Adenovirus Susceptibility to Interferon: Sensitivity of Types 2, 7, and 12 to Human Interferon* (33506)

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There is a striking lack of published data on the susceptibility of adenoviruses to interferon. Despite the fact that no definitive evidence is available, adenoviruses have been generally regarded as being relatively insensitive to interferon mediated interference (1–5). A single exception is the report by Glasgow *et al.* (6) which presented some evidence that adenovirus Type-4 cytopathic effect (CPE) could be somewhat inhibited by interferon, but no infectivity titers were reported, leaving the issue less than resolved.

The present report describes experiments designed to examine the interferon sensitivities of representative adenovirus (Ad.) types from each of the three subgroups based on their oncogenic potential proposed by Green (7). Accordingly, Ad. Type-2, a "nononcogenic" type; Ad. Type-7, a "weakly" oncogenic type; and Ad. Type-12, a "strongly" oncogenic adenovirus were tested by means of plaque reduction assays. Each virus type showed substantial plaque reduction in the presence of human interferon. In addition, Ad. Type-2 produced reduced yields of infectious virus in the presence of rabbit interferon. The findings demonstrate that at least some adenovirus types are susceptible to interferon although to a clearly lesser extent than are most other viruses.

Materials and Methods. Interferon induction. The Barnes strain of mumps virus (obtained from Dr. Freidrich Deinhardt) was used to induce interferon in cultured human embryo fibroblasts (HEF). The HEF cell cultures in 32-oz prescription bottles were infected at a multiplicity of infection of approximately 2 EID_{50}/cell. After 2 hr adsorption at 37° the cultures were washed twice with maintenance medium (MEM supplemented with 2% fetal bovine serum) and finally overlaid with 25 ml of this medium. Interferon was harvested 24 hr after adsorption of mumps virus.

Influenza A (strain WSN), originally obtained from Dr. George K. Hirst and passaged repeatedly in embryonated eggs by Dr. J. Emerson Kempf, was used to induce interferon *in vivo* in domestic rabbits. The virus was first irradiated as a thin layer of fluid in 100 mm petri dishes under a Westinghouse Sterilamp. A dose of 5.8×10^3 ergs/cm^2 for 2 min was optimum for interferon induction and sufficient to destroy all infectivity. An influenza inoculum equivalent to 5×10^{10} EID_{50} before irradiation was injected intravenously into 5-kg rabbits and serum was obtained 12 and 24 hr later. Both human and rabbit interferons were nonsedimentable at 110,000*g* for 2 hr, nondialyzable, stable at pH 2 for 48 hr at 4°, trypsin sensitive, and heat (70°) stable.

Interferon assays. Human tissue culture interferon and rabbit serum interferon preparations were acidified and dialyzed at 4° for 48 hr before being neutralized, again dialyzed, and assayed. Human interferon was assayed in human embryonic kidney (HEK) cell cultures by vesicular stomatitis virus (VSV) plaque reduction. Rabbit serum interferon preparations were assayed in cultures of a rabbit heart cell line, RHF-1 (8), by vaccinia plaque reduction.

Challenge viruses. Adenovirus Type-2 was isolated by one of us (N.K.) from a patient with diarrhea at Indiana Univ. Medical Center, Type-7 (strain Pinckney) was obtained from Dr. M. R. Hilleman; prototype-12 (strain Huie) was obtained from Dr. John J. Trentin. These strains have been passaged numerous times in HEp-2 or KB cell cul-

* Some aspects of this investigation were presented at the 68th Annual Meeting of the American Society for Microbiology, Detroit, Michigan, May 6, 1968. Supported in part by the American Cancer Society, (Illinois Div.)

tures to obtain high titer virus stocks. Vesicular stomatitis virus (Indiana strain) was used in titrating human interferons. The VSV stocks were prepared and assayed in HEp-2 cells. A calf lymph strain of vaccinia virus, obtained from Dr. Joel Warren, was used in rabbit interferon assays. Vaccinia stocks were prepared and titered in RHF-1 cultures.

