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Effects of Heat on the Infecting, Antibody-Absorbing, and Interfering Powers of Vaccinia Virus

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ABSTRACT

GALASSO, G. J. (University of North Carolina School of Medicine, Chapel Hill), AND D. G. SHARP. Effects of heat on the infecting, antibody-absorbing, and interfering powers of vaccinia virus. J. Bacteriol. **89**:611-616. 1965.—At 56 C the infectivity of vaccinia virus particles is destroyed rapidly, but even when it is reduced by a factor of 10^6 the particles are capable of producing strong interference in L cells. The rate constant K for thermal inactivation of plaque-forming power is greater than that for interfering power by the factor $e^{3.8}$ or about 45 times. At 37 C both properties of the virus decline more slowly and at equal rates. The temperature coefficient of K is discontinuous in the region of 40 C, indicating quite different activation energies for the reactions above and below this critical point. The degradation of interfering power exhibits a similar discontinuity, although less in magnitude, but none has been found in the antibody-reactive power of the virus, which is much more heat resistant.

Among the many kinds of activity exhibited by virus particles, the one most sensitive to heat is infectivity. The rate of loss of infectivity is dependent upon temperature, but the function expressing this dependence is discontinuous, for some viruses, in the region of 40 C. This was discussed in review by Woese (1960) and by Hiatt (1964), both of whom referred particularly to the observations of Bachrach et al. (1957) on foot-and-mouth disease virus. Subsequent reports by Dougherty (1961) and Krugman and Goodheart (1964) have shown similar discontinuities for Rous sarcoma and human cytomegalovirus, respectively. Kaplan (1958) showed, for vaccinia virus, a complex time decay curve composed of two straight lines. The slope of the steeper of the two lines was dependent on temperature, with an indication of discontinuity in the same temperature region as with the other viruses described above.

There is ample evidence that the antigenic activity of viruses is generally more stable than the infectivity (Woese, 1960), but little has been published on the heat sensitivity of the power to produce homologous interference. Interference can often be established in a culture of cells by exposing them to heated virus with "slightly damaged" nucleic acid (Isaacs, 1959). It might be supposed, then, that the power to induce homologous interference would be generally less heat-sensitive than infectivity. We present additional data which, together with previously published results (Sharp, Sodhukhan, and Galasso, 1964*a*), provide a fairly complete description of the decay of infectivity of the WR strain of vaccinia virus in the temperature range 56 to −62 C. The decay rate of homologous interfering power has now been determined at 56 and at 37 C, and the antibody-absorbing power of variously heated samples of the virus has been measured as well.

Inasmuch as interference is critically dependent on multiplicity of inoculation of the test cells, and the determination of antibody-absorbing power is likewise dependent on the amount of virus involved, we have counted, by electron microscopy, the number of particles used in each experiment. By this procedure, it is hoped, the results will be directly comparable with those obtained later with other viruses.

MATERIALS AND METHODS

Virus. The vaccinia virus employed was the WR (mouse neurotropic) strain from the American Type Culture Collection. Its adaptation to and

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subsequent passage in L cells have been described elsewhere (Sharp, Sadhukhan, and Galasso, 1964*b*). For these experiments, virus from L-cell passages 22 and 347 were employed. They will be called early-passage and late-passage virus, respectively. Both grow well in sealed slant tube cultures of L cells, yielding more than 3,000 new virus particles per cell in 72-hr infected cultures. They differ markedly, however, in plaque-forming power on monolayers of L cells.

Cells. Earle's L cells were used in two ways. For measurement of the growth of virus, with or without interference, the cultures consisted of 1 ml of growth medium (Hanks balanced salt solution, supplemented with Yeastolate and 16% horse serum) containing 10^6 inoculated cells, sealed with a screw cap and incubated slantwise at 37 C. Groups of identical cultures of this kind were prepared in each experiment, and individual tubes were withdrawn at appropriate times, to be held at −20 C for subsequent virus particle count by electron microscopy. Plaque titrations were made on monolayer cultures of L cells prepared as previously described (Galasso and Sharp, 1962).

