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Effect of Adenosine Deaminase upon the Antiviral Activity In Vitro of Adenine Arabinoside for Vaccinia Virus

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This study determined that the effect of 9-β-D-arabinofuranosyl-adenine (adenine arabinoside, Ara-A) upon vaccinia virus plaque development in the stable monkey kidney line, LLC-MK_2, was increased approximately 40-fold when an inhibitor of adenosine deaminase (ADA) was added to the tissue culture media along with infective inocula. The concentration of Ara-A required to completely suppress plaque development (total plaque inhibitory concentration$_{100}$; $TPIC_{100}$) was greater than 10 μg/ml. However, when ADA activity was inhibited, the $TPIC_{100}$ was 0.5 μg/ml or less. Chromatographic assay of arabinosylpurines in the media provided evidence that adenine arabinoside was rapidly deaminated to 9-β-D-arabinofuranosylhypoxanthine by the cellular monolayers, in the absence of animal serum, and that the rate of deamination, at 5 μg/ml, by the cells was equal to the rate of diffusion of Ara-A across the cellular membrane. The half-life of Ara-A in the media, starting with 5 μg/ml, was 2 to 3 h and shorter at lower concentrations. The study demonstrates the profound effect that an indicator system, acting as an intact biological unit, can have upon a potential antiviral compound.

Inhibitory activity of 9-β-D-arabinofuranosyl-adenine (adenine arabinoside Ara-A) for large deoxyribonucleic acid viruses has been demonstrated in cellular infection in vitro and in animal infections of various types (18). The drug is now in clinical trials against herpes simplex and varicella-zoster virus infections (3, 4). Our interest in pharmacology of Ara-A led to development of a method which measures plaque reduction activity at concentrations much lower than previously reported. This has been accomplished by inhibiting completely the activity of adenosine deaminase (ADA) (13) generated by animal cells of the plaque indicator system, thus preventing deamination of Ara-A to the metabolite, 9-β-D-arabinofuranosylhypoxanthine (hypoxanthine arabinoside, Ara-Hx), reported (15) to have less antiviral activity. The effect of ADA upon the antiviral activity in vitro of Ara-A for vaccinia virus (VV) is reported herein.

MATERIALS AND METHODS

All plaquing experiments were performed with a mycoplasma-free (11) stable monkey cell line LLC-MK_2 (12). The cells were subcultured in Eagle minimal essential medium (MEM) with 10% bovine serum and seeded into 30-mm wells of plastic tissue culture plates (Linbro). Before experimental use, media with serum was removed by vacuum and MEM without serum was added for maintenance. Lyophilized standard vaccine virus (Dryvax-Wyeth Laboratory) was put into suspension with MEM containing 10% skim milk. Two-tenths milliliter of a dilution containing $10^{1.5}$ to $10^{1.7}$ plaque forming units was added with a micropipette to each well.

Adenosine deaminase inhibitor (P. W. K. Woo, H. W. Dion, S. M. Lange, L. F. Dahl, L. J. Durkham, and H. F. Mosher, J. Heterocycl. Chem., in press) (ADAI; inhibitor) [(R)-3-(2-deoxy-β-D-erythropentofuranosyl) - 3,6,7,8 - tetrahydroimidazo(4,5 - d)(1,3)diazepin-8-ol] was kindly supplied by Parke-Davis & Co. in 1 mg/ml aqueous solution. With a micropipette, 0.025 ml of a 1:10 dilution was added to appropriate wells after addition of Ara-A. Ara-A (Parke-Davis & Co.), was weighed on a standard pan balance and put into solution with water at 500 μg/ml. It was further diluted with maintenance media appropriate to concentrations of 5, 4, 3, 2, 1, 0.5, and 0.1 μg/ml before an experiment. In some experiments with and without virus infection, 0.5-ml portions were removed from three replicate well cultures, pooled, and frozen at −10 C for chromatographic assay.