Plaque assays. Adenovirus plaque assays were carried out in HEp-2 cells (stock virus titrations) or in HEK cells (interferon assays) using a modification of the procedure of Rouse *et al.* (9). Confluent monolayer cultures were obtained 5 days after plating 2 $\times$ 10^6 cells in 5 ml of growth medium in 60-mm plastic petri dishes. Cultures were washed once with Hank's balanced salt solution (BSS) before they were inoculated with 0.2-ml volumes of appropriate dilutions of stock virus. Adsorption was allowed to proceed for 2 hr at 37°. The monolayers were then washed twice with BSS and overlaid with 5 ml of a 1% agar solution containing 4.5% calf serum, 0.3% lactalbumin hydrolysate (LAH), and 0.1% yeast extract. The plates were incubated at 37° in a humidified 5% CO_2–air mixture. At intervals of 4 days, plates were overlaid with additional aliquots of the same medium. A final 2.5-ml aliquot of this medium containing 0.0075% neutral red was added to the plates 24 hr prior to the expected development of adenovirus plaques. Plaques were counted on the first, third, and fifth days after addition of neutral red. Plaque counts usually increased by approximately 10% from the first count to reach a plateau on the fifth day following the addition of neutral red. Ad. 2 plaques in HEp-2 cell cultures first appeared 8 days after inoculation and Ad. 7 plaques appeared after 15 days incubation, while Ad. 12 plaques were not observed until 16 days after inoculation. All adenovirus types tested produced plaques in HEK cell cultures which appeared approximately 48 hr earlier than in HEp-2 cell cultures, and which spread more rapidly.

Vaccinia plaque assays were done in HEK or RHF-1 cell cultures in 60-mm plastic petri dishes according to the method described above except that the overlay contained 0.6% agar and plaques appeared after 48-hr incubation. The VSV plaque assays were done in HEK cell cultures in 3 oz prescription bottles. Plaques were counted 16 hr after VSV adsorption. The staining procedure of Holland and McLaren (10) was used to demonstrate vaccinia and VSV plaques.

Cell cultures. The HEp-2, RHF-1, and HEK cell cultures were grown in medium 199 supplemented with 8% calf serum and 0.1% LAH. The HEF cell cultures were grown in MEM supplemented with 15% fetal bovine serum, and were maintained on the same medium supplemented with 2% fetal bovine serum.

Results. Sensitivity of adenoviruses to human interferon. Before examining the sensitivity of adenoviruses to human interferon, stock tissue culture interferon preparations were titrated in HEK cell cultures using standard plaque reduction assays and 200 pfu challenge doses of either VSV or vaccinia. Titers were expressed as the reciprocal of the highest interferon dilution which reduced by 50% the number of plaques formed in control cultures. By this method human interferon stocks titered approximately 4000 units/ml against a VSV challenge and 64 units/ml when a vaccinia challenge was used. While human interferon preparations were titrated by VSV plaque reduction during each experiment, only the plaque reduction curves for vaccinia virus which is moderately resistant to interferon are included in all of the graphs. Quantities of human interferon which partially reduced adenovirus plaque counts totally inhibited VSV plaque formation, thus rendering comparison of the effects of various doses of human interferon on different adenovirus types most difficult.

To study the sensitivities of adenovirus types to interferon, experiments were carried out using groups of five replicate HEK cultures, treated with 2-fold dilutions of human interferon or control fluid (spent tissue culture medium) for 16 hr at 37°. These cultures were then challenged with 100 pfu of an adenovirus or vaccinia. Significant reduction in plaque formation was observed in all cultures protected with 125 or more units

