# HOMOLOGOUS INHIBITION, TOXICITY, AND MULTIPLICITY REACTIVATION WITH ULTRAVIOLET-IRRADIATED VACCINIA VIRUS[1]

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ABSTRACT

GALASSO, G. J. (University of North Carolina, Chapel Hill) AND D. G. SHARP. Homologous inhibition, toxicity, and multiplicity reactivation with ultraviolet-irradiated vaccinia virus. J. Bacteriol. **85**:1309–1314. 1963.—Vaccinia virus whose plaque-forming capacity had been destroyed by ultraviolet rays (2,537 A) was shown to retard the growth of L cells in tube cultures. At input multiplicities (M) of $0 < M < 10$, no interference was detected, but at $M \geqq 100$ the irradiated virus particles exerted a strong toxic effect on the L cells in monolayer cultures, affecting the plaque formation by active virus which was added. Multiplicity reactivation occurs in sublethally irradiated vaccinia, as shown by virus particle counts via electron microscopy and plaque counts. It is clearly demonstrated in this system because there is no complicating interference. It sets in at a total virus particle multiplicity of about one, even though the multiplicity of the original plaque-forming particles is much below one.

Vaccinia virus has several kinds of activity in addition to infectivity. Among these are the ability of heated virus to exert a toxic effect upon Earle's L cells in culture and to induce in these cells an interference to subsequent infection with fully active virus (Galasso and Sharp, 1961*a*, *b*, 1962*b*, Virology, *in press*). In this published work, the magnitude of the effects was found to be critically related to the total multiplicity or ratio of the numbers of virus particles to cells in the reaction. Ordinarily M (multiplicity of total virus particles) is not known, because virus quantity is usually measured in plaque-forming units (PFU) or similar units of infectivity, which are usually much smaller in number than total virus particles (Galasso and Sharp, 1962*a*; Isaacs, 1957; Dumbell, Downie, and Valentine, 1957; Overman and Sharp, 1959; Easterbrook, 1961; Sharp, Ergeb. Mikrobiol., *in press*). Accurate multiplicity data can be obtained by counting the virus particles in an electron microscope, and the above-mentioned work on homologous interference with vaccinia virus and L cells was based upon these measurements. It was done with heated virus which exhibited marked interfering properties when applied to L cells at $M = 1$. At $M = 100$, general toxic effects were observed.

The present paper describes analogous experiments with vaccinia virus which had been irradiated with ultraviolet light having a wavelength of 2,537 A.

## MATERIALS AND METHODS

*Virus.* The virus employed was the WR (mouse neurotropic) strain from the American Type Culture Collection. Its culture and adaptation to L cells have been described previously (Galasso and Sharp, 1962*a*). It has been in continuous passage in L cells since September 1960, and the experiments described here were made with passages 114 to 169.

*Cells.* L cells were obtained through the courtesy of W. Earle, and they have been in continuous passage in this laboratory since 1958. All methods for culture and preparation of these cells in monolayers for virus titrations, and for virus growth experiments in screw-capped tubes, were described in the above reference and in a more recent paper by Galasso and Sharp (*in press*).

*Inoculation of cells.* All cell inoculations with fully active or with irradiated virus were done by

[1] A part of these results was presented (Galasso and Sharp, 1962*b*) at the meeting of the American Society for Microbiology, Kansas City, Mo., 6–10 May 1962.

the sedimentation method of Sharp and Smith (1960), using known numbers of both cells and virus particles.

*Irradiation of virus.* Cultures (48 hr) of infected L cells were scraped from the glass and sedimented in glass tubes (16 × 50 mm) in a horizontal centrifuge rotor (type HS, Ivan Sorvall, Inc.) at 12,800 × *g* for 30 min. This was sufficient to sediment infected cells and cell-free virus from the 3-ml samples. The pellets were resuspended in phosphate-buffered saline (PBS) and sedimented again. The second resuspension in PBS was sonically treated for 3 min, 9 kc per sec, at full power of a Raytheon sonicator (model S-102A) to break up cells and disperse the virus. In an open 9-cm petri dish, 3 ml of this suspension were exposed to the unfiltered radiation of a germicidal lamp (15 w, General Electric Co.) which gave at the liquid surface a flux of 77 μw per cm², consisting mostly of 2,537-A wavelength from the prominent resonance emission line of mercury. The dish was frequently agitated by hand during exposure.

*Counting of virus particles.* Virus particles were counted by the agar sedimentation method of Sharp (1960), employing low magnification (1,200 ×) in an electron microscope.