N^6-methyldeoxyadenosine (6-MedAdo) (Terra-Marine, Inc.) was put into solution with MEM at a concentration 10^{-2} M (molecular weight 265) and further diluted in maintenance media. 6-MedAdo and then infective inocula (VV) were added to well cultures to initiate an experiment. After inoculation, cultures were incubated for 46 h at 36.5 C in 5% CO_2. At the end of this period, monolayers were stained with crystal violet, plates were dried, and plaques

were counted under a dissecting microscope at ×10 magnification. Plaque development was calculated from appropriate replicate plates comparing the total number of plaques in three 30-mm cultures of a single set in the presence of inhibitor alone or without inhibitor, with matched cultures containing Ara-A. Inhibitor alone was not found to effect plaque development at the concentrations used.

The arabinosylpurines were quantitatively analyzed by using a Chromatronix liquid chromatograph with a flow rate of 1 ml/min and by recording the absorbance at 254 nm. For analysis of Ara-A only, a column (100 by 0.6 cm) of Sephadex G-25 Superfine was eluted with 0.4 M ammonium acetate, pH 7.0. Ara-Hx eluted at 33 ml with components of the culture media and Ara-A eluted at 47 ml, free of media components. For analysis of both Ara-Hx and Ara-A in culture media, a second column of Aminex A-6 (25 by 0.6 cm) was added. The A-6 column was equilibrated with 0.4 M ammonium acetate, pH 4.82, and the G-25 column with 0.4 M ammonium acetate, pH 7.0. The sample was applied to the G-25 column only and eluted with 0.4 M ammonium acetate, pH 4.82. After 25 ml was eluted, the A-6 column was connected in series with the G-25 column and the elution was continued. Ara-Hx eluted at 42 ml and Ara-A at 68 ml, free of media components.

Quantitation was by peak area (height times width at half-height) compared to peak areas of weighed standards.

RESULTS

The effect of Ara-A or Ara-Hx upon plaque development of VV in the absence of inhibitor is shown in Table 1. It is apparent that there is little difference in the antiviral activity of the two nucleosides at concentrations over a 10-fold range. In one experiment (1 of 5), in 10 μg/ml concentration, Ara-A completely prevented plaque development and the same occurred with 10 μg/ml Ara-Hx in 1 of 4 experiments. At 10 μg/ml there was a plaque development range of 0 to 26%. In later experiments, we have determined that the technical accuracy of achieving a desired concentrations of nucleoside by dilution was no greater than 80%. By chromatographic assay, the concentration of Ara-A was 421 μg/ml, not 500 μg/ml, in preliminary experiments. These factors may have influenced the plaque results.

TABLE 1. *Plaque development*[a] of VV in presence of Ara-A and Ara-Hx

Concn[b] (μg/ml)	Plaque development (%)	
	Ara-Hx	Ara-A
10	11.2	11.8
5	32.7	38.4
1	57.8	58.5

[a] Calculated from total number of plaques in three 30-mm well cultures containing nucleoside at a single concentration, compared to controls without nucleoside. In Table 2, compared to controls with ADAI alone. Based upon averages from two or more experiments using each concentration.

[b] Calculated from appropriate dilution of 500 μg/ml stock in MEM. Chromatographic assay of Ara-A stock diluted to concentration of 500 μg/ml was 421 μg/ml. In later experiments, dilutions of Ara-A stock to be put into culture media were first assayed and then adjusted to exact concentration in order to eliminate as much variability as possible.

The effect of ADAI upon the antiviral activity of Ara-A and Ara-Hx is shown in Table 2. There was no significant difference in plaque development with or without ADAI in cultures treated with Ara-Hx over a 10-fold range. There was no significant effect of ADAI upon plaque development when used alone, in comparison to infected cultures without ADAI in any experiment.

In contrast, ADAI added to cultures treated with Ara-A had a marked effect upon plaque development. The susceptibility of plaque development to Ara-A increased approximately 50-fold. Similar results were found in replicate experiments.

