Cytosine Arabinoside Kills Postmitotic Neurons in a Fashion Resembling Trophic Factor Deprivation: Evidence That a Deoxycytidine-Dependent Process May Be Required for Nerve Growth Factor Signal Transduction

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Cytosine arabinoside (AraC) is a pyrimidine antimetabolite that kills proliferating cells by inhibiting DNA synthesis. In this paper we report that AraC kills postmitotic rat sympathetic neurons in a fashion similar to the neuronal death that follows nerve growth factor (NGF) deprivation.

Postmitotic rat sympathetic neurons were cultured for 1 week in the presence of NGF and then treated with AraC, still in the presence of NGF. AraC killed neurons after 4 d with an EC_{50} of 50 μ M. The morphological and temporal characteristics of neuronal death that began around 3 d after addition of AraC were indistinguishable from those observed beginning 24 hr after NGF deprivation. Death caused by AraC was prevented by the same agents that prevent the death of NGF-deprived neurons, which included inhibitors of RNA and protein synthesis, a cAMP analog, and depolarizing concentrations of KCl. In contrast, neuronal death caused by ultraviolet irradiation, ricin toxin, and a variety of other toxic insults did not share these morphological, biochemical, or temporal characteristics.

Other antimetabolic drugs, including adenine arabinoside, thymine arabinoside, fluorodeoxyuridine, hydroxyurea, and aphidicolin, did not kill neurons. AraC caused neurons to behave as if deprived of NGF by interfering with deoxycytidine (dC) metabolism distinct from DNA biosynthesis. dC entirely prevented the neurotoxicity of AraC, even when present at a concentration 1000-fold less than that of AraC. Other deoxynucleosides, and cytidine, did not prevent AraC neurotoxicity. dC could not, however, substitute for NGF and thus is unlikely to be a direct mediator of NGF action. It is hypothesized that dC may participate in a pathway, distinct from DNA synthesis, that is necessary for neurons to respond to exogenous trophic factors.

Cytosine arabinoside (AraC) is a pyrimidine antimetabolite used clinically for the treatment of acute leukemia (for reviews, see Chabner, 1982; Kufe and Major, 1982; Pallavicini, 1984). It is also commonly used in the culture of neurons or muscle *in vitro*

to kill proliferating cells such as fibroblasts. The general rationale behind these uses is that AraC inhibits DNA synthesis and thereby kills dividing cells, while sparing nonmitotic cells. Despite extensive study, the precise mechanism of cellular death caused by AraC is not understood (Calabresi and Parks, 1985). While AraC clearly kills dividing cells, there is some evidence that it may also affect cells that are not proliferating. Patients treated with high doses of AraC for leukemia occasionally suffer from a cerebellar toxicity syndrome (Sylvester et al., 1987) in which Purkinje neurons die (Winkelman and Hines, 1983), despite the fact that these cells do not divide. Furthermore, there exists a "folk wisdom" in tissue culture that AraC is more toxic to neurons than other antimetabolic drugs (Bustos et al., 1980; Oorschot and Jones, 1986; Smith and Orr, 1987).

We have recently reported (Wallace and Johnson, 1989) that AraC is unique among antimetabolic agents in its ability to kill postmitotic neurons. In that study, AraC killed both postmitotic chick ciliary and dorsal root ganglion neurons *in vitro*. Several other antimetabolites, including adenine arabinoside (AraA), thymine arabinoside (AraT), and aphidicolin, had no effect on neuronal survival, even at very high concentrations. The neurotoxicity of AraC was inhibited by low concentrations of 2'-deoxycytidine (dC) or elevated concentrations of cytidine (which was 40-fold less potent than dC), but not by very high concentrations of other ribo- or deoxyribonucleosides.

In a separate study (Martin et al., 1988), we have shown that the death of cultured rat sympathetic neurons caused by nerve growth factor (NGF) deprivation is prevented by inhibitors of RNA or protein synthesis. These data strongly suggest that neuronal death caused by trophic factor deprivation is an active process requiring the synthesis of new mRNA and protein, rather than a passive atrophy secondary to the loss of anabolic stimulation by NGF. Our working hypothesis is that NGF (and presumably other trophic factors) normally suppresses a genetic program that is designed to kill the neuron under appropriate physiological circumstances, such as during development when competition for target-derived neurotrophic support deprives superfluous neurons of adequate trophic factor.

