Combined Antiviral Effects of Interferon, Adenine Arabinoside, Hypoxanthine Arabinoside, and Adenine Arabinoside-5'-Monophosphate in Human Fibroblast Cultures

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Adenine arabinoside and human interferon are currently being evaluated in clinical trials against herpes- and poxvirus infections. Interferon production is also a normal antiviral response. It is therefore important to examine the combined actions of interferon and antiviral arabinosides for possible synergy or antagonism. We have examined the antiviral activities of human fibroblast interferon, adenine arabinoside, hypoxanthine arabinoside, and adenine arabinoside 5'-monophosphate individually, using plaque inhibition of vaccinia and herpes simplex type 2 viruses in human skin fibroblast cultures. By combining doses of interferon and arabinosides that, acting alone, give intermediate degrees of plaque inhibition, we were able to compare the combined antiviral activity with that calculated from the activity of each inhibitor alone, assuming that the activities are statistically independent. Our results show that the plaque-inhibitory activities of interferon and the arabinosides tested are statistically independent. The results also show that the arabinosides do not destabilize the antiviral state previously induced by interferon, and that interferon pre-treatment does not interfere with subsequent arabinoside action in infected cells. We have also found that arabinosides do not affect the induction of interferon synthesis by either Newcastle disease virus or double-stranded ribonucleic acid, and are not themselves interferon inducers.

Adenine arabinoside (9-β-D-arabinofuranosyladenine, Ara-A) and human interferon are currently being evaluated in clinical trials for therapeutic use against several deoxyribonucleic acid virus infections, including herpes simplex types 1 and 2 (HSV-1 and -2), varicella-zoster, cytomegalovirus, and vaccinia (2 to 9, 14). Hypoxanthine arabinoside (Ara-Hx) is produced in vivo and in vitro by deamination of Ara-A, catalyzed by the enzyme adenosine deaminase; it has significantly lower antiviral activity than the parent compound (4, 5, 11). Ara-AMP, the 5'-monophosphate of Ara-A, also has inhibitory activity against the large deoxyribonucleic acid viruses and is presently in phase I clinical trials. For brevity, we refer to all three of these compounds as "arabinosides."

The interferon system is an important component of the vertebrate host's natural antiviral defenses (3). Therefore, it seemed desirable to examine the effects of any potential chemotherapeutic agent on both the production and the antiviral activity of interferon. We have done so using human diploid foreskin fibroblasts (HSF) infected with either HSV-2 or vaccinia virus. Our results show that the arabinosides tested (Ara-A, Ara-Hx, and Ara-AMP) neither antagonize nor enhance the antiviral activity of HSF interferon; that is, the antiviral activities of the arabinosides and interferon are statistically independent. Our results show that the arabinosides do not interfere with the establishment of the antiviral state induced by interferon, do not destabilize the antiviral state induced by previous exposure of cells to interferon, and do not interfere with the induction of interferon synthesis by either virus or synthetic double-stranded ribonucleic acid.

(A preliminary report of these results has been presented [Y. Bryson, J. D. Connor, and L. H. Kronenberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, p. 239, S211].)

MATERIALS AND METHODS

Cell cultures. The Bae strain of HSF was obtained from the Genetics Center, School of Medicine, University of California, San Diego. The cells were...
propagated as monolayers with weekly passage in Eagle minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). For plaque assays, 10^6 cells in 1.0 ml were seeded in each well of 24-well culture trays (Falcon no. 3008) and incubated for 3 to 5 days at 37°C under 5% CO2 until confluent. Maintenance medium for experiments refers to MEM containing 2% FBS.

Viruses. A clinical isolate of HSV-2 was kindly provided by A. Nahmias. Virus stocks were prepared by passage in HSF and were stored at -70°C. Vaccinia virus was obtained from Wyeth as Dryvax calf lymph vaccine. The lyophilized virus was reconstituted in MEM containing 10% skim milk and stored at -70°C. Newcastle disease virus (NDV, L-Kansas strain) was originally obtained from J. J. Holland and was propagated in 10-day embryonated chicken eggs as previously described (13). Virus-containing allantoic fluids were titrated in primary chicken embryofibroblast cultures and were stored at -20°C. A wild-type strain of encephalomyocarditis (EMC) virus, also obtained from J. J. Holland, was used for the isolation of small- and large-plaque clones by four serial plaque purifications in monolayer CCL-1 cells. The virus stocks used herein were obtained by passage in CCL-1 cells and were stored at -20°C.