To determine that the marked increase in antiviral activity was, in fact, due to presence of Ara-A in media of the infected cultures at various concentrations throughout the total period of a plaquing experiment, we measured the two nucleosides at intervals after incorporation of Ara-A into the media of infected monolayers at a concentration of 5 μg/ml. In Table 3, it may be observed that deamination by the cultures occurred rapidly, no Ara-A being detectable after 24 h. Ara-Hx was present at 2 h and comprised 100% of measurable arabinosylpurine at 24 h. The half-life was approximately 2 to 3 h for Ara-A, incubated in MEM without serum in presence of LLC-MK_2 cell monolayers in resting stage at 36.5 C (Fig. 1).

TABLE 2. *Plaque development of VV in presence of nucleosides and an inhibitor of ADA*

Concn (μg/ml)	Plaque development (%)			
	Ara-Hx		Ara-A	
	Without ADAI	With ADAI	Without ADAI	With ADAI
5.0	23.3	16.6	22.2	0
1.0	76.6	73.3	65.1	0
0.5	76.6	56.5	81.8	0
0.1			98.2	30
0.05			100.0	70

TABLE 3. *Deamination of Ara-A by VV-infected LLC-MK$_2$ monolayer cultures*[a]

Time in culture[b] (h)	Concn (μg/ml)[c]		
	Ara-A	Ara-Hx	Ara-A + Ara-Hx
2	4.30 (86)[d]	0.70 (14)	5.00
4	1.47 (29)	2.83 (57)	4.30
8	0.37 (7)	4.02 (80)	4.39
24	<0.10 (0)[e]	3.98 (80)	3.98

[a] Five micrograms per ml in maintenance media. Initial concentration calculated by dilution from 500 μg/ml stock.

[b] Time from addition of media containing Ara-A to removal from contact with the cell culture. Immediately frozen at −10 C until assay.

[c] Concentration determined by liquid chromatographic assay.

[d] Percent of calculated original concentration of Ara-A.

[e] Detectable range ≥0.10 μg/ml.

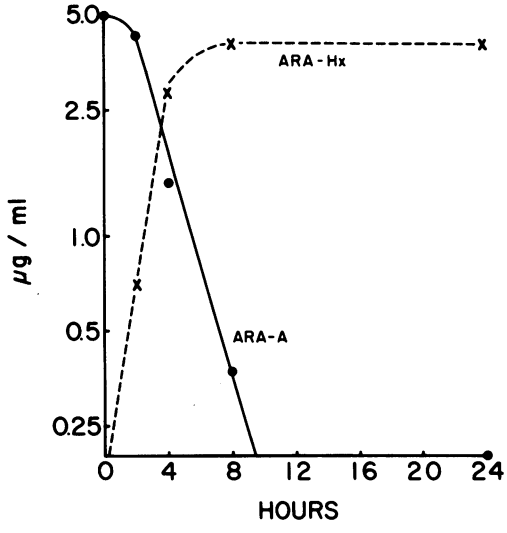

FIG. 1. *Deamination of Ara-A by VV-infected LLC-MK$_2$ monolayers. Ara-A at 5 μg/ml was contained in original maintenance media. Three 0.5-ml portions were removed from each of three well cultures, pooled, and used for chromatographic assay of nucleoside at each time period.*

In Table 4 the effect of ADAI upon deamination of Ara-A is shown. When portions were removed from cultures containing 5 μg of Ara-A per ml without ADAI and assayed, all Ara-A was deaminated by 24 h. In contrast, in cultures containing ADAI, no deamination occurred in 24 h (Table 4). We also examined the effect of ADAI upon deamination by conditioned media alone (Table 4). After 24 h of incubation upon a cell monolayer, conditioned media were removed and centrifuged to remove any cells or cellular debris. Ara-A with or without ADAI then was added to the supernatants. Deamination also occurred, but at a slower rate in conditioned media without ADAI than in intact monolayers.

In associated studies, we have determined that neither Ara-A at 5 μg/ml nor VV infection alters the production of ADA by LLC-MK$_2$ cells, over a period of 48 h at 36.5 C.