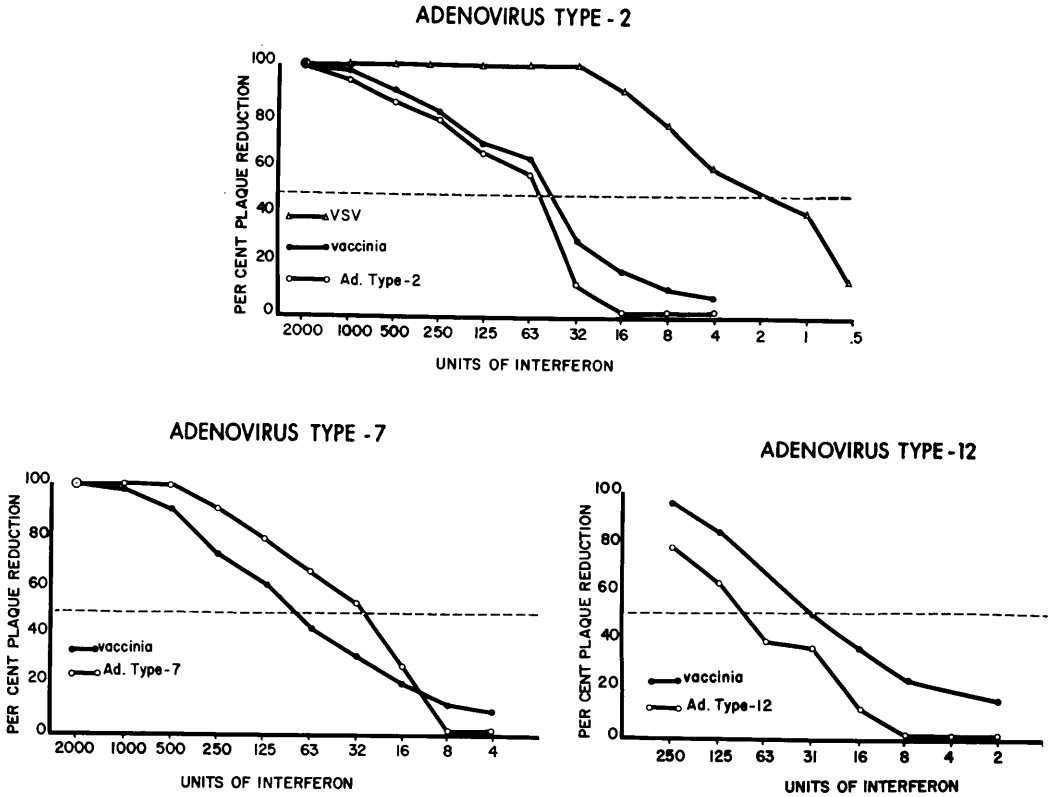

FIG. 1. Sensitivity of adenovirus Types 2, 7, and 12 to human interferon in HEK cell cultures. Comparison of interferon sensitivities of adenovirus Types 2, 7, and 12, (○); vaccinia virus, (●); and vesicular stomatitis virus, (△); plotted as percentage of plaque reduction in interferon-treated cultures. Three to five cultures were used for each interferon dose. Each culture received a given number of units of interferon contained in 2 ml of maintenance medium. Each adenovirus exhibits a relatively low susceptibility to interferon similar to that of vaccinia, another DNA virus moderately resistant to interferon-mediated interference.

(based on VSV plaque reduction) of interferon. Figure 1 shows the patterns of interferon-mediated interference for Ad. Types 2, 7, and 12. Similar patterns of interference were noted for each virus type and remained relatively constant when different lots of human interferon were used in numerous separate experiments.

An unusual observation was made with respect to the development of Ad. 2 plaques in interferon-treated HEK cell cultures. While the plaques in control plates appeared on day 6 and increased slightly in number until day 10 after infection, the Ad. 2 plaques did not appear in HEK cultures treated with large quantities of interferon (125 or more units) until about day 10, by which time plaques in control cultures had reached their final counts and had begun to spread somewhat. Thus moderate concentrations of interferon not only reduced the number of adenovirus plaques, but also delayed the appearance of plaques by at least 4 days. While viral interference mediated by interferon is usually manifested by reduction in number and size of plaques (11), the long delay in appearance of Ad. 2 plaques seemed to be but another manifestation of this interference. Delays of only 1 day were observed in the appearance of Ad. 7 and Ad. 12 plaques in interferon-treated cultures. The explanation for this difference in findings is unknown, but may be related to the fact that both Ad. 7 and Ad. 12 require more than twice the time required

by Ad. 2 for plaque formation.