Here we report that AraC kills postmitotic rat sympathetic neurons maintained *in vitro* with NGF. Of particular interest, AraC-treated neurons behaved as if deprived of NGF. Additional data suggest that AraC exerts its neurotoxicity by inhibiting a dC-dependent process other than DNA synthesis. It is hypothesized that a dC-dependent process is necessary, but not sufficient, for neuronal responses to trophic factors. These ob-

Received May 17, 1989; revised July 10, 1989; accepted July 13, 1989.

We thank Patricia Lampe and Jenny Columbo for excellent technical assistance, and Patricia Osborne for help in editing the manuscript. This work was supported by NIH grants NS18071 and GM07085.

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servations provide a basis for the "folk wisdom" that AraC adversely affects nonproliferating cells and suggest a possible new scheme for signal transduction.

A preliminary report of this work has appeared in abstract form (Wallace et al., 1988).

Materials and Methods

Cell culture. Primary, dissociated cultures of sympathetic neurons were prepared from the superior cervical ganglia of embryonic-day-21 rats as described previously (Johnson and Argiro, 1983). The vast majority of these neurons are postmitotic at this age. Cells were plated onto rat-tail collagen at a typical density of 20,000 cells/well (0.75 ganglia/well) in 24-well tissue culture plates (Costar, Cambridge, MA). Cultures were grown for 7 d in culture medium (0.6 ml/well) which consisted of 90% Eagle's minimal essential medium (Gibco Laboratories), 10% fetal bovine serum (JR Scientific, Woodland, CA), and 50 ng/ml 2.5S NGF (prepared by the method of Bocchini and Angeletti, 1969). Cultures were fed every 3–4 d. During the first 7 d after plating, the medium also contained 20 μ M fluorodeoxyuridine (FdU) and 20 μ M uridine to kill nonneuronal cells. Thereafter, the cultures were fed medium lacking the FdU and uridine.

Adenylate kinase (AK) assay. To measure AK activity, a 50- μ l aliquot of sample was added to 1 ml of assay reagent in a 10 $\times$ 75 mm fluorometer tube. Assay reagent consisted of 50 mM imidazole-HCl buffer, pH 7.0, containing 0.5 mM ADP, 1 mM glucose, 2 mM $MgCl_2$, 100 μ M NADP⁺, 0.02% bovine serum albumin, 2 μ g/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 10 μ g/ml yeast hexokinase. Fluorescence was measured on a fluorometer (Farrand Optical Co., New York, NY) set to a sensitivity equivalent to 15 μ M NADPH full scale with a quinine sulfate standard. Readings were not made until at least 5 μ M NADPH had been formed (usually 10–20 min). Corrections were made for the AK content of culture medium from parallel collagen-coated wells lacking cells.

Methionine incorporation assay. Each culture well received 33 μ Ci/ml [³⁵S]methionine (ICN Radiochemicals, Irvine, CA) in normal medium for 10–20 hr. TCA-precipitable counts were assessed by the method of DiStefano et al. (1985). Data were corrected by subtracting the counts nonspecifically bound to collagen-coated wells lacking cells and were expressed as percentages relative to [³⁵S]methionine incorporated by control (untreated) neurons on the same plate.

Reagents. To effect rapid and total NGF deprivation, polyclonal guinea pig antiserum against NGF (Rich et al., 1984) was added to the culture medium at a final concentration of 1%. Chlorophenylthio-cAMP and hexokinase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. All other reagents were purchased from the Sigma Chemical Company, St. Louis, MO.

Results

AraC kills rat sympathetic neurons

Dissociated sympathetic neurons were plated onto collagen in the presence of NGF and the antimetabolite fluorodeoxyuridine (FdU). Within 24 hr of plating, the neurons began to extend neuritic processes, which fasciculated and covered the dish by 5–6 d. The neuronal cell bodies tended to aggregate in clusters and were phase bright, with prominent nuclei and nucleoli. Over a period of 1 week, the neurons hypertrophied and established a lush carpet of neurites, but never divided. Nonneuronal cells (mostly Schwann cells and fibroblasts) were killed by the FdU between days 3–5, so that after 7 d >95% of the cells in culture were neurons. The FdU had no apparent adverse effects on the neurons, even at 1 mM, over many weeks. However, in these experiments FdU was removed from the culture medium on the seventh day and thereafter.