All virus plaque assays were carried out as follows: confluent 16-mm HSF cultures were washed once with 1 ml of tri(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4 (18), and infected in 0.06 ml of Tris buffer containing virus diluted to give 50 to 60 plaque-forming units (PFU)/culture. The inoculum was adsorbed for 60 min at room temperature and was redistributed at 15-min intervals. After adsorption, the cultures were drained and replenished with 0.6 ml of maintenance medium containing antiviral substances as described below. For EMC plaques, the medium postinfection also contained 0.6% agarose (Calbiochem), which was removed by aspiration before the cells were fixed. Cultures were incubated at 37°C under 5% CO2 for 36 h after infection with EMC or for 48 h after infection with HSV-2 or vaccinia virus, then washed with 1 ml of Tris buffer, fixed in 100% methanol, and stained with 1% aqueous crystal violet. Plaques were counted using a dissecting microscope at ×10 to 15 magnification.

Interferon. A single interferon preparation was used for all experiments in which arabinosides and interferon were used together. This preparation was produced by inducing diploid human fetal fibroblasts with polyinosinic-polycytidylic acid (poly I:C) (18) and was previously characterized as nondialyzable, non-sedimentable (3 h at 100,000 × g), stable at pH 2.2, trypsin sensitive but ribonuclease and deoxyribonuclease insensitive, and active against a variety of virus types in human and monkey cells but not in chicken, rabbit, mouse, or cat cells.

For experiments in which interferon was induced in combination with arabinosides (Table 1), confluent 35-mm HSF cultures in Linbro FB-6 dishes were induced as follows: for NDV, the cultures were washed once with 2 ml of Tris buffer and infected at approximately 300 PFU/cell in 0.2 ml of NDV-containing allantoic fluids for 90 min at room temperature. After adsorption, the cultures were washed twice with 2 ml of Tris buffer and refed 2 ml of maintenance medium. For poly(I:C), confluent 35-mm cultures were washed once with 2 ml of MEM and refed 1 ml of MEM, 1% FBS, 100 μg of diethylaminoethyl dextran (Pharmacia) per ml, and 10 μg of poly(I:C) (P-L Biochemicals) per ml. After incubation for 3 h at 37°C, the cultures were washed three times with 2 ml of MEM and refed 2 ml of maintenance medium. Twenty-four hours after the beginning of induction, culture fluids were removed, pooled for duplicates, and dialyzed at pH 2.2 and pH 7.4 as described (18). The media were then clarified by centrifugation and stored at -20°C. Interferon yields were measured by a modification of the micro dye-uptake method previously described (1): confluent HSF monolayers in Falcon microtest II plates were incubated overnight in 0.1 ml of maintenance medium per well containing serial threefold dilutions of interferons. The cultures were then washed with Tris buffer, infected with 10 PFU of large-plaque EMC virus per cell and refed 0.12 ml of maintenance medium per well. After 24 h at 37°C, the cultures were fixed in methanol and stained with 1% aqueous crystal violet. Interferon titers given in Table 1 are the reciprocal of the dilution giving approximately 50% protection of the microtest cultures from destruction by EMC virus.

Arabinosyl compounds. Ara-AMP, arabinosyl adenine deaminase inhibitor (ADA1) (24) were obtained from Parke-Davis and were the kind gifts of James D. Connor. The Ara-Hx was further purified by high-pressure liquid chromatog-
RESULTS