The effect of a competitive inhibitor of ADA, 6-MedAdo, upon the antiviral activity of Ara-A is shown in Table 5. At 10^{-3} M concentrations, the inhibitor alone had a substantial effect upon plaque development but increase in total antiviral effect with Ara-A could be observed at all Ara-A concentrations. At 10^{-5} M concentration, there was little or no effect when compared to Ara-A alone. At 10^{-4} M concentration, a marked effect could be noted.

Assays of nucleoside in media of cultures containing 6-MedAdo demonstrated that the half-life of Ara-A in presence of 10^{-4} 6-MedAdo was, on the average, 22 h, compared to average 2 to 3 h without 6-MedAdo.

DISCUSSION

These in vitro experiments have shown clearly that resting mammalian cells enzymatically alter 9-β Ara-A and markedly reduce its

TABLE 4. *Effect of ADAI upon deamination of Ara-A by uninfected LLC-MK$_2$ cultures or by conditioned culture media alone (5.0 μg/ml[a])*

A. Cultures[a]	Ara-A concn (μg/ml)[b]	
	Without ADAI	With ADAI
Time in culture (h)		
2	3.41 (68)	4.90 (98)
6	1.50 (30)	4.90 (98)
24	<0.10[c] (0)	5.24 (104)
B. Conditioned media alone[d]		
Time of incubation (h)		
2	3.49 (70)	4.72 (94)
6	3.50 (70)	4.95 (99)
24	1.60 (32)	4.82 (96)

[a] Culture media contained Ara-A at 5 μg/ml with or without ADAI at time experiment was initiated (0 time). Initial concentration was calculated by dilution from 500 μg of stock per ml.

[b] Concentration of Ara-A by liquid chromatographic assay; ( ) = percent of input concentration of Ara-A.

[c] Detectable range ≥0.10 μg/ml.

[d] Conditioned media was removed from cultures after a 24-h incubation and Ara-A at 5 μg/ml was then added. The incubation was at 36.5 C for the time indicated in fresh culture plates without cell monolayers, then freezer portions were stored until assay was performed. ADAI was added at same time as Ara-A.

TABLE 5. *Effect of 6-MedAdo upon antiviral activity of Ara-A*[a]

Concn of Ara-A (μg/ml)	Plaque development (%)			
	Without 6-MedAdo	6-MedAdo (10^{-3} M)	6-MedAdo (10^{-4} M)	6-MedAdo (10^{-5} M)
5	11.8[b]	0	0	4.2
2	27.0	0	0	18.2
1	48.5	0	0	44.1
0.5	66.5	0	11.1	64.2
0.1	96.5	10.6	41.3	95.4
None		42.3 (34.2)[c]	80.1 (86.0)	92.8 (96.4)

[a] Data from a single typical experiment, demonstrating protection of the antiviral effect of Ara-A, in presence of 6-MedAdo.

[b] No. of plaques in three 30-mm of VV-infected cultures with indicated compounds/no. of plaques in three 30-mm VV-infected cultures without additives. ×100.

[c] ( ) = Development in presence of 6-MedAdo, concentration indicated, average of 2 to 4 experiments.

antiviral activity by deamination. The enzyme ADA, originated in the cells of the monolayer, not in mammalian serum, since the media used was serum free. Production of ADA was sufficient to deaminate Ara-A at a rate which prevented demonstration of antiviral activity any greater than the deaminated product, Ara-Hx, at nucleoside concentrations up to 10 μg/ml. That loss of, not native lack of, antiviral activity of Ara-A was the case could be demonstrated by inhibiting ADA with ADAI, and then comparing antiviral activity to cultures without inhibitor. Under such conditions, in cultures with ADA inhibitor, Ara-A retained antiviral potency 40 to 50 times that in cultures without inhibitor where Ara-A activity was essentially that of Ara-Hx.

