# Role of Heated Adenovirus 2 in Viral Interference.* (30582)

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The isolation of a viral inhibitor from adenovirus 2-infected RHF-1 cells in which the cells were not rapidly destroyed by this agent but the inhibitor was produced during the sublethal infection of the cells has been previously reported(1). The inhibitory activity of this material could not be removed by ultracentrifugation, but approximately 60% of its activity could be abolished by treatment with adenovirus 2 hyperimmune serum (unpublished). It was later discovered that the inhibitor contained moderate amounts of adenovirus complement-fixing antigen, indicating perhaps its analogy to that of Pereira's(2) inhibitor or the type-specific antigen.

In the present study, adenovirus 2 heated at 2 different temperatures (56°C and 100°C) for various times elicits resistance in normal RHF-1 cells to infection with vaccinia. However, the resistance of 100°C heated adenovirus 2-RHF-1 system seems to be mediated by an active principle (interferon?), while the 56°C heated adenovirus 2-RHF-1 model fails to induce the formation of an interfering principle. The investigation reported here was, therefore, undertaken: (1) to determine the basis of resistance to vaccinia in RHF-1 cells induced by adenovirus 2 heated at 56°C and 100°C; and (2) to describe the relationship between time of exposure of adenovirus 2 to 2 different temperatures and its inhibitory effect on vaccinia in cells of RHF-1 cultures.

*Materials and methods.* The procedures and materials employed for cultivation of RHF-1 cultures and for assay of vaccinia plaques as well as infective center formation have been described(1).

*Heating of adenovirus 2.* Adenovirus 2 infected fluids (1st RHF-1 passage) were placed in tightly stoppered tissue culture tubes and immersed in an artificially heated water bath (56°C or 100°C) for various periods. Control fluids consisting of RHF-1 cell lysates heated in a similar manner were also included. After heating, the fluids were slowly cooled at room temperature, followed by light centrifugation (1000 rpm; 20 min) before use.

*Assay of interferon.* The assay was performed in RHF-1 plaque bottles (3 ml of undiluted or diluted interferon/bottle) using vaccinia as the test virus according to the method described previously(1). Percentage plaque inhibition was expressed on the basis of plaque counts in control cultures.

*Results. Inhibitory effect of adenovirus 2 heated at 56°C for various times.* It was observed on several occasions that the addition of heat-inactivated (56°C, 1 hr) adenovirus 2 fluids (1st RHF-1 passage) to RHF-1 cell cultures would result in protection against vaccinia plaque formation. Experiments were performed to see whether resistance to vaccinia could also be induced in these cells by treating the cultures with adenovirus samples heated at the same temperature for much shorter periods. Briefly, the RHF-1 plaque bottles were exposed to adenovirus 2 preparations heated at 56°C (an equivalent of 0.1 to 0.8 $TCD_{50}$ of inactivated virus/cell) for 5, 15, 25, 35, 45, and 65 minutes, respectively for 20-24 hours at 37°C. Comparable control cultures treated with RHF-1 cell lysates heated at 56°C for various times were also included in these experiments. The heated virus preparations and control fluids were removed, and the cultures were thoroughly washed prior to challenge with vaccinia (50-70 PFU) at 37°C for 4 hours. After decanting the unadsorbed vaccinia and washing the monolayers, the plaque bottles were overlaid with overlay-nutrient mixture and then incubated at 37°C for 3 to 5 days. The inhibitory activity of the test samples was

* Supported by USPHS under General Research Support Grant #127, the Institutional Grants Committee of Am. Cancer Soc. (IN-9F), and Univ. of Illinois Graduate Trust Grants.

† USPHS postdoctoral Fellow (1-F3-CA-19,581-01).