It was a first thought that the late-appearing Ad. 2 plaques which develop in the interferon-treated HEK cultures might represent an interferon-resistant "persistent fraction" as described for encephalomyocarditis virus, VSV, and vaccinia by Takemoto and Baron (12). However, no such long delay in virus production was noted by these authors, and the persistent fraction was a constant for each virus-cell system. From observations thus far on the adenovirus-HEK cell system, the final number of plaques appeared to depend solely on the interferon concentration.

Sensitivity of Ad. Type-2 to rabbit interferon. In attempts to extend the findings with adenoviruses and human interferon to another species, experiments were designed to examine the susceptibility of Ad. 2 to rabbit serum interferon in yield-inhibition studies in an established line of rabbit heart cells, strain RHF-1. The characteristics of Ad. 2 infection of RHF-1 cells have been described (13). Confluent RHF-1 monolayers in 60-mm plastic petri dishes were drained of growth medium and washed twice with BSS before addition of rabbit interferon. Each culture received approximately 350 units of rabbit interferon (as determined by vaccinia plaque reduction). Sera obtained from rabbits prior to interferon induction were acidified, dialyzed, and diluted to serve as control fluid. Rabbit interferon preparations titered 512–1024 units/ml by vaccinia plaque reduction. None of the preparations contained any influenza hemagglutinins after acidification and dialysis.

After overnight incubation of cultures at 37° in a 5% CO_2–air mixture, the interferon and control fluids were removed by aspiration and the cultures were washed three times in BSS. Virus infection was initiated by inoculating plates with an appropriate dilution of stock Ad. 2 contained in 0.2-ml volumes. After 4 hr adsorption at 37° the monolayers were again washed with BSS before receiving 5 ml of maintenance medium, followed by incubation at 37°.

Adsorbed multiplicities were determined by measuring the difference in plaque titer

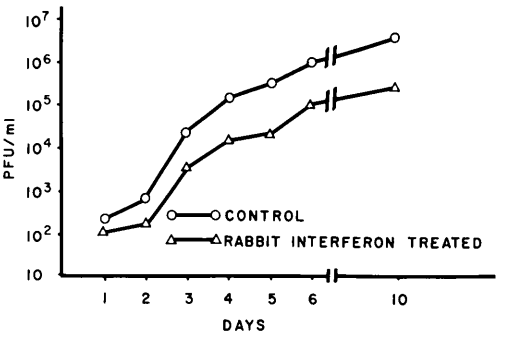

FIG. 2. Adenovirus Type 2 synthesis in RHF-1 cells. Course of Ad. Type-2 production in control RHF-1 cultures, (○); and in cultures protected with 350 units (by vaccinia plaque-reduction) of rabbit interferon, (△). Cultures received an input multiplicity of 3 pfu/cell. Approximately 1 $\log_{10}$ unit reduction in yield of infectious virus is apparent.

before and after exposure to cells. Cell counts were done on trypsinized monolayers using a hemocytometer. Control and interferon-treated RHF-1 cultures were observed for CPE daily for 10 days. Groups of five cultures each were harvested daily and pooled before being assayed for adenovirus pfu. The cells were scraped into the medium and then frozen and thawed six times. Cell debris was removed by centrifugation at 1000*g* for 15 min before assaying the virus-containing supernatant in HEp-2 cell cultures. Figure 2 is a plot of a representative experiment in which the Ad. 2 multiplicity was 3 pfu/cell. In this experiment the estimated adenovirus CPE in interferon-treated cultures was reduced by 75% or more when compared with control RHF-1 cultures. Approximately a tenfold reduction in pfu titer was also observed during most of the course of the infection. Similar reductions in titer were observed when Ad. 2 multiplicities of 10 and 20 were used in experiments with the same interferon dose as described above. However, little or no inhibition of CPE or virus titer was observed when the Ad. 2 multiplicity was 50 or greater.

Discussion. The findings of this report are the first to demonstrate significant interferon-mediated interference against several representative adenovirus types using plaque-reduction assay techniques. Moreover, similar

patterns of interferon sensitivity were noted for each type. These studies are being extended to numerous other adenovirus types and strains. Preliminary results suggest that many adenoviruses share a similar limited susceptibility to moderate amounts of human interferon. It is possible that differences in interferon sensitivity among the adenoviruses might be detected using a more sensitive assay method such as yield-inhibition studies during single cycle growth in highly sensitive cells, or by examining other parameters of virus infection such as T antigen formation or viral structural protein synthesis.