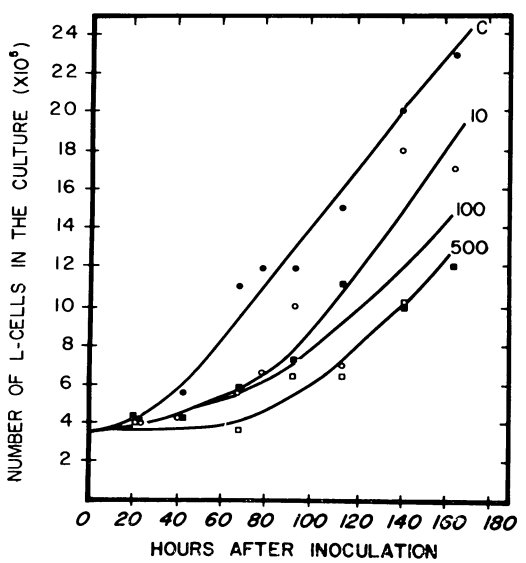


FIG. 1. *Effect of ultraviolet-inactivated vaccinia virus on the increase of L cells in screw-capped culture tubes. The numbers indicate the multiplicity of virus particles used. The control culture is indicated by C.*

## RESULTS

*Effect of ultraviolet-inactivated virus particles on cultures of L cells.* Vaccinia virus exposed for 3 min to ultraviolet rays (2,537 A) at an incident intensity of 77 μw per cm² has been found devoid of plaque-forming ability (Galasso and Sharp, *in press*). Several million L cells were inoculated, by sedimentation, with this irradiated virus at a multiplicity of $M_i$ [multiplicity of treated (partially or completely inactivated) virus particles] = 10. After resuspension in fresh growth media, they were divided into screw-capped culture tubes, each containing 3.5 × $10^5$ cells in 1-ml volume. These were incubated slantwise at 37 C along with other groups of cells that had received irradiated virus at $M_i$ = 100 and $M_i$ = 500 as well as control cells at $M_i$ = 0. At various times up to 176 hr, as shown in Fig. 1, the cell population of one tube culture of each group was resuspended and counted in a hemocytometer.

The growth of control cells, after the experience of scraping from the glass and centrifugation at 8,000 × *g* for 7 min, was delayed only briefly. It became steady after about 24 hr of incubation, and the population reached 2.3 million cells per ml at 176 hr. All cultures exposed to irradiated virus, even as few as ten particles per cell, were delayed in growth and the amount of delay was increased with increasing multiplicity. Nevertheless, the rates of growth in cell number in all cultures approached that of control cultures when the delay period was past. Apparently this ultraviolet-inactivated virus makes measurable changes in the growth of L cells in culture although it has no detectable infectivity and no power, in multiplicities up to $M_i$ = 100, to inhibit the growth of active virus given to such cultures 4 hr later (Galasso and Sharp, *in press*).

*Effect of inactivated virus particles on plaque titration of active virus on L cells.* Culture bottles containing 3 million L cells in monolayers were inoculated, in triplicate, with mixtures of fully active and irradiated virus particles. All cultures received 300 particles of the active virus. Multiplicities of irradiated (2.5 min) particles ranged from 0 to 200. In Fig. 2 are shown the numbers of plaques observed, relative to the numbers found on control cells without irradiated virus. These are the points of the upper curve, and they show no tendency toward declining titer in the multiplicity range $M_i$ = 1 to 10. With virus inactivated at 56 C for 45 min in similar circum-

stances, a sharp decline in titer of the infectious virus added to the mixture was observed (Galasso and Sharp, *in press*). The lower curve was plotted from the data of that paper for comparison.

In the present experiments, as we have just seen, there was no decline; there was instead a slight rise in titer in the region of $0 < M_i < 10$, followed by a sharp decline in the region of $M_i = 100$ accompanied by a decrease in plaque size and a general degraded appearance of the cells in the monolayers. The slight rise led to the reactivation experiments described in the next section. The sharp decline, at high multiplicity of irradiated virus, indicates a toxic effect which survives irradiation but which is not as strong as that previously shown for the heated virus.

*Plaque titration of virus partially inactivated with ultraviolet rays.* Virus preparations used in the experiments of the preceding two sections were irradiated for 3 and 2.5 min, respectively. Neither of these had any demonstrable plaque-forming power. Preparations irradiated 0 to 1 min have been shown to decline in titer ($\log_{10}$ PFU) at a rate strictly linear with dosage (Galasso and Sharp, *in press*). The interval of dosage between 1 and 2 min produces a mixed population of inactive and plaque-forming particles that can be difficult to titrate, because the number of plaques produced is in some cases proportional to dilution and in others it is not. The following two experiments serve to clarify this situation.