Established cultures (7 d after plating) were treated with 100 μ M AraC. The NGF concentration remained 50 ng/ml (approximately 2 nM) throughout the experiment. No changes were observed over the first 48 hr, but by 72 hr, the neuronal cell bodies began to atrophy. Between 72 and 96 hr the neurons

Figure 1. AraC kills postmitotic neurons with an EC_{50} of 50 μ M. Sympathetic neurons were grown for 1 week in the presence of NGF (50 ng/ml) without AraC whereupon AraC was added at the indicated concentrations. Five days later the cultures were assayed for released AK as described in Materials and Methods. Each point represents the mean $\pm$ SD of 3 wells.

degenerated dramatically. The neurites became thin and discontinuous, followed by condensation and degeneration of the cell bodies. By 96 hr of AraC treatment, the culture dish was covered with the degenerated, phase-dark remnants of cell bodies and fragmented residua of neurites. This debris gradually lifted off the culture dish over the next several days. The same results were obtained if the FdU was not removed after the initial culture period and AraC was added in addition to the FdU.

To determine the relationship between AraC dose and neuronal death, established cultures of neurons were incubated for 5 d in various concentrations of AraC. By visual inspection, sympathetic neurons cultured in 100 μ M or more AraC were dead, with fragmented neurites and condensed remnants of cell bodies lacking nuclei. At concentrations of AraC less than 10 μ M, the neurons were alive and healthy, indistinguishable from control cultures. When incubated in concentrations of AraC between 10 μ M and 100 μ M, the neurons appeared atrophic and partially fragmented after 5 d, but some of the neurons still appeared to be alive. This dose-response relationship was quantified by measuring the release of intracellular AK into the medium, which occurs when the integrity of the plasma membrane is lost as cells die. The released AK activity was expressed as a percentage of the sum of the initial culture medium sample and the activity extracted from remaining live cells by an equal volume of 0.1% Triton X-100. This percentage thus represents the fraction of total available intracellular AK that was released during the experiment and is proportional to the number of dead neurons. Typically, about 40% of the total Triton-extractable AK is released upon death of 100% of the neurons. The magnitude of this percentage can vary between experiments; therefore, each experiment is controlled such that all data are compared within the same plate. Figure 1 shows that the EC_{50} of AraC neurotoxicity is about 50 μ M, consistent with the result obtained above from morphological observation.

AraC neurotoxicity resembles NGF deprivation

The morphology of AraC-treated neurons closely resembled that of neurons deprived of NGF. To compare these deaths in more detail, the time course of death caused by AraC was quantitatively compared to that caused by trophic factor deprivation. Figure 2 shows the amount of AK released into the culture

Figure 2. Both NGF deprivation (○—○) and AraC (●—●) cause the rapid demise of neurons over a 24-hr period following an initial lag in which the neurons appear unaffected. Neurons were grown for 1 week in the presence of NGF without AraC. AraC (100 μ M) was then added to 1 group while the second group received antiserum to NGF. At times indicated on the abscissa, death was quantified by measuring the fraction of intracellular AK released into the culture medium. Each point represents the mean $\pm$ SD of 3 wells.

medium as a function of time after either NGF deprivation or addition of AraC. The kinetics of death caused by AraC were similar to NGF deprivation, though delayed in onset. In both cases, the release of AK correlated with the morphological degeneration of the cells. The morphological features of neuronal demise resulting from either NGF deprivation or AraC were indistinguishable, executing rapidly over an approximately 24-hr period that commenced after a delay during which the neurons remained apparently unaffected. For NGF deprivation this delay period lasted about 24 hr, whereas for AraC the delay period was about 72 hr. Hence, once initiated, the deaths caused by AraC and NGF deprivation appeared similar by criteria observable under phase-contrast microscopy and occurred with the same rate as measured by the release of AK into the culture medium. The death caused by AraC, however, began about 48 hr later than that caused by NGF deprivation.