Antiviral activity of arabinosides against vaccinia virus and HSV-2. Initially the antiviral activities of Ara-A, Ara-Hx, and Ara-AMP were measured individually, using plaque inhibition against vaccinia virus or HSV-2. These experiments were similar to those reported previously (5, 10) for Ara-A and Ara-Hx, except that plaque assays were carried out in multwell tissue culture dishes containing 24 cultures 16 mm in diameter. Confluent HSF monolayers were infected with approximately 60 PFU of either vaccinia virus or HSV-2 per culture as described in Materials and Methods and then refed maintenance medium containing serial twofold dilutions of the arabinosides. At 48 h postinfection, the plaques were scored and expressed as percentage of plaque number in drug-free controls. Points representing intermediate degrees of plaque inhibition were fitted to a least-squares line, and from this line concentrations of inhibitors giving 50% plaque inhibition were calculated. For vaccinia virus (Fig. 1) the median inhibitory doses were:

0.038 μg of Ara-A per ml (0.14 μM); 0.077 μg of Ara-AMP per ml (0.22 μM); and 0.45 μg of Ara-Hx per ml (1.7 μM). For HSV-2 (Fig. 2) the median inhibitory doses were: 0.18 μg of Ara-A per ml (0.67 μM); 0.41 μg of Ara-AMP per ml (1.2 μM); and 15.4 μg of Ara-Hx per ml (57 μM). Expressing in terms of median inhibitory doses, vaccinia plaque development was approximately five times as sensitive to Ara-A, and approximately 34 times as sensitive to Ara-Hx, as was HSV-2 plaque development.

These activity curves (Figs. 1 and 2) were used as a reference for choosing drug doses appropriate to give approximately 25% plaque inhibition for the combined experiments that follow.

Combined antiviral activities of arabinosides and interferon. The strategy of these experiments was as follows: confluent HSF monolayers were incubated overnight in maintenance medium containing serial threefold dilutions of human fibroblast interferon. The cultures were then washed, infected with 60 PFU of either vaccinia virus or HSV-2 per culture, and replenished with maintenance medium containing doses of arabinosides chosen from the data in Fig. 1 and 2 to give approximately 25% plaque inhibition. "Drug controls" refers to cultures pretreated with interferon-free medium and maintained postinfection in medium containing the individual arabinosides. "Interferon controls" refers to cultures pretreated with interferon dilutions but maintained postinfection in drug-free medium. "Virus controls" refers to cultures pretreated with interferon-free medium and maintained postinfection in drug-free medium. At 48 h postinfection, the plaques were scored and expressed as percentage of virus controls. Results representative of the experiments with vaccinia virus are shown in Fig. 3. Panel A shows the interferon controls,

![Fig. 1. Inhibition of vaccinia plaque development (as percentage of control plaque number) by Ara-A, Ara-Hx, and Ara-AMP. Confluent HSF monolayers were infected with 50 to 60 PFU of vaccinia virus and incubated postinfection in maintenance medium containing serial dilutions of arabinosides, and plaques were scored at 48 h postinfection as described in the text. ADAI (0.83 μg/ml) was included with Ara-A and Ara-AMP. Symbols: (△) Ara-A; (□) Ara-AMP; (○) Ara-Hx. Vertical bars represent standard deviation in plaque count for triplicate samples. Linear regression lines calculated from the points are indicated.](http://aac.asm.org/)

![Fig. 2. Inhibition of HSV-2 plaque development by Ara-A (△), Ara-AMP (□), and Ara-Hx (○). Other details as in Fig. 1.](http://aac.asm.org/)
Fig. 3. Inhibition of vaccinia plaque development by interferon pretreatment alone (A) or in combination with arabinosides postinfection (B, C, D). See text for experimental details. Vertical bars represent standard deviation in plaque counts for triplicate samples. Plaque inhibition due to arabinosides alone (drug controls) is indicated in B, C, and D by horizontal dotted lines. Symbols: (O) observed plaque development expressed as percentage of virus controls; (D) results calculated from the data in panel A and from the arabinoside controls, assuming statistical independence. (A) Interferon pretreatment, drug-free medium postinfection. (B) Interferon pretreatment, 0.04 µg of Ara-AMP per ml (0.11 µM) postinfection. (C) Interferon pretreatment, 0.025 µg of Ara-A per ml (0.094 µM) postinfection. (D) Interferon pretreatment, 1.0 µg of Ara-Hx per ml (3.7 µM) postinfection.

i.e., cultures pretreated with interferon dilutions and post-treated in drug-free medium. The results are equivalent to a plaque-inhibition assay of our interferon preparation, using vaccinia virus for challenge. In panels B, C, and D the drug controls are shown by the horizontal dotted line. Thus the dose of Ara-AMP used (0.04 µg/ml) produced approximately 15% inhibition of plaque development, Ara-A (0.025 µg/ml) produced approximately 21% plaque inhibition, and Ara-Hx (1.0 µg/ml) produced approximately 30% plaque inhibition. In panels B through D, circles indicate the observed results for cultures pretreated with interferon dilutions (shown on the abscissa) and post-treated with these doses of arabinosides.