Fresh virus was released from infected cells and dispersed by a 3-min treatment with 9-kc sound waves. The degree of dispersion was 92% active units (Galasso and Sharp, 1962*a*). (An "active unit" is a single virus particle *or* a group of particles, as seen in electron micrographs made particularly for dispersion studies. The percentage of active units in a given virus preparation is used as a measure of dispersion.) When this preparation was placed upon monolayers of L cells, the number of plaques produced by 3,000, 1,000, and 300 virus particles was strictly proportional to these particle numbers (Fig. 3); they indicated a quality of 26 PFU per 1,000 particles, and the scatter of points indicates tolerable precision of titration of fresh, unirradiated virus.

Fresh virus was irradiated for 1 min and titrated upon the same kind of monolayer bottles (3 million cells per bottle). Bottles received different numbers of virus particles in the range

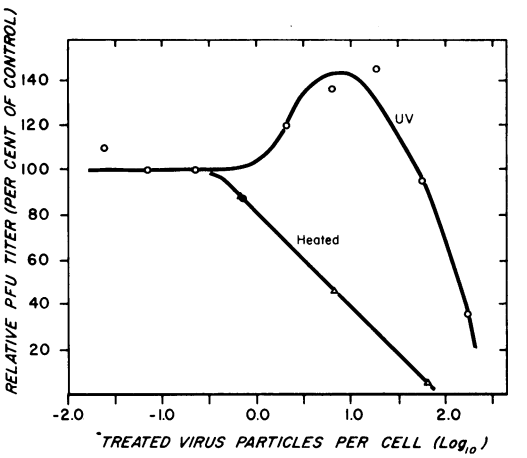


FIG. 2. *Plaque titrations of a constant number of fresh vaccinia virus particles in the presence of varying numbers of inactivated particles. In the region of multiplicity between 1 and 30 ($0 < \log M_i < 1.5$), the irradiated (—○—○—○—) and the heated (—△—△—△—) virus particles produce different anomalous results, revealing distinctly different activities which remain after infectivity has been destroyed.*

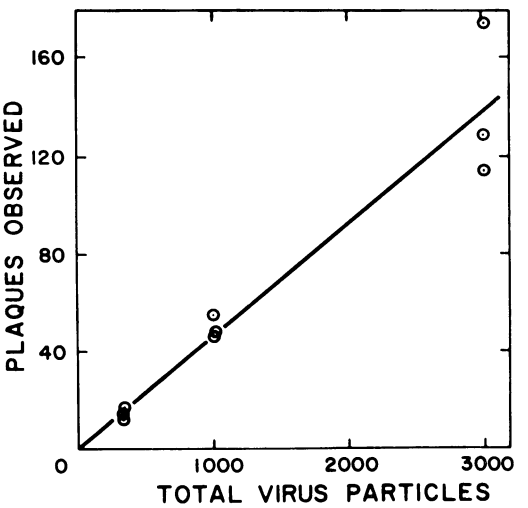


FIG. 3. *Chart of the linear dependence of PFU on dilution in titration of fresh vaccinia virus on L cell monolayers. There was an average of 21 virus particles per plaque, 47 PFU per 1,000 particles.*

$10^{5.5}$ to $10^{8.0}$, and the observed plaque numbers are plotted in Fig. 4. Bottles that received about $10^{5.5}$, $10^{6.0}$, and $10^{6.5}$ virus particles produced plaque numbers proportional to these numbers. All others produced more plaques than would be

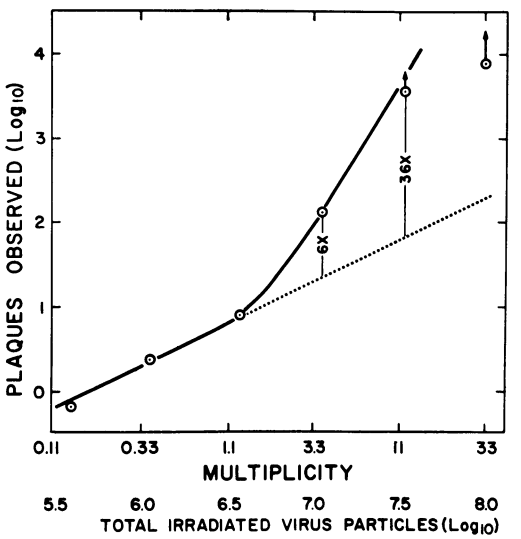


FIG. 4. *Chart showing departure from linearity in titration of vaccinia virus partially inactivated by ultraviolet rays. Abscissas indicate the total number of virus particles and the corresponding multiplicities on the L cell monolayers. Multiplicity reactivation is evident in the region of M > 1.*

predicted from this linear trend, and the departure from the line became increasingly greater with three further increases in inoculum. At $10^7$ particles per bottle there were six times more plaques than expected; at $10^{7.5}$ the factor was 36; and at $10^8$ there were more plaques than could be accurately counted. All inocula containing more than one virus particle per cell of the titration monolayers gave excessive titers.