That Ara-A was not deaminated by ADA in presence of inhibitor was shown by measuring nucleoside concentrations after separation by liquid chromatography. Disappearance of Ara-A (deamination) corresponded with appearance of Ara-Hx in tissue culture media of VV-infected or -uninfected monolayers when ADAI was not present. When inhibitor was added to original media of cultures, the concentration of Ara-A remained high (or the same) throughout the total period of an experiment, regardless of Ara-A concentration, thus indicating sufficient inhibitor in the cultures to inactivate all ADA produced during a 46-h period at 36.5 C. ADAI neither interfered with measurement of nucleosides in the media nor displayed antiviral activity. Since Ara-A appeared at the same effluent volume from the chromatography column, whether inhibitor was present or not, it is assumed that it was not inhibitor bound.

Since deamination occurred in conditioned media in the absence of cells, it may be assumed that ADA escaped from cells in the absence of serum. An alternative explanation may be that ADA originating in 10% calf serum, contained in growth media, was incompletely removed at time of change to serum-free maintenance media; the enzyme ADA is heat stable.

In any case, the rate of deamination in conditioned media alone was at approximately 25% the rate of that in cells. This fact strongly implies that ADAI acts primarily intracellularly, but, as well, may act extracellularly.

Ara-Hx was the only compound present in tissue culture fluids after 24 h at 37 C since LLC-MK_2 cell purine nucleoside phosphorylase was demonstrated in our laboratory not to cleave Ara-A to Ara-Hx. However, a human diploid cell strain contaminated with mycoplasma rapidly cleaved either nucleoside as compared to no cleavage by a mycoplasma-free human diploid line. We take this as further evidence that the LLC-MK_2 cells were free of mycoplasma. The relationship between mycoplasmal contamination and phosphorolytic cleavage requires further examination.

In the presence of ADAI, media concentrations of Ara-A between 0.5 to 5 μg/ml resulted in intracellular Ara-A concentrations which were effective in totally preventing plaque development. At lowest media concentration, limited or incomplete viral replication may have occurred since, microscopically, there was minimal evidence of cellular changes in a few areas of the monolayers without plaques. However, we cannot be sure since "treated" monolayers were not examined beyond 46 h (the total duration of an experiment). It would be interesting to know at what concentration, if any, infection fails to progress after Ara-A is removed from the cultures.

Cultures "pulse-treated" with ADA inhibitor before infection or incorporation of Ara-A into the media failed to prevent deamination. This strongly suggests that the inhibitor had no lasting effect upon ADA production by the cell.

In the absence of ADA inhibitor, deamination required several hours; at 5 μg/ml input concentration, 0.37 μg/ml remained at 8 h (see Table 3). If the susceptible step in viral replication occurred at or before this time, then Ara-A at that input concentration should have resulted in viral inhibition, since that concentration in the cultures containing ADA inhibitor caused plaque reduction (see Table 2).

There seem to be two hypothetical explanations for this, both depending upon the rate of diffusion (2, 17) of Ara-A across the cell membrane, at varying extracellular concentrations

of nucleoside and the rate of intracellular deamination of Ara-A, by LLC-MK_2 cells. First, since the rate of diffusion of nucleosides varies directly with concentration (19), it is possible, at the higher input media concentrations, that the rate of diffusion initially exceeded the rate of intracellular deamination. Thus, Ara-A may have reached some intracellular concentration initially. However, as the concentration in the media declined with time, as a result of deamination, then the rate of diffusion may have fallen below the rate of deamination and the only intracellular Ara-nucleoside, at that time, would have been Ara-Hx. This point must have been reached before the susceptible step in the replication cycle; or else, the level of Ara-A intracellularly must have been lower than the inhibitory level.

Alternatively, the rate of intracellular deamination may be greater than the rate of diffusion across the membrane at any media concentration (≤ 10 μg/ml). In that case, in the absence of ADA inhibitor, Ara-A was deaminated as rapidly as it reached the intracellular space.

Either hypothesis would be consistent with the observations that the PIC_{100} (concentration at which Ara-A completely prevented plaque development, i.e., zero survivors; plaque inhibitory concentration) of Ara-A (≥ 10 μg/ml) in the absence of ADAI was the same as the PIC_{100} of Ara-Hx; or allow for complete protection of Ara-A by ADA inhibitor.