Our criterion for antagonism or synergy between the antiviral actions of arabinosides and interferon is as follows: if \( P_1 \) is the fraction of control plaques observed in the presence of a given amount of interferon and \( P_2 \) is the fraction of control plaques observed in the presence of a given amount of arabinoside, then the fraction of control plaque number observed in the presence of both substances is the product \( (P_1)(P_2) \) if the inhibitory activities are statistically independent. We use antagonism to denote plaque survival in excess of that calculated assuming statistical independence, and we use synergy to denote plaque survival less than that calculated assuming statistical independence. Thus the expected results (shown in Fig. 3 by squares) are calculated by multiplying the fraction of control plaque number observed at a given dose of interferon and the fraction of control plaque number observed at the dose of arabinoside used. In Fig. 3 it is evident that the expected and observed results coincide quite closely. Hence we conclude that the antiviral activities of interferon and each of the arabinosides tested are statistically independent.

Identical experiments were performed using HSV-2. Comparison of the abscessae in Fig. 3A and 4A shows that the median inhibitory dose of interferon was approximately eightfold higher for HSV-2 than for vaccinia virus. In Fig. 4, the inhibition due to arabinosides alone (drug controls) is again indicated by horizontal dotted lines. In this case, the dose of Ara-A (0.1 µg/ml) produced approximately 11% plaque inhibition, Ara-AMP (0.2 µg/ml) produced approximately 23% plaque inhibition, and Ara-Hx (6.0 µg/ml) produced approximately 53% plaque inhibition. In each case, the experiments with combined inhibitors (Fig. 4B to D) gave observed results that were experimentally indistinguishable from the expected results calculated assuming statistical independence of the inhibitory activities. For both vaccinia virus and HSV-2, results identical to those obtained for the interferon controls (panel A) were given by cells treated only with ADAI postinfection. We conclude, insofar as HSV-2 and vaccinia virus are concerned, that the plaque-inhibitory activities of human fibroblast interferon and these arabinosides are statistically independent and show no evidence of antagonism or synergy in their actions.

Two further conclusions can be drawn from the results given in Fig. 3 and 4. In these experiments the cells were pretreated overnight with interferon dilutions, infected, and then maintained postinfection in interferon-free medium containing arabinosides. Since the observed results for combined inhibitors were virtually identical with the results calculated on the basis of statistical independence, it follows that neither the arabinosides nor ADAI acts to destabilize the antiviral state induced in the cells by previous exposure to interferon; otherwise, the results would indicate antago-
**Fig. 4.** Inhibition of HSV-2 plaque development by interferon pretreatment alone (A) or in combination with arabinosides postinfection (B to D). Vertical bars represent standard deviation in plaque counts for triplicate samples. Plaque inhibition due to arabinosides alone (drug controls) is shown by horizontal dotted lines. Symbols: (○) Observed plaque development as percentage of virus controls; (□) results calculated from the data in panel A and from arabinoside controls, assuming statistical independence. (A) Interferon pretreatment, drug-free medium postinfection. (B) Interferon pretreatment, 0.1 μg of Ara-A per ml (0.37 μM) postinfection. (C) Interferon pretreatment, 6.0 μg of Ara-Hx per ml (22 μM) postinfection. (D) Interferon pretreatment, 0.2 μg of Ara-AMP per ml (0.58 μM) postinfection.

...nism between the inhibitory actions of interferon and arabinosides. Conversely, prior exposure of cells to interferon does not interfere with the antiviral action of subsequent doses of arabinosides.