Death caused by AraC requires protein and RNA synthesis

Since the death of NGF-deprived neurons requires the synthesis of RNA and protein (Martin et al., 1988), we determined whether the same dependency existed for AraC neurotoxicity. Figure 3*a* illustrates healthy sympathetic neurons grown for 12 d in the presence of NGF, without AraC. Figure 3*b* shows neurons at the same age that were treated with AraC between days 7 and 12 of culture. AraC caused the destruction of these neurons, characterized by neuritic fragmentation and condensation of the phase-dark cell bodies. We previously found that these neurons can withstand total inhibition of protein or RNA synthesis for 5 d (Martin et al., 1988). Figure 3, *c*, *e*, illustrate neurons treated with cycloheximide and actinomycin-D, respectively, from days 7 to 12 of culture. Although the neurons have atrophied somewhat, they are still alive. Inhibitors of RNA and protein synthesis prevented neuronal death caused by AraC. This is demonstrated by comparing Figure 3, *d* and *f* with Figure 3*b*. Neurons treated with AraC along with cycloheximide or actinomycin-D were alive, whereas those treated with AraC alone were dead and degenerated.

The prevention of AraC neurotoxicity by RNA and protein synthesis inhibitors was quantified by measuring AK release. Figure 4, bars 1 and 2, shows that AraC treatment causes a

dramatic release of AK into the culture medium, indicating the loss of neuronal integrity associated with cell destruction. In contrast, if protein or RNA synthesis was inhibited with cycloheximide or actinomycin-D, respectively, AK was not released into the medium. This indicates that the neurons remained intact despite treatment with AraC.

Implicit in the experiment described above is the fact that neurons can withstand total inhibition of protein synthesis for 5 d without dying. Because cycloheximide is a reversible inhibitor, it was possible to rescue the cells by washing out the drugs. If the cells that appeared alive were truly viable, then they should incorporate amino acids into TCA-precipitable protein after a sufficient rescue period. To test this, established cultures were treated with AraC and/or cycloheximide for 5 d as in the previous experiment. The cultures were then washed several times with normal culture medium (no AraC or cycloheximide) and cultured for an additional 4 d. At this point all cultures were fed fresh medium supplemented with 35 S-Methionine for 17 hr. TCA-precipitable counts were measured and are presented in Figure 5 as a percentage of that incorporated by the control culture which was never exposed to AraC or cycloheximide.

Neurons that were treated with AraC alone for 5 d had died and thus were unable to synthesize protein. The neurotoxicity of AraC alone is quantified by the 90% difference between control and AraC (bars 1 and 2 of Fig. 5). The majority (about 65%) of neurons that were treated with cycloheximide alone for 5 d recovered from the total inhibition of protein synthesis. These surviving neurons, after removal of cycloheximide, were able to incorporate methionine into TCA-precipitable protein, as represented by bar 3 of Figure 5. Bar 4 of Figure 5 shows that cycloheximide prevented the neurotoxicity of AraC since these cultures incorporated comparable amounts of [35 S]methionine into protein as did cultures treated with cycloheximide alone.

AraC neurotoxicity resembles NGF deprivation by other criteria

Neuronal death caused by NGF deprivation can also be prevented by *p*-chlorophenylthio-cAMP (Rydel and Greene, 1988; unpublished observations from this laboratory). Established cultures were treated with 100 μ M AraC in medium containing 400 μ M *p*-chlorophenylthio-cAMP. After 5 d the neurons were alive; although they did not look as healthy as controls, they appeared comparable to neurons treated only with the analog. The fact that cAMP prevented the death of AraC neurons is quantified by the low level of AK release in Figure 4.

Elevated levels of potassium in culture medium can prevent the death of neurons that normally occurs in the absence of trophic factors (Scott and Fisher, 1970; Phillipson and Sandler, 1975; Wakade and Thoenen, 1984; Koike et al., 1989). Established neurons were treated with 100 μ M AraC in culture medium containing 35 mM KCl. These neurons were alive and healthy after 5 d, showing no signs of atrophy or neurite degeneration. Figure 4 shows that no AK was released from the neurons treated with AraC in the presence of elevated potassium.