We used a known competitive inhibitor of ADA, 6-MedAdo (14), to confirm the evidence that reduction in level of deamination of Ara-A would correlate with increased antiviral activity. This was so, and we were able to demonstrate again that concentrations of Ara-A in media were maintained in presence of 6-MedAdo for greatly prolonged periods. However, there could have been some added or synergistic activity between Ara-A and 6-MedAdo since 6-MedAdo had some antiviral activity alone.

Previously, experiments to determine the susceptibility of VV (7, 10, 15) and of herpesvirus (7, 8, 10, 15) to Ara-A have not taken into account deamination of the nucleoside to Ara-Hx by ADA produced by indicator cells and present in bovine serum. Therefore, those reports probably found inhibitory levels of Ara-A at concentrations higher than actual susceptibility or reflect susceptibility to Ara-Hx, not Ara-A. The susceptibility of VV to Ara-A in the absence of ADA activity is probably a reflection of the real relationship between the nucleoside and VV replication, rather than a level of susceptibility determined in a system where deamination is occurring at a rapid rate. Using the LLC-MK_2 cell indicator system, the latter would represent Ara-Hx activity; in a system where the rate of deamination was slower, perhaps Ara-A activity at a level lower than calculated input would be determined. Thus, in vitro susceptibility of VV to Ara-A would be inversely proportional to deaminase activity in the indicator host cell.

In other words, fate of a prospective antiviral compound depends upon the number of enzyme-producing cells per test unit (well, dish, etc.), the state of the cell culture (resting or growth phase), the total period of a test procedure (incubation period), the concentration and species of mammalian serum in the test system, the kind of cell comprising the indicator culture, and probably, the presence or absence of biological contamination (bacterial or mycoplasmal). The total effect of a system upon a test compound in vitro can be determined by its quantitation in the media of the indicator cells; this information should be the minimum required to introduce a system proposed to test the susceptibility of a virus to a compound. In LLC-MK_2 cells Ara-A is rapidly deaminated. In human diploid fibroblastic cells, deamination of an equivalent amount of Ara-A requires approximately twice as long (L. Sweetman, J. D. Connor, M. A. Stuckey, R. Seshamani, S. Carey, and R. Buchanan, Symposium Adenine Arabinoside, in press). In ADA-deficient cells, deamination should be minimal or absent. Such factors probably are important in the differences that have been reported for the susceptibility of VV and herpesviruses to Ara-A and in respect to the ratio of antiviral activity between the parent and deaminated compound.

It is, therefore, apparent that in vitro viral susceptibility is determined by the effect of a compound upon viral replication, limited by the extent to which the compound is altered by the indicator system, acting as an intact biological unit, and the effect such alteration has upon the basic antiviral property of the compound (Fig. 2). Recently, we have reported the susceptibility of 20 sero-typed strains of *Herpesvirus hominis* to Ara-A using a similar method to that reported herein, and found it lower than previously recognized (1).

It is important to recognize the direct effect of a biological system upon a compound put into test as a potentially useful antiviral agent if we are to accurately develop data relating doses and therapeutic effect, both in in vitro studies and in clinical trials. Further, adventitial bio-