While the use of interferon as an adenovirus inhibitor has previously met with little success (1, 2, 4), a few noninterferon inhibitors of adenovirus replication have been described. Pereira (14) was the first to describe the viral inhibitory activity of the Ad. Type-5 fiber antigen against poliovirus, vaccinia, and the homologous adenovirus. This finding was confirmed and extended by Levine and Ginsberg (15) who elucidated the mechanism by which Ad. Type-5 fiber antigen inhibited adenovirus multiplication. The profound effects of the fiber antigen were not due to interferon induction. A group of defective viruses, the adeno-associated viruses (AAV) have been shown to inhibit plaque formation and yields of helper adenoviruses (16, 17). Inhibition of adenovirus by AAV did not appear to be due to interferon.

A number of DNA viruses including adenoviruses and some members of the herpesvirus group seem to elicit little if any interferon during infection, and at the same time exhibit limited or minimal susceptibility to interferon (2, 5, 6). The mechanisms for such phenomena are at present unclear. It is by no means certain that the two characteristics are more than coincidentally found in the same virus. It is possible that many of the "interferon resistant" viruses might be shown to be susceptible to interferon provided that adequate concentrations are employed in an appropriate assay system.

The *in vitro* studies of adenovirus susceptibility to interferon described here suggest a number of applications where the use of interferon might aid in advancing the understanding of adenovirus-cell interactions during latent and acute infections of the respiratory tract and during viral transformation and tumorigenesis. A number of adenovirus serotypes have been repeatedly implicated in latent infections of tonsils and adenoids of children despite the presence of humoral antibody (18, 19). Since these infections seem to resolve eventually with no evidence that the immune response is the sole determinant in the outcome, it is possible that interferon induced by any of a number of agents including live virus vaccines might play a significant role in quenching these inapparent infections.

The safety of parenteral adenovirus vaccines has been challenged by the discovery of the oncogenic potential in laboratory animals of eight adenovirus serotypes, and by the demonstration of a "hybrid" between the vaccine strain (L.L.) of Ad. Type-7 and simian $virus_{40}$, an oncogenic papovavirus (20, 21). Because of the uncertain state of adenovirus vaccines, the control of acute respiratory diseases produced by adenoviruses in military recruits and school populations remains a continuing problem. Should immunoprophylaxis prove unsuitable for any reason it seems within the realm of possibility that interferon might in the future find use in controlling adenovirus infections.

Interferon is also a potentially useful tool for the study of the mechanisms of adenoviral oncogenesis. Oxman and his associates (22) used the differential effects of interferon on SV_{40} and adenovirus T antigen formation to obtain further evidence of the physical linkage between adenovirus and SV_{40} DNA in the Ad. 7–SV_{40} hybrid virus ($E46^+$). Another interesting phenomenon which remains to be further investigated is the association of the DNA's of several adenoviruses (Types 4, 12, and 18) with human chromosomes, the association resulting in breaks and chromosomal anomalies (23, 24). If virus-induced chromosomal anomalies play a prime role in oncogenesis, then it would be relevant to the problem of preventing transformation *in vitro* or tumor formation *in vivo* to determine

whether interferon can inhibit the induction of these chromosomal aberrations.

Summary. The sensitivity of adenovirus Types 2, 7, and 12 to moderate amounts of human tissue culture interferon was demonstrated in plaque-reduction assays using primary and secondary HEK cell cultures. The findings for Ad. Type-2 were extended to another species using rabbit serum interferon in yield-inhibition assays done in an established rabbit heart fibroblast cell line. These results indicate that at least some members of the adenovirus group are indeed susceptible to interferon, although to a clearly lesser extent than are many other viruses. The implications of these findings for the possible use of interferon in examining *in vitro* the mechanisms of viral oncogenesis are considered.

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Received Sept. 5, 1968. P.S.E.B.M., 1969, Vol. 130.