# Inhibitory Effect of Heated Vaccinia Virus on Growth of Vaccinia Virus in Earle's L Cells.* (26808)

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When cultures of L cells were inoculated with vaccinia virus at several different multiplicities (M), the yield of virus particles per culture cell varied in an inverse manner with multiplicity in the range M = 50 to M = 400(1). In order to learn whether this was caused by an excessive number of infectious particles(2) or to inhibitory effects of noninfectious virus in the inocula(3,4,5,6) experiments were done, and reported here, to test the latter possibility. Cultures of L cells have been inoculated with fresh virus preparations to which have been added known numbers of heat-treated virus particles. Other cultures have been treated with heated virus and later seeded with a challenge dose of active virus. Multiplicities and yields have been determined precisely by counting the virus particles in the electron microscope.

The observed inhibitory effects of the heated virus preparations are of such magnitude as to offer partial, if not complete, explanations of the reciprocal relationship cited above. They also introduce doubt regarding the validity of some titration methods for detecting residual active virus in heat-treated and possibly in otherwise degraded virus preparations.

*Materials and methods.* The vaccinia strain WR (mouse neurotropic) was obtained from the American Type Culture Collection and adapted to growth(1), in L strain cells obtained from Dr. Earle. Virus was kept in continuous passage in the cells with transfer every 48 or 72 hours. Inoculum was prepared by subjecting infected cells to 3-minute treatment with 9 kc waves.

Growth medium and details of the L cell culture have been previously described(1). Cells were scraped from the screw-capped tubes or prescription bottles in which they were grown and their number determined by means of a counting chamber. Virus particles were diluted with PBS and counted by the agar sedimentation method(7) in the electron microscope. For inoculum at a given multiplicity the virus and cells were mixed, in growth medium, in proportions calculated from the counts. These mixtures were sedimented immediately to bring virus and cells together in somewhat less than 7 minutes time(8). Heated virus was prepared by treating active virus in one ml quantities in screw-capped tubes immersed in a 56°C water bath for 45 minutes.

Just prior to inoculation all virus suspensions were treated 1½ minutes with 9 kc waves to disperse aggregates that form with standing. After sedimentation inoculation in the several centrifuge tubes, the supernatant fluid was decanted, the cells were then pooled, washed and replicate cultures set up in screw-capped tubes, each containing $10^6$ cells in 1 ml of growth medium. After various intervals of incubation at 37°C tubes were removed and placed at −20°C to arrest virus growth. At the end of the experiment all tubes were thawed. The cells were scraped off, sonic treated to release and disperse the virus which was then counted in the electron microscope.

The term "active virus" will mean fresh, unheated inoculum.

*Experiments and results.* Freshly prepared seed virus was heat treated and applied to L cells at a multiplicity of 10. One hour later the same cells received active virus at the same multiplicity. Control cells received active virus at M = 10 at the same time. All cultures were incubated together and the virus particle development can be seen in the graph, Fig. 1. Active virus increased to nearly 9000 particles per cell at 95 hours incubation. The same virus at the same multiplicity in cells pretreated with 10 M heated virus reached a maximum production of only

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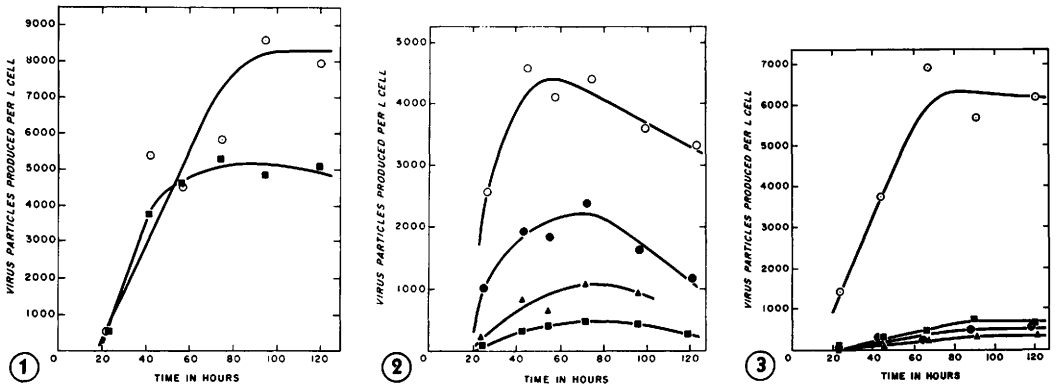


FIG. 1. Inhibitory effect of heated vaccinia virus on growth of active virus in L cells. Cultures of upper curve received active virus alone, M = 10. Those of the lower curve received M = 10 heated virus followed, 1 hr later, by M = 10 active virus.