Only NGF deprivation and AraC cause active deaths

We have tested a number of diverse chemical agents for their ability to prevent neuronal death caused by NGF deprivation (Martin et al., unpublished observations). In each of these experiments, a control group of neurons received the drug in the presence of NGF. Many drugs were toxic at high concentrations,

Figure 3. RNA and protein must be synthesized for AraC to kill neurons. Neuronal cultures were grown for 1 week in medium containing NGF and then treated as follows. The neurons on the right side of the panel (*b*, *d*, and *f*) were treated with 100 μ M AraC for 5 d, whereas those on the left (*a*, *c*, and *e*) served as untreated controls. Cultures *a* and *b* received no inhibitors, *c* and *d* were treated with 1 μ g/ml cycloheximide, and cultures *e* and *f* were treated with 0.1 μ g/ml actinomycin-D. The neuronal destruction caused by AraC was prevented by cycloheximide and actinomycin-D. Neurons *in vitro* were photographed with a phase-contrast microscope using a green interference filter and Kodak T-Max film, ASA 400. Scale bar, 100 μ m.

Figure 4. Inhibitors of protein or RNA synthesis, as well as potassium depolarization or *p*-chlorophenylthio-cAMP, prevent neuronal death caused by AraC. Sympathetic neurons were grown for 1 week in medium containing NGF and then treated as follows. *Unfilled bars* represent cultures that received only the indicated drug, without AraC. *Filled bars* represent cultures that received both AraC (100 μ M) and the indicated drug. *No Drug*, Normal culture medium; *CHX*, 1 μ g/ml cycloheximide; *AMD*, 0.1 μ g/ml actinomycin-D; *K⁺*, normal culture medium supplemented with 35 mM KCl; *CPT*, 400 μ M *p*-chlorophenylthio-cAMP. After 5 d the fraction of intracellular AK released into the culture medium was measured. Each bar represents the mean $\pm$ SD of 3 wells.

but in most cases, the morphology of death differed from that caused by NGF deprivation and AraC neurotoxicity. It was far more typical for dying neurons to swell and accumulate vacuoles, exhibiting toxic effects immediately and progressively after the insult rather than degenerating abruptly after a delay period of apparent health. AraC and NGF deprivation were the only treatments that caused neuronal condensation and rapid neuritic fragmentation after an initial period of time wherein the neuron appeared normal. Treatments that kill neurons without requiring protein synthesis include A_{23187} , antibody + complement, azide, colcemid, extreme pH, heat shock, 6-hydroxydopamine, melittin, ouabain, ricin, UV irradiation, and vincristine.

Figure 5. AraC-treated neurons remain viable if protein synthesis is inhibited. Cultures were grown for 1 week in culture medium, followed by incubation for 5 d in the presence of AraC (100 μ M) and/or cycloheximide (1 μ g/ml) as indicated. After treatment with these drugs, all cultures were washed thoroughly with normal culture medium and allowed to recover for 4 d. At this point [³⁵S]methionine was added to the medium for 17 hr and TCA-precipitable protein was assayed as described in Materials and Methods.

Figure 6. Of several antimitotic drugs, only AraC kills sympathetic neurons. Neurons were cultured for 1 week in normal culture medium and then treated with the following antimitotic drugs. *Cont*, Normal culture medium without antimitotic; *AraC*, 100 μ M cytosine arabinoside; *AraA*, 1 mM adenine arabinoside; *AraT*, 1 mM thymine arabinoside; *FdU*, 1 mM fluorodeoxyuridine; *HU*, 1 mM hydroxyurea; *APC*, 100 μ M aphidicolin. After 5 d the fraction of intracellular AK released into the culture medium was measured. Each bar represents the mean $\pm$ SD of 3 wells.

Other antimitotic drugs are not neurotoxic

Generally, AraC kills proliferating cells by inhibiting DNA synthesis. The established neurons in this tissue culture system are not proliferating, however, suggesting that the site of AraC action in this case is distinct from DNA synthesis. Established neuronal cultures were incubated in the presence of a variety of other antimitotic drugs at a high concentration (1 mM) for 5 d. These drugs included AraA, AraT, FdU, and hydroxyurea, all of which have the ability to kill proliferating cells comparable to AraC. Aphidicolin, an inhibitor of DNA repair, was used at 100 μ M instead of 1 mM because of its limited solubility. None of these treatments had any observable adverse effects on the neurons. Figure 6 documents this observation by showing that these cultures remained intact as indicated by the very low amount of AK in the medium. Note that at a 10-fold lower concentration than these agents, AraC had caused the total destruction of the neurons by morphological criteria and concomitant release of AK.