**Effect of arabinosides on establishment of the antiviral state induced by interferon.** The preceding experiments (Fig. 3 and 4) tested the plaque-inhibitory activities of arabinosides in cells in which an antiviral state was already established by previous exposure of the cells to interferon. The results show that the antiviral arabinosides do not destabilize the antiviral state induced by interferon, but do not rule out the possibility that concomitant exposure of cells to interferon and arabinosides can interfere with establishment of the antiviral state induced by interferon. To test this possibility, confluent HSF monolayers were pretreated overnight with maintenance medium containing interferon dilutions plus relatively high doses of arabinosides (5 μg of Ara-A, 8 μg of Ara-AMP, or 10 μg of Ara-Hx per ml). Controls contained interferon dilutions without arabinosides, with and without ADAI. The cultures were then washed, infected with 60 PFU of small-plaque EMC virus per culture, and scored at 36 h postinfection. Since the arabinosides were washed out of the cultures before infection, and since EMC is ribonucleic acid virus insensitive to the inhibitory activities of these arabinosides (not inhibited in cells even when treated concurrently with arabinosides; our unpublished data), any inhibition of EMC plaque formation in this experiment must be attributed to the antiviral state established in the cells before infection, in response to interferon. The results are shown in Fig. 5. The inhibition curves were identical whether the cells were pretreated with interferon alone or in the presence of Ara-A, Ara-AMP, or Ara-Hx. The results were unchanged for cells treated with interferon and ADAI (data not shown). Hence, neither ADAI nor high concentrations of arabinosides inhibited the establishment of the antiviral state induced by interferon.

**Effect of arabinosides on induction of interferon synthesis.** A final set of experiments was designed to test whether ADAI or arabinosides interfere with the induction of interferon synthesis by virus or poly(I:C), or might themselves be interferon inducers. Confluent HSF monolayers were induced by NDV or poly(I:C) as described in Materials and Methods and

**Fig. 5.** EMC plaque development in cultures pretreated with interferon alone or in combination with arabinosides. Cultures were pretreated overnight with serial twofold dilutions of interferon, infected, and maintained postinfection in interferon-free, arabinoside-free medium. The plaque numbers at 36 h postinfection were expressed as percentage of virus controls. Symbols: (○) Pretreatment with interferon alone; (◇) pretreatment with interferon and 8 μg of Ara-AMP per ml (23 μM); (●) pretreatment with interferon and 5 μg of Ara-A per ml (19 μM); (□) pretreatment with interferon and 10 μg of Ara-Hx per ml (37 μM). ADAI (0.83 μg/ml) was included with Ara-A and Ara-AMP.
ferred maintenance medium containing Ara-A (5 µg/ml) or Ara-AMP (8 µg/ml), each with and without ADAI (0.83 µg/ml). Ara-Hx was not tested in these experiments, but was produced by deamination of Ara-A in cultures not containing ADAI. Controls contained no additions. At 24 h postinduction, the extracellular media were removed and the interferon activity was quantitated as described in Materials and Methods (Table 1). Neither arabinosides nor ADAI alone or in combination caused synthesis of any detectable interferon. Control NDV-induced cultures produced 1,200 U of interferon, and neither arabinosides nor ADAI markedly affected this yield. Control poly(I:C)-induced cultures produced about 4,000 U of interferon, and this also was not significantly affected by arabinosides or ADAI. Thus arabinosides and/or ADAI do not impair the production of interferon by human fibroblasts in response to virus or poly(I:C).

**DISCUSSION**

The experiments reported herein were designed to test whether the antiviral arabinosides Ara-A, Ara-Hx, and Ara-AMP demonstrate antagonistic or synergistic effects upon the production and antiviral activity of human interferon. Interferon production and action are important components of the early antiviral response in vivo (3), and there is increasing evidence that interferon production also serves a regulatory purpose in both cell-mediated and humoral immunity (12, 15–17, 20–22). Therefore, it seems desirable to examine the effects of any potential antiviral chemotherapeutic agent on both aspects of the interferon response. Our experiments provide a model by which the combined activities of interferon and antiviral drugs can be examined in vitro. Such information may prove to be important in the chemotherapy of viral diseases since a rational approach to antiviral chemotherapy would require that drugs do not impair the endogenous interferon response. In addition, combined therapy with antiviral drugs and exogenous interferon may provide increased clinical efficacy, provided that the activities are not antagonistic.