Inoculation of cells. All L cells used for slant cultures to determine growth of virus were inoculated by the sedimentation method (Sharp and Smith, 1960). A homogeneous suspension of cells and virus was made in growth medium. The relative numbers of virus particles and cells were chosen to give the desired multiplicity, and the absolute number of cells was just that needed to cover the flat bottom of the tube in which the mixture was sedimented. This sedimentation adsorption was completed in 7 min or less, and the cells were then resuspended and divided equally among the growth tubes as described above.

Sera. Early-passage L-cell virus was used to inoculate 12-day embryonated eggs. The infected chorioallantoic membranes were harvested 48 hr later, and the virus was extracted in phosphate-buffered saline and counted in an electron microscope. Albino rabbits, weighing about 2.3 kg, were inoculated intradermally with 8×10^6 virus particles. Typical vaccinia lesions appeared 3 days later and subsided by the third week when a second inoculation of 8×10^6 virus particles was made. This gave the typical immune response, and 3 weeks later 8×10^7 virus particles were given intravenously (in the ear). The rabbits were bled 5 days later. Normal sera were obtained from all the rabbits prior to the inoculation series.

Enumeration. Cells were dispersed by pipetting, and were counted in a counting chamber. Virus particles were released from infected L cells and dispersed with 9-kc sonic waves. They were inspected for aggregation and counted by the agar sedimentation method (Sharp, 1960), by use of an electron microscope.

Ultraviolet treatment of virus. Ultraviolet treatment was done at a concentration of 10^9 virus particles per milliliter in an open petri dish under conditions described previously (Galasso and Sharp, 1963*b*), except that the radiant flux used here was 33 $\mu w/cm^2$ and the exposure time was 5 min.

RESULTS

A sensitive system for the detection of homologous interference of vaccinia virus in L-cell cultures consists of cells inoculated with just enough interfering virus particles to insure the presence of at least one per cell, and a number of fresh test virus particles large enough to produce maximal yield in control cultures but small enough to require more than one generation of virus to achieve this. Thus, the initial inoculum of fresh virus can reach only a small known fraction of the cells, the remainder of which may then develop interference during the delay and eclipse period of the active virus. Both viruses are administered together in the sedimentation inoculation, thus eliminating all questions of similar handling of control cells which arise in the usual delay experiments for measurement of interference. Past experiments with our system have shown that about two virus particles per L cell are sufficient to cause interference when the virus is heated at 56 C for 45 min (Galasso and Sharp, 1964). The present experiment consisted of heating the fresh late-passage virus at 56 C for times ranging from 25 min to 3 hr and inoculating cells with a mixture of this interfering virus at a particle multiplicity (M_i) of 5 and fresh active virus at a multiplicity (M_a) of 0.1. Growth of new virus in these cultures, as followed by particle count, was detectable in both control and interfered cultures at 24 hr, but the rate of increase and the maximal yield from the cultures at 120 hr of incubation were clearly dependent upon the time of exposure of the interfering virus to the 56 C water bath (Fig. 1). Inasmuch as the curves are of approximately the same shape, the choice of an incubation time for comparison of yields does not seem to be critical. At 120 hr the yield from cultures receiving interfering virus heated for 25 min is about 10% of that of control cultures. Because the multiplicity of active virus was 0.1, it would seem that there was complete suppression of virus particle growth in all cells other than those reached initially by the fresh virus. Interference by virus particles heated for longer times is progressively less. With interfering virus heated 180 min, the active inoculum increased to 69% of that in control cultures.

The data from several growth experiments, such as that of Fig. 1, have been accumulated to determine the rate of deterioration of interfering power of the virus at 56 C (Fig. 2). Inasmuch as all cultures received active virus at multiplicity 0.1, it might be expected that no culture would produce less than 10% of maximal yield. If such

a correction were applied to the data (Fig. 2), the maximal ordinate would be 2.00, instead of 1.95 as shown. This, however, requires that interference be complete in all cells that do not receive active virus at once, and that all cells that do recieve it will become infected and produce a normal yield of progeny despite the presence of interfering virus. Although we think this is true we prefer to present the data uncorrected until such time as more direct evidence is available. In either case, the calculation of the rate constant will be affected little.