Deoxycytidine blocks AraC neurotoxicity

Established cultures were treated with 100 μ M AraC in the presence of the conventional deoxynucleosides. After 5 d the amount of AK released into the medium was measured (Fig. 7). dC (100 μ M) entirely blocked the neurotoxicity of AraC, while the other 3 deoxynucleosides at a 10-fold higher concentration (1 mM) did not prevent the death caused by AraC. While the AK data suggest that dT partially prevents AraC neurotoxicity, these neurons appeared dead by morphological criteria. None of these deoxynucleosides was toxic to neurons at 1 mM, in the absence of AraC (data not shown). This result suggests that AraC neurotoxicity involves a process that is specific for dC.

The potency of dC inhibition of AraC neurotoxicity was determined by treating established cultures with 100 μ M AraC for 5 d in the presence of various concentrations of dC. After 5 d, neurons treated with 50 nM dC or less were dead, appearing similar to those treated with AraC alone. Cultures treated with AraC and 1 μ M or more dC, however, were alive and healthy. This dose-response relationship was quantified by measuring

Figure 7. Of several deoxynucleosides, only deoxycytidine prevents the neurotoxicity of AraC. Cultures were grown for 1 week in normal culture medium and then treated with 100 μ M AraC and deoxynucleosides as indicated. dC, 100 μ M deoxycytidine; dA, 1 mM deoxyadenosine; dT, 1 mM deoxythymine; dG, 1 mM deoxyguanosine. After 5 d the fraction of AK released into the culture medium was measured. Bars are mean $\pm$ SD of 3 wells.

AK release into the culture medium. These data are presented in Figure 8 and show that dC has an EC_{50} of 100 nM for preventing the neurotoxicity of 100 μ M AraC.

The ability of cytidine to prevent AraC neurotoxicity was evaluated by incubating established cultures in the presence of 100 μ M AraC with various concentrations of cytidine. Released AK was measured after 5 d and is presented in Figure 9. These data show that doses up to 1 mM cytidine are not capable of preventing the death of all the neurons, although high doses of cytidine are partially able to prevent AraC neurotoxicity. It is possible that this protective ability of cytidine might result from its intracellular reduction to dC. To test this, we inhibited the responsible enzyme, ribonucleoside reductase, with hydroxyurea. In the presence of hydroxyurea, cytidine was unable to prevent the toxicity of 100 μ M AraC, while the ability of dC to prevent the death remained unaffected (Fig. 10). This indicates that the partial ability of cytidine to block AraC neurotoxicity stems from its conversion to dC. These data are most consistent with AraC (or its phosphorylated nucleosides) competing for dC (or its phosphorylated forms) at a site other than DNA synthesis or repair.

Deoxycytidine cannot substitute for NGF

The previous data show that AraC causes neurons to behave as if deprived of NGF. Since AraC neurotoxicity is prevented by dC, it was determined whether dC could also prevent the death of NGF-deprived neurons. Established neurons were deprived of NGF by adding anti-NGF antiserum, with and without dC. After 48 hr all the cultures were dead regardless of the presence of dC. This result was quantified by measuring released AK and is presented in Figure 11. These data suggest that, while a dC-dependent process is critical for neuronal survival in the presence of NGF, dC itself does not promote survival in the absence of trophic factor.

Discussion

AraC kills postmitotic neurons

In this report we have presented evidence that AraC, an analog of dC, kills postmitotic rat sympathetic neurons. This result

Figure 8. The neurotoxicity of 100 μ M AraC is completely inhibited by dC, with an EC_{50} of 100 nM. Cultures were grown for 1 week in medium containing NGF without AraC, whereupon they received 100 μ M AraC in conjunction with the indicated concentration of dC. After 5 d the cultures were measured for released AK. Each point represents the mean $\pm$ SD of 3 wells.

extends to rat sympathetic neuron our previous observation that AraC also kills postmitotic chick sensory and parasympathetic neurons (Wallace and Johnson, 1989). The observation that AraC kills multiple neuronal types in different animal species suggests that it interferes with a conserved and fundamental process that is necessary for neuronal survival. The fact that AraC is also neurotoxic *in vivo* (Winkelman and Hines, 1983; Sylvester et al., 1987) suggests that the ability of AraC to kill neurons *in vitro* is not an artifact of the culture conditions or the state of the neurons after dissection. Furthermore, AraC (but not AraA or FdU) reduced or eliminated the response of muscle cells to a factor that induces incorporation of acetylcholine receptors (F. Johnson and G. D. Fischbach, personal communication), thus suggesting that AraC may be selectively toxic to postmitotic muscle cells as well.