We chose HSV-2 and vaccinia viruses as representatives of the herpes- and poxvirus groups. It would also be of interest to examine other herpesviruses such as varicella-zoster and cytomegalovirus. Our experiments measured the antiviral activities of interferon and arabinosides alone and in combination; by combining doses of each that, acting alone, would give intermediate degrees of plaque inhibition, we were able to compare the combined antiviral effects with those calculated from the activity of each inhibitor alone, assuming that the activities are statistically independent. Our results are indistinguishable from those calculated on this basis and lead to the conclusion that the plaque-inhibitory activities of interferon and each arabinoside tested are statistically independent. It should be noted that in these experiments cell cultures were first treated with interferon to establish an antiviral state; the interferon was removed at the time of infection, and infected cultures were then maintained in interferon-free medium containing arabinosides. Therefore, the results also allow us to conclude that treatment with arabinosides postinfection does not destabilize the antiviral state previously induced by interferon, and also that interferon pretreatment does not interfere with the subsequent action of these arabinosides in infected cells.

Our results show that at doses of arabinosides giving intermediate plaque inhibition, there is no synergy at any dose of interferon tested. These results do not preclude possible synergy or antagonism between interferon and higher doses of arabinosides. However, the doses of arabinosides used in our experiments are comparable with serum levels of Ara-A and Ara-Hx achieved after intravenous administration of Ara-A in clinical trials (Y. Bryson and J. D. Connor, in preparation).

In addition, measurement of plaque inhibition is equivalent to measurement of inhibition of viral infectivity, but does not address the yield of progeny virus per infected cell. Hence, our data do not rule out the possibility of synergy between the effects of arabinosides and interferon on virus yields.

Lerner and Bailey (19) reported that the plaque-inhibitory effects of human leukocyte interferon and Ara-A were synergistic in a system using Vero cells infected with HSV-1. Our results do not corroborate this report, and we presume that the discrepancy may arise from the following facts. (i) Lerner and Bailey used a definition of synergy that is not meaningful in the absence of more complete dose-response information and depends, in particular, on the slope of the dose-response curves. When their data (19, Table 2) are recalculated according to our criterion of synergy (statistical independence), some of the data do not, in fact, support the conclusion of synergy. (ii) Lerner and Bailey used Vero cells in their study, whereas we have used human diploid foreskin fibroblasts. (iii) ADAI was not used in their study, necessitating the use of much higher concentrations of Ara-A than were used in the experiments re-
ported here. Hence the plaque inhibition observed by Lerner and Bailey may have been due, in part, to nonspecific cytotoxicity. The doses of inhibitors used in our study are not cytotoxic according to the criteria of trypan blue staining and efficiency of plating of the cells upon subculture (Y. J. Bryson, unpublished data). Also, as demonstrated in Fig. 5 and Table 1, even the highest doses of inhibitors used here were not cytotoxic as measured by the ability of cultures to support virus replication and interferon synthesis, which are the indexes of cytotoxicity most pertinent to our conclusions. (iv) Lerner and Bailey applied interferon at the same time as Ara-A, i.e., immediately after infection of the cultures. Hence the observed antiviral activities must depend on the ability of interferon to establish resistance in cells treated simultaneously with Ara-A and virus, and on the relative kinetics of virus maturation and the development of resistance. Our results show that the arabinosides do not interfere with the establishment of the antiviral state induced by interferon in uninfected cells, but the situation in infected cells may be more complex.

In summary, our results indicate that for vaccinia virus and HSV-2 in HSF, the plaque-inhibitory activities of arabinosides and interferon are statistically independent, with no evidence for synergy or antagonism. Arabinosides do not destabilize the antiviral state induced by previous exposure to interferon, and previous exposure to interferon does not antagonize the subsequent activity of the arabinosides. In addition, neither ADAI nor the arabinosides affect the induction of interferon synthesis by either virus (NDV) or poly(I:C), and are not themselves interferon inducers.

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LITERATURE CITED


