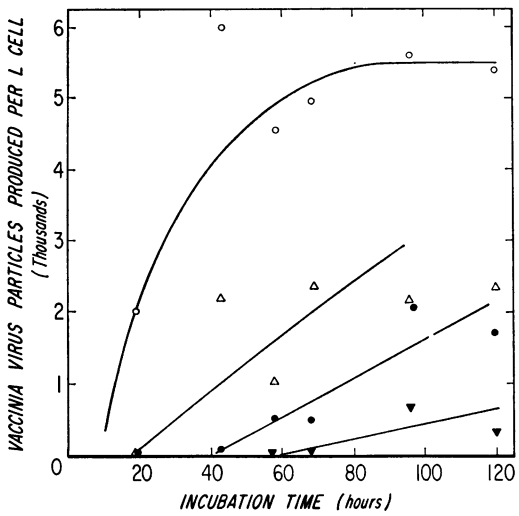

FIG. 3. *Increase of vaccinia virus in L cells inoculated with a mixture of heat-inactivated and fresh (fully active) virus. The multiplicity of inactivated particles was M_i = 20 for all. Active virus was M_a = 100 (○), 10 (△), 3 (●), and 1 (▼).*

Another means of learning, from kinetic studies, whether or not a given M_a is sufficient to infect all the cells of a culture was suggested by the work of von Magnus (1946). It consisted of inducing interference in all the cells that do not receive fresh virus immediately. We have already shown that this vaccinia virus, when heated at 56 C for 45 min, is reduced over 6 log units in plaque titer and that the virus so treated produces interference in L cells (Galasso and Sharp, 1963*a*). In Fig. 3 are growth curves of virus in cell cultures inoculated with mixtures of heated and fresh virus. All the cultures received heated virus at M_i = 20 mixed with fully active virus. Four sets of cultures were tested in which the active component, M_a, was 100, 10, 3, and 1. All were suppressed in yield. Without interference, they should have reached 7,000 particles per cell. Although these data confirm the earlier findings that strong interference is present, they show that a sufficiently high M_a (100) will produce an almost normal yield of virus. They indicate further that the dose of interfering virus (M_i = 20) was chosen too high for sensitive detection of the critical M_a just necessary to infect all the cells initially.

Mixed inocula containing M_i = 10 and various values of M_a revealed a sensitive dependence of maximal new virus yield on input multiplicity. A graphical resume of several such growth experiments appears in Fig. 4. Each point on the graph shows the ratio of the maximal yields of two cultures inoculated with the indicated M_a, one of which received an additional M_i = 10 at the same time. The data are consistent with the concept that a competition for the cells exists between fresh and heated virus. Those that receive fresh virus at the outset seem capable of full virus production, but those that do not receive fresh virus have sufficient time to develop interference before the progeny of the first are ready to infect them. The probable multiplicity of adsorption has been calculated (see Discussion), and the fraction of cells adsorbing one or more virus particles, as predicted by the theory of Poisson, has been plotted as a curve (VP) which falls reasonably well through the experimental points. The corresponding curve for PFP does not. The substantial variation among the data is probably due to the critical balance established between interference and infection, which is of a highly dynamic nature. The minimal input multiplicity capable of producing full yield in the presence of M_i = 10 is about 5. This agrees quite well with the results of the less sensitive experiments of Fig. 1

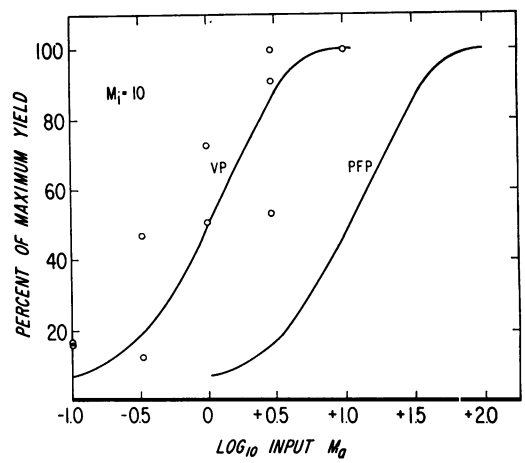

FIG. 4. *Summary of ten growth experiments involving mixed inocula containing a constant amount of heat-inactivated virus (M_i = 10) and varying amounts of fully active virus (M_a). The curve (VP) is the fraction of cells that would be expected to have adsorbed one or more virus particles. PFP is the corresponding curve for plaque-forming particles.*

and 2, and together these data provide strong evidence that essentially all the virus particles do infect these L cells.