The neurotoxicity of AraC was steeply dose dependent, with an EC_{50} of about 50 μ M. This value is higher than that found for chicken neurons, which died when exposed to AraC con-

Figure 9. Cytidine partially blocks the neurotoxicity of 100 μ M AraC. Cultures were grown for 1 week in culture medium containing NGF and then treated with 100 μ M AraC and the indicated concentration of cytidine for 5 d. Then the fraction of AK released into the culture medium was measured. Cytidine could only partially prevent the neurotoxicity of AraC and exhibited an EC_{50} of 100 μ M, which is 3 orders of magnitude greater than that of dC. Each point represents the mean $\pm$ SD of 3 wells.

Figure 10. The ability of cytidine to prevent AraC neurotoxicity stems from its intracellular conversion to dC. Neurons were grown for 1 week in medium containing NGF and then treated with 100 μ M AraC and drugs as indicated. C, 1 mM cytidine; HU, 1 mM hydroxyurea (inhibits reduction of cytidine to deoxycytidine); dC, 1 mM deoxycytidine. After 5 d the fraction of AK released into the medium was measured. Each bar represents the mean $\pm$ SD of 3 wells.

Figure 11. dC does not prevent the death of NGF-deprived neurons. Neurons were grown for 1 week in medium containing NGF and then deprived by adding anti-NGF antisera and/or treated with 1 mM dC as indicated. After 3 d the fraction of AK released into the culture medium was measured. NGF deprivation caused neuronal death regardless of the presence of dC. Each bar represents the mean $\pm$ SD of 3 wells.

centrations as low as 1 μ M (Wallace and Johnson, 1989). The difference might be species specific and/or related to the culture conditions in which the neurons were grown. The latter possibility is likely in light of the fact that the toxicity of AraC was potentially blocked by exogenous dC in the culture medium. Differences in the dC concentration (due to variable serum lots, for example) might result in marked differences in the apparent sensitivity of neurons to AraC. It is also possible that each neuronal type contains differing amounts of the key enzymes, cytidylate kinase and cytidine deaminase, which determine the steady-state level of activated AraCTP in the cell.

AraC is commonly used in tissue culture as an antimitotic drug to prepare relatively pure populations of nondividing cells, such as neurons or muscle. The rationale is that AraC will kill proliferating cells like fibroblasts or Schwann cells while sparing the cells that are not dividing. The fact that AraC, but no other commonly used antimitotic drug, kills nondividing cells indicates that this practice should be reconsidered. We suggest that AraC is a poor choice as an antimitotic because it adversely affects both nonproliferating cells as well as dividing cells. In fact, there is a "folk wisdom" that makes this same point (Bustos et al., 1980; Oorschot and Jones, 1986; Smith and Orr, 1987). Other antimitotics, like FdU, AraA, or AraT, had no adverse effects on the neurons but kill dividing cells comparable to AraC. Any of these antimitotics would be more appropriate than AraC for the preparation of relatively pure nondividing cultures.

A consequence of the use of AraC in tissue culture is that it may confuse the interpretation of experiments designed to study agents that promote the survival or differentiation of nonmitotic cells. When culture medium contains AraC to control proliferating cells, the AraC may also exert negative effects on the nondividing cells under study. Hence, an observed positive effect on the nondividing cells might result either from a true positive influence relevant to the normal biology of the cell or, alternatively, from the inhibition of the toxic effects of AraC. In fact, it was such an "artifact" that led to our initial appreciation of the selective neurotoxicity of AraC and its prevention by exogenous dC (Wallace and Johnson, 1989).