The data reveal substantial fluctuation, which is indicative of the difficulty of determining end points under these dynamic conditions. Nevertheless, they give unmistakable evidence that the interfering power of the heated particles is greatest when heating is least. It would seem that heating at 56 C is not an essential step in transforming an infecting particle into an interfering particle but that it reveals the property of interference simply by destroying infectivity at a more rapid rate.

To compare the rate of inactivation of infectivity with that just measured for interfering power, the virus was heated at three different temperatures (56, 50, and 45 C) in complete growth medium at a concentration of approximately 5×10^9 particles per milliliter. Plaque titrations of the virus, heated for various lengths of time at these three temperatures, yielded the three rate constants shown in Fig. 3 (filled circles). These data are plotted against the reciprocal of the temperature, according to the method of Arrhenius. Rate constants for the inactivation of virus infectivity at temperatures of 37 C and less (Sharp et al., 1964*a*) are plotted as open circles on the same chart. The break in the plot is conspicuous. The rate of inactivation of infectivity at 56 C is about 400-fold greater than that indicated by the dotted extrapolation of the line through the low-temperature data. Inactivation of interfering power at 56 C (open square) is clearly intermediate, fitting neither line and lying below the value for infectivity by a factor of 45.

All attempts to detect interference by virus inactivated at 37 C have failed. Evidently, at this temperature the interfering power is destroyed at the same rate as infectivity.

Antibody-absorbing power of the virus was measured by adding 5×10^8 particles to 1 ml of dilute antiserum, incubating the mixture for 30 min at 37 C, and then removing the virus by passage of the serum through a 0.22-μ Millipore filter. The strength of the filtrate serum was measured by plaque suppression according to the method of Dulbecco, Vogt, and Strickland (1956). Relative absorbing power of the various virus samples is expressed in terms of the final dilution of the filtrate serum necessary to reduce the plaque yield from 8×10^5 late-passage virus particles to one-half.

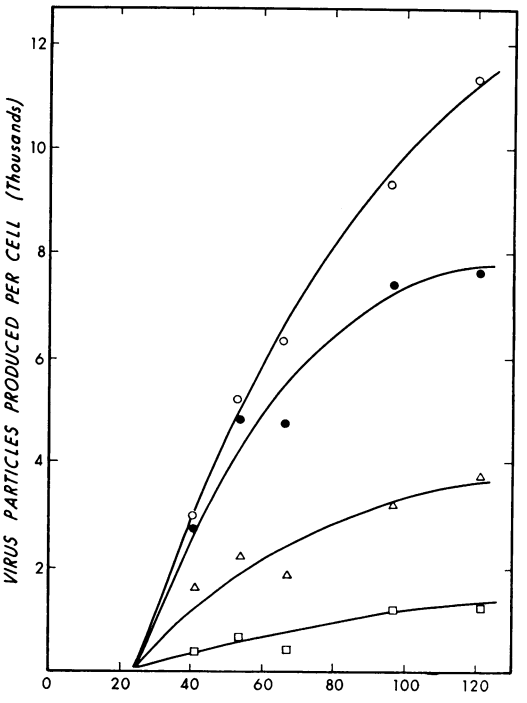

FIG. 1. *Interference by virus heated at 56 C for different times. Yield of vaccinia virus particles from cultures of L cells inoculated with a mixture of fresh and heated virus. The total input multiplicity was 5.1 particles per cell. Fresh (fully active) virus-particle multiplicity was 0.1. Control cultures, with only fresh virus* (○); *cultures with virus heated at 56 C for 25 min* (□), *90 min* (△), *and 180 min* (●).