Inasmuch as the previous experiment indicated a minimal input M_a necessary to provide full yield in the presence of interfering virus, it seemed that we might determine the minimal M_i necessary to cause interference in all the cells. Again, cells were inoculated with mixtures but now the M_a was kept low (0.1) and different values of M_i were used. A typical series of growth curves is shown in Fig. 5 in which maximal yields are clearly dependent, in an inverse manner, upon the level of M_i in the inoculum. The data from a series of such experiments are gathered for comparison in Fig. 6. The ordinate is the percentage of control maximal yield plotted against the logarithm of the input multiplicity of heated virus ($\log_{10} M_i$). This curve does not end at zero because even at $M_i = 30$ there is virus production in the cells which receive active virus immediately. Drawn on the chart are curves calculated from input multiplicity, with proper allowances for incomplete adsorption and residual particle aggregation, predicting the fraction of cells that received one or more, two or more, and four or more heated virus particles. These were calculated from the Poisson function. They show the experimental points lying nearest the two-particle curve through most of its course.

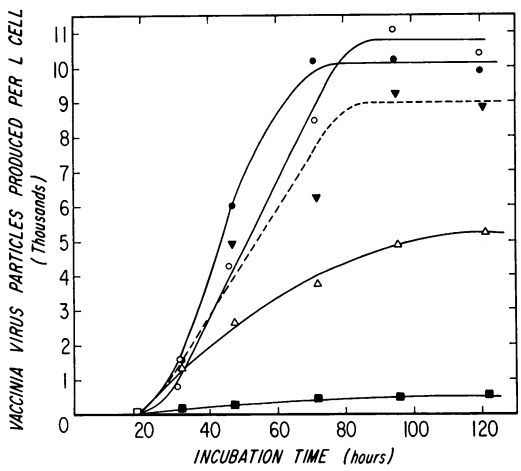

FIG. 5. *Increase of vaccinia virus in L cells receiving a limited inoculation of fully active virus (M_a = 0.1) mixed with varying amounts of heat-inactivated virus (M_i). M_i = 0 (○), 0.03 (●), 0.3 (▼), 3 (△), and 30 (■).*

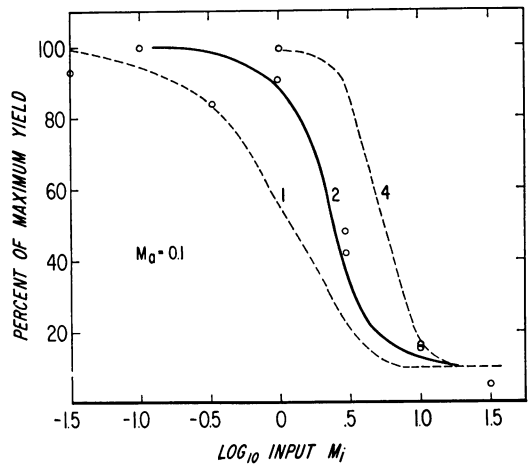

FIG. 6. *Summary of ten growth experiments involving mixed inocula containing a constant limited amount of active virus (M_a = 0.1) and varying amounts of heat-inactivated virus (M_i). The numbered curves represent the fraction of cells that would be expected to adsorb 1 or more, 2 or more, and 4 or more heated particles, respectively.*

Quite clearly, every heated virus particle does not cause complete interference in these L cells, but the indication is that two are enough, either because only one in two is capable or possibly because two per cell are required.

The fresh, fully active virus used in these experiments was titrated frequently during the time of the experiments. An average of 22 such titrations made over a period of 6 months was 94 plaques per 1,000 virus particles. This is an average of one plaque for 10.6 particles; this is close to the value published for an earlier set of titrations with this virus which gave 92 plaque-forming units per 1,000 (Galasso and Sharp 1963*a*, *b*), and it provides evidence for the stability of the virus.