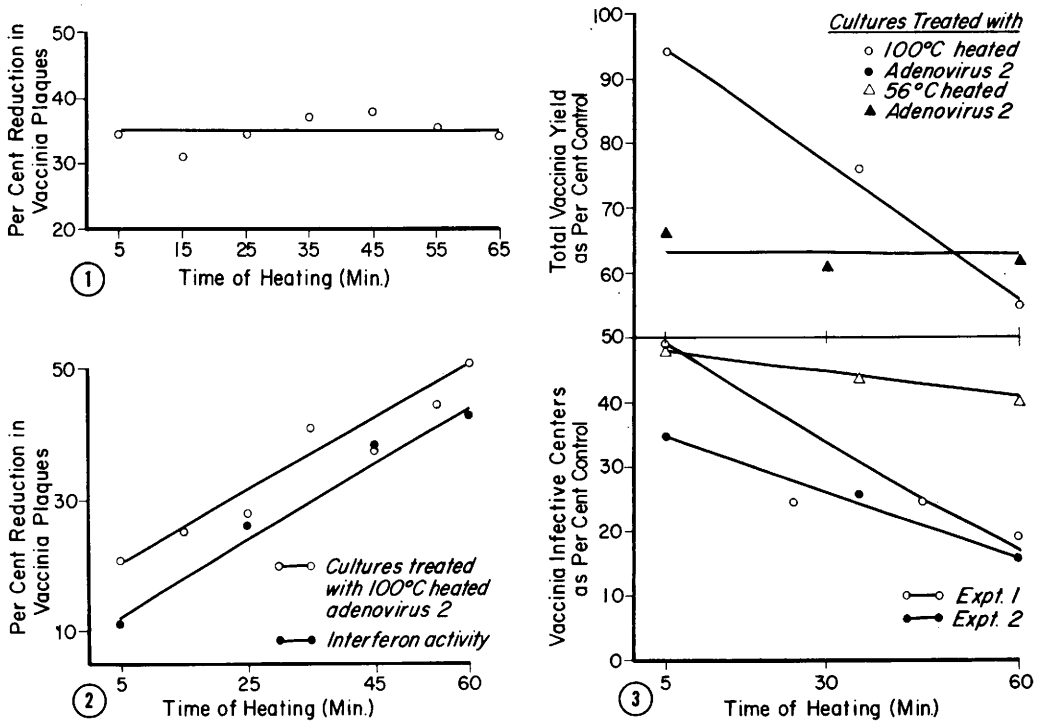


FIG. 1. Effect of heating time of adenovirus 2 at 56°C on development of resistance against vaccinia in cells of RHF-1 cultures. Two separate experiments with identical protocol were conducted using 3-4 RHF plaque bottles per sample in each experiment. Each point represents the average mean of plaque inhibition of 2 experiments.

FIG. 2. Percentage of vaccinia plaque inhibition in adenovirus 2-RHF-1 model as a function of heating time of virus at 100°C (upper curve). Increase in interferon activity as a function of heating time of adenovirus 2 at 100°C for various times based on experiments described in text (lower curve). The points for each curve represent average mean of plaque inhibition of 2 separate experiments with identical protocol.

FIG. 3. Resistance induced against vaccinia in RHF-1 cells pretreated with adenovirus 2 heated at 56°C or 100°C for various times. See text for details. Upper portion of graph: Inhibition of 24-hr total vaccinia yield in resistant cells relative to their controls. Each point represents the average mean of 2 separate experiments with identical protocol. Lower portion of graph: Inhibition of vaccinia yielders of resistant cells in relation to their controls. Values of 2 separate experiments for 100°C-heated adenovirus-RHF-1 model are given to show that inhibition rate appeared to be somewhat different from one experiment to another. Values for 56°C-heated adenovirus-RHF-1 model are from a single experiment.

assayed by the plaque reduction method. It can be seen from Fig. 1 that viral suspensions heated at 56°C exerted virtually the same degree (34 to 37%) of inhibition against vaccinia plaque formation regardless of the length of time they were exposed to heat. Inhibitory activity of 12 to 20% was also present when the heated fluids were diluted 1:4. These experiments clearly indicate that the degree of protection achieved in RHF-1 cells by a given heated adenovirus sample is not dependent on the duration of treatment of virus at 56°C.

Further experiments were carried out to determine whether the interfering effect of 56°C heated adenovirus noted was a property of the virus particles *per se* or the soluble substance(s) in the virus suspension. It was found that supernatant obtained after heating and ultracentrifugation (110,000 × *g* for 2 hours) inhibited vaccinia plaque formation to the same extent as the non-ultracentrifuged material. This could only mean that direct participation of virus particles was not necessary for the establishment of interference. That 56°C heated virus fluids or fluids free of virus particles exert their viral inhibitory activity at an intracellular site was

verified by the fact that the per cent of challenge vaccinia adsorbed was virtually the same in both treated and susceptible cultures.

*Absence of an interferon-like substance in cells treated with 56°C heated adenovirus 2.* To determine whether the virus inhibitory effect of 56°C heated adenovirus 2 preparations might possibly be due to cellular interferon production in response to the heated virus, a series of experiments was performed to test this hypothesis. It was found that no interferon-like substance could be recovered from monolayers by lysing the RHF-1 cultures 20-24 hours after the treatment of cells with the heated virus for 4½-5 hours at 37°C, nor could its production in cells be stimulated when the incubation time was extended from 24 to 48 or 72 hours following initial treatment of cells with the heated virus. Furthermore, negative results were also obtained when the initial incubation of heated virus with the cells was extended to 24 hours.