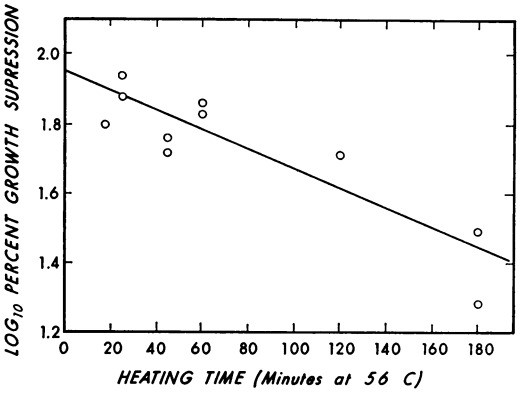

FIG. 2. *Effect of heat (56 C) on the power of vaccinia virus to induce homologous interference in L cells.*

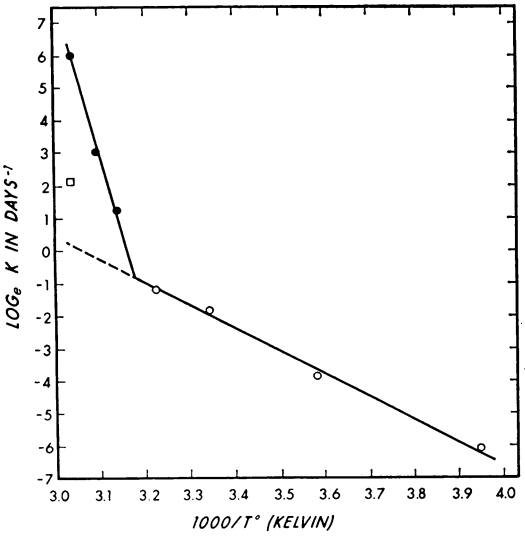

FIG. 3. *Effect of heat on the infectivity of vaccinia virus in the region of high (●) and low (○) temperature coefficient (k), the inactivation constant. The value of K for homologous interfering power is shown by the open square at the critical value of 56 C. It is the same as that for infectivity at 37 C.*

TABLE 1. *Effects of heat on vaccinia virus*

Virus	Heat treatment	Infectivity*	Interfering power†	Antibody-absorbing power‡
			%	
Late passage	Unheated	77	—	1:95
	56 C, 20 min	10^{-1}	76	—
	56 C, 45 min	10^{-4}	67	1:123
	56 C, 180 min	10^{-4}	31	—
	60 C, 15 min	10^{-4}	ND	1:170
	37 C, 29 days	10^{-4}	ND	1:180
Early passage	Unheated	1.4	—	1:80
	56 C, 45 min	10^{-4}	ND	—

* Plaques per 1,000 virus particles.

† Percentage reduction in yield of new virus from L cells inoculated at multiplicity M = 5.1 with a mixture of M = 5 heated virus and M = 0.1 fresh late-passage virus.

‡ Dilution of antiserum which, when treated at 37 C for 30 min with 5 × 10^8 virus particles, is still able to suppress, by half, the plaque formation by late-passage virus on L cells.

The strengths of antisera after absorption by equal numbers of early- and late-passage unheated virus particles were very similar, although the quality difference between these viruses was great (1.4 and 77 plaque-forming units per 1,000 virus particles, respectively). Although heating of late-passage virus for 45 min at 56 C leaves the particles with strong interfering power and reduces the plaque titer by 10^{-6}, it makes only a slight change in the antibody-absorbing powers (1:95 vs. 1:123, Table 1). The sensitivity of the test was checked by absorbing the serum with 10^8 instead of 5 × 10^8 virus particles per milliliter. Dilutions of these filtrate sera, required to suppress the plaque formation of test late-passage virus to one-half, were 1:563 and 1:123, respectively, which shows that the change in antibody absorption wrought by heating is small indeed. The samples heated at 60 C retained about half of their absorbing power after losing all detectable infectivity and all interfering power as well. Degradation at 37 C made no change in antibody-absorbing power. These data are summarized in Table 1.