DISCUSSION

Certain bacterial viruses have been found to be so uniformly infectious that essentially all the particles produce plaques (Luria, Williams, and Backus, 1951; Kellenberger and Arbor, 1957). The failure of animal viruses in general to produce one plaque per particle has usually been attributed to failure of the particles to infect the cells that start the growth of a plaque. Our experiments indicate a different explanation of this phenomenon with vaccinia virus in L cells. It appears that essentially all of the particles in a

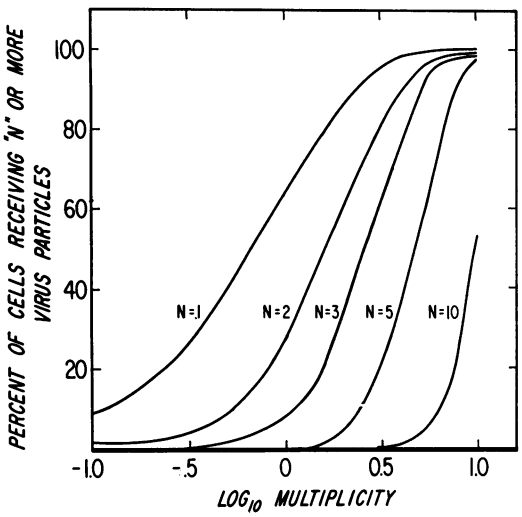

FIG. 7. *Frequencies predicted, by the theory of Poisson, for the fraction of cells in a perfectly dispersed mixture of virus particles and cells, that receive 1 or more, 2 or more, etc., virus particles. In practice, these values are reduced by incomplete adsorption and by particle aggregation.*

fresh lysate of a 48- to 72-hr culture of cells infected with this virus are capable of infecting cells, even though only about one-tenth of this number of plaques are producers on monolayer cultures.

It is possible to calculate, from the Poisson distribution of small numbers, the fraction of cells in a mixture of cells and virus particles that should receive one or more, two or more, etc., virus particles when the average number, N, is known. This should apply as well to cells that receive virus by sedimentation in a centrifuge as to those in suspension or in monolayer cultures where they receive it by random kinetic collision. These ideal frequency curves are shown in Fig. 7 for the range of multiplicities $0.1 < M < 10$, where M is the ratio of the numbers of virus particles to cells and perfect dispersion is assumed for both. With data on the fraction actually adsorbed and the actual degree of aggregation, one can calculate what actual fraction of the cells, at a given multiplicity that would adsorb zero, one or more, two or more, etc., active units of virus. These data indicate that the mean multiplicity of adsorption in these experiments was $MA = 0.82 \times 0.84\ M = 0.69\ M$.

This factor was employed in calculation of the predicted curves on Fig. 4 and 6. Although the data of Fig. 4 are somewhat scattered about the curve for one or more virus particles, there is no doubt that they are far separated from the corresponding curve for PFP that is drawn on the same chart for comparison. The same argument applies in the case of interference (Fig. 6). In this case, the best fit is to the 2-particle curve and clearly not to a 10.6 (or greater) particle curve, as it would be if only particles that were PFP before heating could induce interference. The evidence is strong in both cases, that the activities of cell infection and interference are not restricted to that fraction of virus particles that is capable of producing plaques.

It is very doubtful that the interference observed here is the result of exclusion of active virus from cells that have received heated virus. The observations fit better the concept as stated by Isaacs (1959) that the damaged virus particle does enter the cell and there it sets up a reaction that leads to a block at the site of virus synthesis. The yield of new virus in our experiments with mixed inocula seems to be determined by the outcome of a competition between fresh and interfering virus particles for these sites.

These data show this virus to be very efficient in cell infection. The reasons for its relative inefficiency in plaque production may now be sought, with more conviction, in other directions. It seems likely that new virus production in infected cells under agar overlay may be restricted. Furthermore, the requirements for successful plaque formation extend beyond the infection of the first cell and include an invasive power sustained through several generations. This involves the repeated production and release of high-quality virus from a succession of infected cells. Thus, the quantity and quality of progeny virus at each step, as well as the time of its release from each cell, may strongly influence the probability of plaque formation.

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