*Presence of an interferon-like substance in cells treated with 100°C heated adenovirus 2.* In order to see if an interferon-like substance could be implicated in the establishment of interference in the 100°C heated adenovirus 2-RHF-1 system, experiments were performed to test this hypothesis. In this connection, it was interesting to discover that an interferon-like substance normally undetectable in the cells of RHF-1 cultures could be released from the cells by rapidly freezing and thawing the cultures (6 times) 20 hours after incubation of cells with the heated adenovirus preparations (100°C for 1 hr) at 37°C for 4½-5 hours. Interferon obtained under these conditions though usually of low activity, was capable of imparting resistance in normal RHF-1 cells to infection with vaccinia but had no *in vitro* effect on the virus. No such antiviral interferon could be found either in normal RHF-1 cells or in cells pretreated with normal RHF-1 lysates heated at 100°C for 1 hour. These findings strongly suggested that a specific viral inhibitor (interferon?) rather than a preformed normal inhibitor was most likely responsible for the resistance of RHF-1 cultures.

To elucidate further the nature of the interference phenomenon observed in this system, and possibly to consider the fact that such findings might well reflect a definite difference in the capacity of cells to synthesize interferon depending upon the duration of heating time of the adenovirus, the following experiments were conducted:

Virus suspensions derived from the first RHF-1-passaged adenovirus 2 were treated at 100°C for specific times ranging from 5 to 60 minutes. Samples were allowed to react on RHF-1 monolayers (equivalent of 0.2 to 0.5 $TCD_{50}$ of inactivated virus/cell) for 4½-5 hours at 37°C. Each virus sample was accompanied by an appropriate control sample (heated RHF-1 lysates were used in place of heated virus). Following incubation, the heated viral or control inocula were decanted and the cells were washed before maintenance medium (10 ml/bottle) was added to each culture, followed by incubation at 37°C for 20 hours. The interferon content of each appropriately treated culture was then released by freezing and thawing the cells in their media. The antiviral activity of these undiluted fluids was assayed by the plaque reduction method.

Analysis of this phenomenon indicated that 100°C heated virus stimulated the RHF-1 cells to produce varying amounts of an interferon-like substance. The plaque inhibitory effect of the interferon was linearly increased when the time of heating was extended (Fig. 2). That the rate of vaccinia inhibition was dependent on the concentration of interferon in the cells was borne out by the fact that by diluting the interferon preparations obtained from cells treated with virus heated at 100°C for one hour inhibitory activity could be proportionately reduced. In spite of these findings, the possibility still remains that virus heated at 100°C for 5 minutes might be inducing interferon in a small fraction of the cell population as compared with virus which has been heated at 100°C for one hour. Unfortunately, it would be difficult to devise experiments to test this postulate. Nevertheless, the present results strongly suggest that an interferon-like substance is primarily responsible for the resistance of the RHF-1 cells, and its formation is no doubt due to a reaction of cells to a particular

constituent(s) of virus particles. This point will be elaborated further in the discussion.

*Comparison of proportion of vaccinia yielders of 56°C- and 100°C-heated adenovirus 2-cell system.* To test whether the 2 different patterns of resistance induced by 56°C and 100°C heated adenovirus 2 are reflected in the proportions of the specifically treated cells in the cultures, the following experiments were conducted:

Briefly, replicate RHF-1 culture tubes were incubated at 37°C for 24 hours with adenovirus heated at 56°C or 100°C for one hour (an equivalent of 0.3-0.8 $TCD_{50}$ of inactivated virus/cell). Control fluids heated at the same temperatures for one hour (RHF-1 lysates 1 ml/tube) were included. After removing the inocula, the cultures were washed and then infected with vaccinia (8-12 PFU/cell) at 37°C for 4 hours. The unadsorbed virus was removed and the cells were washed with maintenance medium containing vaccinia antiserum (1:30 dilution) and then again with Earle's BSS. The cells were then dispersed with trypsin (0.125%), centrifuged, washed, counted, and then resuspended in prewarmed maintenance medium. Known numbers of infected cells were plated onto each of triplicate or quadruplicate RHF-1 plaque bottles for vaccinia infective center formation. Per cent inhibition in infective center formation for resistant cells in relation to their specific control cells is given in Fig. 3. It can be seen that with increasing time of heating (100°C) of prechallenge virus there was a moderate but progressive inhibition in vaccinia infective center formation (Exp. 1, 50 to 80%; Exp. 2, 65 to 80%), indicating that vaccinia suppression was greater in cells treated with virus heated at 100°C for one hour than for 5 or 30 minutes. Furthermore, only a slight difference (8%) in inhibition was noted when similar experiments were conducted with 56°C heated adenovirus-RHF-1 cells.