The straight line (Fig. 4) has been drawn at unit slope, and titrations of the virus at $M \leqq 1.1$ fit quite well upon it. Excessive titers in the region of $M > 1.1$ are well outside the range of chance variation, as may be judged from Fig. 3. Although the titration at M = 33 produced more plaques than could be counted accurately, it would not have done so had there been no "activation" of presumably inactive virus. These plaques were difficult to count also because the cell monolayers showed some evidence of the generalized toxic effects which are so prominent at greater multiplicities ($M \geqq 100$).

## DISCUSSION

In this paper, we are concerned chiefly with L cells that have received two or more vaccinia virus particles. Earlier experiments have shown that the ultraviolet-irradiated particles do not produce homologous interference (Galasso and Sharp, *in press*) like that of heated virus, but they do produce a toxic effect on monolayers of L cells if applied in sufficiently high multiplicity. Even in quite low dosages ($M_i = 10$) they exert a marked effect upon the rate of multiplication in tube cultures of L cells. This is evident in Fig. 1 where all cultures treated with noninfectious irradiated virus particles were delayed in growth, and the delay increased as the dosage was made greater. It is significant, though, that even the cultures receiving the greatest quantity of virus ($M_i = 500$) showed ultimate recovery of normal growth rate after several days of delay.

Monolayers of L cells register plaques in expected numbers from fresh virus even though ultraviolet-treated virus particles may be present in multiplicities up to ten (Fig. 2). Heated virus produces strong interference under these same circumstances, resulting in reduced plaque formation from the fresh virus. At higher multiplicities, $M_i = 100$, for example, particles of both treatments are detrimental to plaque formations. Cell monolayers so treated exhibit a generalized toxic response, and even the few plaques that do appear are smaller in size than normal. In general, though, the toxic effect of the heated virus is greater than that of the irradiated virus at the same multiplicity.

Although the irradiated virus at $M_i = 10$ produced no reduction in plaques from fresh virus particles inoculated onto the monolayers with it, there was a slight rise in the number of plaques. The increase was small (Fig. 2) but it suggested the possibility of multiplicity reactivation, which was investigated further with virus that had received somewhat less radiation. Having demonstrated (Fig. 3) that the titration method did indeed produce plaques with fresh virus, which were reproducible in number and consistent with dilution, we titrated virus that had received sublethal ultraviolet treatment (Fig. 4). Plaque numbers were as expected with increasing multiplicity up to 1.1, but the next and subsequent increases produced rising titers above the expected values. It is significant that the increased plaque numbers were observed at the first increment of increased inoculum beyond a multiplicity of 1.1. According to the concepts of Luria (1947) and of Luria and Dulbecco (1949), as reviewed by

Pollard (1953), the probability of infection by a pair of radiation-damaged virus particles is greater than that by either separately, because the damage from radiation is random; therefore, it is not likely to be the same in two particles which reach a single cell. Thus, the cell may find, in the pair, a complete set of stimuli for production of true virus progeny. These concepts were drawn from experiments with bacteriophage for which the assumption is generally made that all particles are plaque formers (are infectious). Vaccinia virus does not produce plaques with such high efficiency. The preparation employed in the reactivation experiment of Fig. 4 produced, before irradiation, 1 plaque per 21 particles. After irradiation, then, the probability that any one particle that meets an L cell will be one of the original plaque formers is 1:21. Likewise, the probability that any two particles which meet at a single cell will be original plaque formers is $(1:21)^2$ or 1:441. Still, the plot of M vs. PFU departs from unity slope near M = 1, not M = 21 or 441. The suggestion is strong that many particles, not just the original plaque formers, may participate in plaque formation at M > 1 in this experiment.

Homologous interference has been reported repeatedly for animal viruses, as reviewed by Schlesinger (1959) in *The Viruses* and more recently for vaccinia virus by Galasso and Sharp (*in press*) and Hanafusa (1960). Much of this work has been done with virus treated with ultraviolet light, and sometimes multiplicity reactivation has been observed. Recent papers in this category are those of Barry (1961*a*, *b*) on influenza viruses and Drake (1962) on Newcastle disease virus. In these cases, the results may become complicated by the presence of both interference and multiplicity reactivation in the same experiment. Both effects are dependent upon the cooperative (or antagonistic) effects of two or more virus particles in one cell. Vaccinia virus seems to be free from this difficulty. Although this virus has been shown to produce interference when heated (Hanafusa, 1960; Galasso and Sharp, 1961*a*, *b*, *in press*), the reactivations observed by Joklik, Holmes, and Briggs (1960) and by Hanafusa (1960) might be put in a quite separate category. Our present experiments have shown no tendency toward interference with ultraviolet-treated virus, so the demonstration of multiplicity reactivation is singularly clear.

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