FIG. 2. Inhibitory effect of ( M = 100) heated vaccinia virus on growth of active virus applied to L cells with several time delay periods. -○-○- Control with active virus only, M = 10. -●-●- No delay (heated and active mixed). -▲-▲- 1 hr delay. -■-■- 2 hr delay.

FIG. 3. Inhibitory effect of (M = 100) heated vaccinia virus on growth of active virus applied to L cells with longer time delay periods. -○-○- Control with active virus only, M = 10. -■-■- 2 hr delay. -●-●- 4½ hr delay. -▲-▲- 21½ hr delay.

5000 particles in 72 hours which did not increase on continued incubation to 120 hours. Pretreatment of L cells with 10 M heat-treated virus 1 hour before inoculation with active virus reduced the yield by almost half.

A second experiment of the same kind but with M = 100 heated virus was performed. Active virus multiplicity was 10. the same as in the first experiment. In one set of cultures it was applied together with the heated virus. In a second set there was a 1-hour delay and in a third set a 2-hour delay between application of heated and active virus. The 6 control cultures receiving 10 M active virus produced a curve (Fig. 2) reaching a maximum yield of 4400 virus particles per cell at about 54 hours. Heated virus at M = 100 applied with the active virus reached a maximum of 2200, about ½ of the first set, in 72 hours with the 1- and 2-hour delay cultures producing 1000 and 500 particles per cell respectively. The mechanics of inoculation are such that only 4 sets of 6 cultures can be handled at once, so investigation of longer delay times had to be done in a separate experiment. For this, fresh virus and cells were used and maximum of growth in the control cultures (6200 virus particles per cell) was reached at about 72 hours, Fig. 3. The yield with the cultures having 2-hour delay time was 700 per cell or 11% of maximum, the same as the result of the first experiment. Two other sets of cultures challenged with 10 M active virus after 4½ and 21½ hours delay reached peaks of yield of 500 and 330 particles per cell respectively.

Cultures inoculated with M = 100 heated virus alone showed no virus increase during 91 hours of incubation. There was, however, some growth of residual virus which reached 172 particles per cell at 115 hours incubation. Clearly, the heat treatment was not sufficient to sterilize the preparation but the amount of remaining activity was negligible compared with the challenge dose of 10 virus particles per cell.

To check the possibility that a very small inoculum of active virus followed by a second at 10 M with a 2-hour delay would produce a growth curve different from the control, such a test was made. The initial inoculum was 0.1 M. Maximum yield of both cultures was the same within counting error, $\sigma$ = 16%.

These results, Fig. 1, reveal the appreciable inhibitory effect exerted by 10 heat-treated virus particles per L cell upon the growth of an active, M = 10 inoculum applied 2 hours later. Greater dosage of heated virus, M = 100, (Fig. 2), has reduced the growth of the challenge virus to ½ when applied with it, to about ¼ when applied 1 hour before and to

about 1/10 when applied 2 hours before. In the separate continuation experiment, Fig. 3, the depression in yield for the 2-hour delay time was again about 90% and further small changes were observed when the delay time was increased to 4½ and to 22 hours. Apparently most of the inhibitory effect of this dosage is manifest at 4½ hours.

Examination of the infected cells in the electron microscope by the methods described previously(9) showed that all cells were grossly infected in control cultures. In cultures subject to inhibitor again all cells were infected but fewer virus particles could be seen per cell. This is not the type of observation that can be quantitated but it was clear that among the cells with 100 M and 2-hour delay time (Fig. 2) there was not one cell per 10 producing virus at maximum rate but rather all cells producing virus at a reduced rate.

Each point plotted in Fig. 1-3 was obtained from the average count from 5 electron micrographs. A total of 300 pictures was made and all were counted and used.

*Discussion.* These are the first data, as far as the authors are aware, which show the inhibitory effect of a preparation containing a known number of heat-treated vaccinia virus particles on a known number of tissue culture cells. Although the results are characteristic of interference as described for ultraviolet-treated virus(3,4) and by others for other virus-cell systems, the possibility that a toxic reaction is involved when 100 virus particles are put upon each cell cannot be overlooked. It is true that a relatively small number, (10 particles) does produce an appreciable inhibition. It is equally apparent that 100 virus particles per cell is a large enough dose to come within the range which is often regarded as toxic. Speculation on this point is probably less profitable than consideration of this inhibitory effect upon tests for residual virus in samples treated with heat and other inactivating agents.