*Suppression of vaccinia synthesis in 56°C- and 100°C-heated adenovirus 2-cell systems.* Experiments were next designed to see if the results obtained in the preceding experiments were a reflection of inhibition or decrease in virus synthesis relative to each specifically treated adenovirus-cell system.

In this series of experiments, replicate RHF-1 culture tubes were treated and challenged in exactly the same manner as those monolayers tested for infective center production except that after decanting the challenge virus and washing cells, the cultures were overlaid with maintenance medium (1 ml/tube) followed by incubation at 37°C on a roller drum. After 24 hours of incubation, the cells were frozen and thawed 6 times and the total virus yield was determined by subinoculation of the appropriate virus dilutions into quadruplicate RHF-1 plaque bottles. It can be seen from the results (Fig. 3) that the rate of viral inhibition in cultures was minimal (10%) when cells were pretreated with adenovirus heated at 100°C for 5 minutes, and maximal (45%) in cells treated with virus heated similarly for one hour. These results could be due only to the level or quantity of interferon in the cells. As heating time increases, virus synthesis is inhibited in proportion to the amount of interferon produced in the cells. On the other hand, the rate of vaccinia inhibition in 56°C heated adenovirus-RHF-1 cells remained virtually unchanged irrespective of length of heating time of the virus.

*Discussion.* Evidence obtained from the foregoing experiments indicates that there are some fundamental differences between the interference patterns of 56°C- and 100°C-heated adenovirus 2-RHF-1 systems. First, the cells treated with 56°C heated adenovirus 2 do not show significant change in degree of resistance to vaccinia despite the length of heating time of adenovirus 2, while the cells treated with 100°C heated adenovirus 2 show decreasing susceptibility to vaccinia as the time of heating of adenovirus is increased from 5 to 60 minutes. Second, an interferon-like substance can be isolated from cells treated with 100°C heated adenovirus 2 but not 56°C heated virus. Although interferon production in this system is relatively low, its formation nevertheless can be consistently demonstrated by the present approach. Third, the rates of inhibition of vaccinia plaques, the total 24-hour vaccinia yield, and the vaccinia yielders in 100°C heated

adenovirus 2-RHF-1 cultures were found to be closely associated with the increase in heating time of pre-challenge virus at 100°C, while the same parameters for 56°C heated adenovirus-RHF-1 system remained virtually unchanged (except vaccinia yielders) irrespective of heating time of the virus at 56°C. On the other hand, a common feature pertaining to inhibition as well as suppression of vaccinia as judged by all criteria was evident in both systems. The central fact remained, however, that adenovirus varies in the manner in which it functions in viral interference after exposure to 2 different heating temperatures. For example, the 56°C heated adenovirus suspensions before or after ultracentrifugation were equally effective in inducing resistance against vaccinia in the cells of RHF-1 cultures. Since heating at such temperatures does not alter the antigenic reactivity of the adenovirus antigens, it is most likely that the E or C antigen as originally reported to have viral inhibitory effect in Hela cultures(2) and recently shown to inhibit viral multiplication by its effect on the macromolecular synthesis(3) is the factor responsible for viral inhibition. It would be interesting to know though whether the adenovirus fiber antigen acts in much the same way as other specific viral inhibitors in blocking the viral synthesis. Conversely, the 100°C heated adenovirus not only triggers the production of an interferon-like substance in the cells but its gradual formation seems to depend on the duration of heating. Why the destruction of virus by increased heating at 100°C is more favorable to the production of interferon may be partially due to the increased availability of the viral DNA in some form or another, followed by an increase in the uptake of the inducing principle(s) by the cells. This might not be an unreasonable explanation since heating of adenovirus preparations at 100°C for 1 hour, despite the presence of non-viral impurities, could well lead to complete separation of the viral DNA strands as well as fragmentation of the strands since its Tm has been reported to be around 92°C(4). The viral DNA molecules, presumably partially renatured molecules, could then stimulate the synthesis of this interferon since interferon production can be stimulated by foreign nucleic acids or nucleotides(5,6,7). On the other hand, it could be argued too that such crude preparations after heating and cooling include more than renatured viral DNA, for example, denatured cellular as well as viral nucleic acids, which might well have a role in interferon production. In order to show that interferon formation is due to the action of viral DNA itself or to a change in the state of viral DNA similar experiments should be conducted using pure viral nucleic acid. Whatever the exact nature of the inducing principle might be, it is interesting to note that the duration of heating of adenovirus is one predominant factor in determining the rate of interferon formation.