Treatment of late-passage virus with ultraviolet rays (2,537 A) at an intensity of 33 $\mu w/cm^2$ for 5 min reduced the number of plaques on L cells by a factor of 10^6. When this irradiated virus was tested for antibody absorption in the same manner as the heated samples of Table 1, it was found to be little changed by the irradiation. End points (50%) were reached at a filtrate serum dilution of 1:127, which closely resembles others in the last column of the table.

DISCUSSION

When the rate constant of a reaction is a linear function of the reciprocal of the absolute temperature, it is generally taken to mean that the mechanism remains the same over that temperature range. Discontinuities such as that shown in Fig. 3 for the heat inactivation of the plaque-forming power of vaccinia virus and those mentioned in the introduction for foot-and-mouth disease virus, Rous sarcoma, and human cytomegaloviruses are clear evidence that the mechanism for decay is distinctly different above and below some critical temperature in the region of 40 C. This seems to be true for both dexoyribonucleic acid and ribonucleic acid viruses. The nature of possible mechanisms has been discussed by Woese (1960) and by Hiatt (1964), who consider the nucleic acid, rather than the protein, to be involved. Further evidence that the protein is not involved appears in Table 1, which shows that the antibody-combining property of the virus is relatively insensitive to heat in this temperature range. No discontinuous-reaction constants for the destruction of antigenic properties of a virus have come to our attention. In fact, the

antibody-combining power is very similar for vaccinia virus of early and late passage in L cells, as well as for that heavily irradiated with ultraviolet rays. These viruses are very different in both infectivity and interfering power (Table 1). The mechanism of homologous interference by heated virus may be very similar to that of infection, differing from it only in that some subtle damage has occurred in the nucleic acid. At temperatures below about 40 C, we found no detectable difference between the reaction constants for these two aspects of viral activity, but both shift to higher values above this temperature in a manner suggesting the involvement of the same structure whose preservation is essential to both activities but in differing degrees.

Some confusion exists with regard to the potency of different preparations of virus, which have been measured only with respect to their infectivity. Hanafusa (1960) stated, for example, that heat-inactivated vaccinia virus does not interfere with the multiplication of active virus if both are added to a cell culture at the same time. This statement, made without qualification, seems to deny the results described here for mixed inocula containing fresh and heated virus. Although Hanafusa doubtless thought that no qualification was needed, we have shown his statement to be true or false, for our virus, depending on the relative particle multiplicities used in the inoculum (Galasso and Sharp, 1963*a*; Sharp, 1963). In addition to relative multiplicities, it is necessary to be aware of the absolute level of virus particles per cell. When 100 or more heated or ultraviolet-treated virus particles are applied to each L cell, the cells exhibit a toxic reaction (Galasso and Sharp, 1963*b*). This is certainly beyond the useful range of multiplicity in which interference phenomena can be studied; still, it is easy to reach this domain inadvertently if virus is measured only in plaque-forming units. If there are, for example, 100 virus particles per plaque-forming unit, then a plaque-forming unit multiplicity of one would be toxic to the cells. Only an accurate virus particle count can insure unequivocal results in studies of homologous interference.

It is not surprising to find that heating of the WR strain of vaccinia virus at 56 C has little effect upon its ability to absorb antibody. The review of Woese (1960) provides ample evidence, of a similar nature, for other viruses. It is noteworthy, however, that heat treatments which reduce the plaque titer of both early- and late-passage viruses to the same extent leave the former inert, whereas the latter retains strong interfering power. Before heating, these viruses reproduce in L-cell cultures at about the same rate. They differ only in that late-passage virus produces 55 times more plaques per 1,000 virus particles than does early-passage virus. Thus, the ability of the early-passage virus to infect and grow well in the L cell is not sufficient to insure its performance, after heating, as an interfering agent. The late-passage virus of high plaquing efficiency is the one with homologous interfering power as well. This may be coincidence, but we suspect, rather, that it is evidence that plaque formation requires some property of the virus particle in addition to the minimal requirements for cell infection, a property which is associated with superior interfering power.

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