In these experiments maximum virus particle yield was depressed as much as 94%. These findings clearly illustrate the difficulty involved in titrating a small residual virus activity in a heated preparation such as might be used for vaccine production. The adverse effect of heated virus upon susceptible L cells has been shown. Tests have shown that this inhibition is not caused by the minute amount of active virus remaining in the heated preparation. Consequently it is necessary to reexamine titration methods designed to detect and measure residual active virus in preparations which have been heat treated or possibly inactivated by other means. A preponderance of heated virus which reduces the ability of the test cells to produce virus will be expected to alter the end point in tissue culture titrations of either $LD_{50}$ or plaque procedures although perhaps not to the same extent. It is probable that, in such titrations, one is not learning simply the number of active virus particles but rather some subtle ratio of active to inhibiting particles; a ratio that may be quite different from one cell type to another and which would be expected to change if the inhibitor effect is itself heat labile. These remarks are not intended to cast doubt upon all titrations but only upon those in which there is opportunity for a preponderance of non-infective but inhibitory virus to predispose the test cells to reduced response.

It should be emphasized that the challenge dose of active inoculum employed in these tests, M = 10, is sufficient to incite new virus production at the rate of 130 particles per cell per hour 20 hours after centrifugal inoculation of susceptible cells. When such infected cultures have reached maximum yield all the cells can be seen, by electron microscopy, to be grossly infected. Inhibited cultures do not have even a few grossly infected cells. All seem to be producing virus but in lesser quantity.

*Summary.* Heated vaccinia virus has been applied to cultures of L cells followed by active virus. Multiplicities, determined by electron microscope particle count, were 10 and 100 for heated virus and 10 for challenge, active virus. Delay times ranged from 0 to 21½ hours. Substantial inhibition was observed with inoculation of heated virus at M = 10. At M = 100 the growth of a challenge inoculum applied 21½ hours later was 94% suppressed. The consequences of these

findings are discussed in connection with titration of residual virus in heat-treated virus preparations.

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1. Smith, K. O., Sharp, D. G., *Virology*, 1960, v11, 519.

2. Brown, A., Mayyasi, S. A., Officer, J. E., *Bact. Proc.*, 1957, p75.

3. Matumoto, M., Shinkawa, E., *C. R. Soc. de Biol.*, 1956, v150, 1062.

4. Nagano, Y., Kojima, Y., Sawai, Y., *ibid.*, 1954, v148, 750.

5. Brown, A., Mayyasi, S. A., Officer, J. E., *J. Inf. Dis.*, 1959, v104, 193.

6. Bernkopf, H., Nishmi, M., Rosin, A., *J. Immunol.*, 1959, v83, 635.

7. Sharp, D. G., *Proc. 4th International Cong. for Electron Microscopy*, Berlin, 1958, pp542-548.

8. Sharp, D. G. Smith, K. O., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 167.

9. Smith, K. O., Sharp, D. G., *Virology*, in press.

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## Biochemical Significance of Serum Glycoproteins.* I. Changes in Rat Serum Following Injury. (26809)

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A wide variety of non-specific traumatic conditions(2) are known to cause alterations in the proportions of serum proteins. Such changes in human serum have been shown by electrophoretic studies to involve a reduction in the albumin level accompanied by elevations in $\alpha_1$ and $\alpha_2$ globulins(2-8). In a number of diseases increased proportions of hexosamines were also observed to migrate electrophoretically as the $\alpha_1$ and $\alpha_2$ globulins(7). Increases have been reported in the seromucoid fraction and in total protein-bound carbohydrates(8). These observations show that the glycoproteins are important factors in the alterations of the serum proteins in the diseased state.

Changes are also known to occur in the serum proteins of laboratory animals after trauma(9-11), thus experimental injury in rats results in elevations of $\alpha_2$ and $\beta$ globulins(12). The total serum hexosamine level reaches a maximum value 2 to 4 days after trauma(13). According to Boas and Peterman(13) this response to injury is eliminated if the animals are kept on a starvation diet. Werner(14) prevented the elevation of total serum hexosamine in rabbits by chemically destroying the liver tissues. The elevated total serum hexosamine levels probably reflect the increased $\alpha_2$ and $\beta$ globulins, much as described by Bollet for various pathological conditions in humans(7). No direct observations, however, of the distribution of hexosamines following experimental injury have been reported. In this paper are described the changes in electrophoretic distribution of the hexosamine-containing glycoproteins in the serum of rats subjected to 2 standardized injuries.

*Methods.* Male rats (Yale, Wistar, and Holtzman strains) weighing between 200 and 300 g were injured for the "open wound" study by removing a 2.5 cm circle of skin just caudal to the scapulae (ether anesthesia) or for the "sponge" study by implanting sub-

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