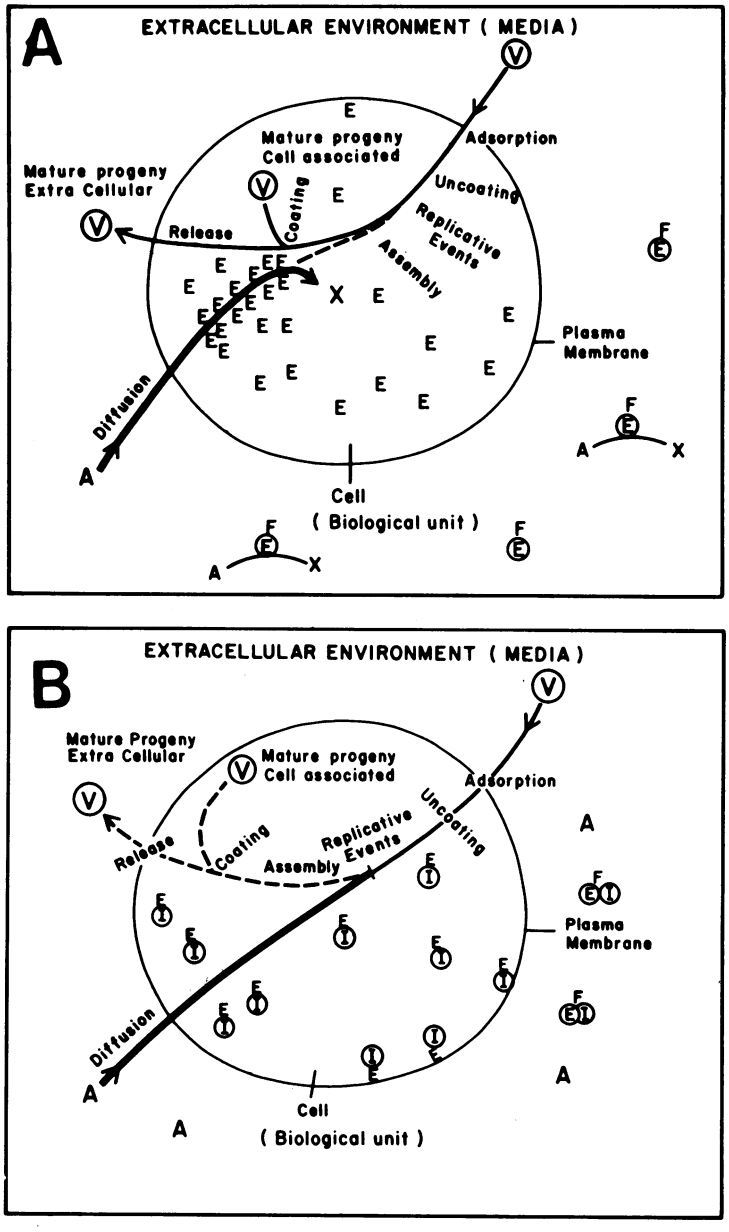

FIG. 2. *Potential effects of indicator system upon antiviral compound. Diagramatic sketch of Compound A altered by cellular process (E) or serum component (E) to inactive form (X). Based upon deamination of ara-A to Ara-Hx by cellular or serum ADA, and protection from deamination by ADA inhibitor. (V) Mature infectious virus; (A) compound with proposed antiviral activity; (X) metabolite of A with same, increased or decreased antiviral activity; (E) enzyme, or other cellular metabolic process, altering A to X; (F) biological fluid, constituent of media, potential source of E, also; (I) compound protecting activity of A; in this case, inactivating E. (A) Loss of antiviral activity; (B) protection of antiviral activity.*

logical systems must be excluded, such as that contributed by bacterial and mycoplasmal contamination.

It is interesting to speculate about the mechanism of antiviral action of Ara-A since it is known that cellular toxicity is due to at least two kinds of anti-deoxyribonucleic acid replicative activity: (i) ara-nucleotide inhibition of

ribonucleotide reductase (16, 21) and (ii) aranucleotide inhibition of deoxyribonucleic acid polymerase (9). Ara-A is known not to inhibit ribonucleic acid polymerase and to be incorporated into new cellular nucleic acid at a rate of 0.2% or less (5). In our experiments, LLC-MK_2 monolayers were at resting stage without serum or growth factor and showed no toxic effects after 48 h of treatment with Ara-A, inhibitor or both, as judged by sub-passage plating, trypan blue, and neutral red staining as compared to untreated controls. Since Ara-A was highly effective against VV at a low concentration, perhaps there is a viral specific pathway such as reported for bromodeoxycytidine in herpes simplex viral-infected cells (6), and for specific binding of 3-methyleneoxindole in poliovirus-infected cells (20). Isolation of the effects of Ara-A in our system may be helpful in establishing the mechanism of its antiviral activity.

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