Obviously, the inhibitory power of 56°C heated adenovirus 2 is due to a different mechanism than that involved with 100°C heated virus. Biochemical experiments presently underway could well permit us to test the validity of this conclusion.

*Summary.* The response of 56°C heated adenovirus 2-protected RHF-1 cells to infection with vaccinia did not appear to depend upon the heating time of the pre-challenge virus, and this resistance was not mediated by an interferon-like substance. A completely different picture was obtained when corresponding experiments were performed with adenovirus heated at 100°C. The 100°C heated adenovirus not only induced the formation of an interferon-like substance in the cells but its production also increased with the duration of heating. The difference in rate of vaccinia inhibition in the cells treated with adenovirus 2 heated at 100°C for increasing time was reflected in the amount of interferon-like substance produced in the cells. Taken as a whole, these data suggested strongly that interferon provides perhaps most of the basis for cellular resistance in this system. The results bring out fundamental differences between the two systems which presumably have different mechanisms.

The authors are grateful to Miss Aldona Avizienis for her capable assistance.

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Received July 19, 1965. P.S.E.B.M., 1965, v120.

## Inhibition of Amino Acid Incorporation *in vitro* by Metabolites of 2-Acetylaminofluorene and by Certain Nitroso Compounds.* (30583)

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Arrhenius and Hultin(1-3) have shown that administration of large amounts of 2-aminofluorene (AF), 2-acetylaminofluorene (AAF), or of certain other carcinogenic amines or amides to rats inhibited the ability of liver preparations to incorporate labeled amino acids into protein when the animals were killed up to 8 hours later. After 8 hours the *in vitro* incorporation of labeled amino acids increased to above normal levels as a result of a corticoid-stimulated mechanism. Inhibition of amino acid incorporation into protein could also be demonstrated when rat liver slices were preincubated with these amines or amides prior to incubation with radioactive amino acids(3,4).

These results suggested that metabolites of these amines and amides were responsible for the inhibition of amino acid incorporation. The present studies were undertaken to determine which metabolites of AAF and AF inhibited amino acid incorporation when added to a rat liver microsome-pH 5 fraction system. 2-Nitrosofluorene, which has been detected as a metabolite of *N*-hydroxy-AAF by Irving(5) and which must therefore also be a metabolite of AAF and AF, inhibited amino acid incorporation strongly. Similar strong inhibition was obtained with nitrosobenzene and 2-nitrosotoluene; these compounds proved not to be carcinogenic when administered subcutaneously under conditions whereby 2-nitrosofluorene is carcinogenic(6).

*Materials and methods.* Weanling male rats (Holtzman Rat Co., Madison, Wis.) were killed by decapitation, and 10 g of liver was homogenized in 40 ml of medium A of Hawtrey *et al*(7) (0.25 M sucrose, 0.005 M $MgCl_2$, 0.025 M KCl, and 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.6). The nuclei and mitochondria were sedimented at 8,500 × *g* for 15 minutes, and the supernatant fraction was recentrifuged at 105,000 × *g* for 1 hour in a Spinco Model L centrifuge. The microsomal pellet was washed, resuspended, and recentrifuged(7), and finally resuspended in 15 ml of medium A. The pH 5 fraction was precipitated from the microsomal supernatant by the addition of 1 N acetic acid to a pH of 5.2, centrifuged, washed(7), and resuspended in 15 ml of medium A. The protein content of each preparation of microsomes and pH 5 fraction was determined according to Cleland and Slater (8).

For the amino acid incorporation assays each tube contained in a total volume of 1 ml 0.5 μmole guanosine triphosphate, 2.25 μmoles adenosine triphosphate, 18 μmoles phosphoenolpyruvate, 50 μg pyruvate kinase, 35 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.6, 9 μmoles $MgCl_2$, 18 μmoles KCl, 190 μmoles sucrose, 10 μl dimethylsulfoxide (containing test compound), and 1.2-2.5 mg each of microsomal and pH 5 fraction pro-

* This investigation was supported by Grants CA-07175 and CRTY-5002 of Nat. Cancer Inst., U. S. P.H.S.; by a grant from the Jane Coffin Childs Memorial Fund for Medical Research; and by the Alexander and Margaret Stewart Trust Fund.