AraC neurotoxicity resembles NGF deprivation

AraC caused neurons cultured in the presence of NGF to behave as if they were deprived of NGF, based on morphological, temporal, and biochemical criteria. First, both instances of death result in condensation of the cell body and fragmentation of neurites. Second, although death caused by AraC commenced about 48 hr later than that caused by NGF deprivation, the time course of each was similar once morphological changes began. Both were rapid, executing over a period of about 24 hr after a lag during which the neurons appeared unaffected. Third, both instances of death required RNA and protein synthesis, indicating that the destructive process was active "suicide" rather than passive attrition. Finally, both instances of death were prevented by the cAMP analog *p*-chlorophenylthio-cAMP and by elevated potassium in the medium. These similarities are unique, since other toxic insults that kill neurons do so with different morphological characteristics, at different rates, and independent of protein synthesis. The similarity between death caused by AraC and NGF deprivation suggests that both treatments ultimately lead to death via the same mechanism.

AraC alters a dC-specific process distinct from DNA synthesis

AraC is commonly classified as an antimitotic drug because, in proliferating cells, it inhibits DNA synthesis by a mechanism it shares with a variety of other purine and pyrimidine analogs. Specifically, AraC enters cells and is phosphorylated to an active intermediate that can substitute for dCTP in DNA strand elongation (Kufe and Major, 1982). Subsequent deoxynucleosides cannot be added efficiently to AraC and DNA synthesis stalls.

It is, therefore, surprising that AraC kills postmitotic neurons because this implies that AraC acts at some site other than DNA synthesis. Evidence for this contention derives from the fact that other antimitotic drugs (that share similar abilities to inhibit DNA synthesis) were not toxic to the neurons. DNA repair is unlikely the target of AraC neurotoxicity because an inhibitor of DNA repair, aphidicolin, did not kill the neurons. Nor is it likely that AraC exerts its neurotoxicity by disturbing the balance of deoxynucleoside pools (Yoshioka et al., 1987; Kunz, 1988) since neurons did not die when the balance of these pools

was perturbed with either FdU, hydroxyurea, or by adding high exogenous concentrations (1 mM) of deoxynucleosides. Additionally, the fact that AraA and AraT did not adversely affect neurons indicates that the toxicity of AraC does not stem solely from the arabinose moiety of the molecule.

dC was able to block the toxicity of AraC in a dose-dependent fashion. The fact that such a low concentration of dC (100 nM) prevented the effects of a much higher concentration of AraC (100 μ M) suggests that the process affected by AraC actually has a much higher affinity for dC than AraC. This process is apparently specific for dC nucleosides because the other conventional deoxynucleosides were unable to prevent AraC neurotoxicity entirely. Although unable to save neurons by morphological criteria, dT was partially able to reduce AK release from AraC-treated neurons. The concentration of dT yielding this partial effect was 1000-fold greater than that of dC providing maximal saving.

Additionally, our data suggest that the process targeted by AraC is selective for 2'-deoxynucleosides rather than the ribonucleosides. Although cytidine could partially prevent AraC neurotoxicity, this effect was completely blocked by hydroxyurea, a drug that inhibits ribonucleoside reductase, the enzyme that converts cytidine to dC. Hence, cytidine itself cannot compete with AraC but can be converted intracellularly to dC, which accounts for the ability of cytidine to prevent AraC neurotoxicity partially.

AraC blocks a dC-dependent process

It is unlikely that AraC competes with dC for cellular uptake because dC blocked AraC toxicity very selectively, relative to the other nucleosides. Deoxynucleosides, as well as nucleosides, gain access to the cytoplasm via facilitated diffusion through a membrane transporter, which exhibits little specificity for any given nucleoside (Cohen et al., 1979; Paterson et al., 1979). This transporter is nonselective for the specific nucleosides and hence not likely to be specifically affected by AraC or dC. Furthermore, we have previously found that a selective inhibitor of nucleoside uptake, nitrobenzylthioinosine, was able to prevent the toxicity of AraC on chick sensory and ciliary neurons, suggesting that AraC must gain entry to the neuron to exert its toxic effect (Wallace and Johnson, 1989). Nitrobenzylthioinosine was not toxic to neurons by itself though, which suggests that neurons do not normally need exogenous dC to live.

Other sites of action, related to but distinct from DNA synthesis, are the enzymes that regulate the pools of activated deoxynucleotides. The rate-limiting enzyme for synthesis is cytidylate kinase and that for degradation is cytosine deaminase. The same enzymes apparently catalyze these reactions for both AraC and dC, although their relative affinities for the two substrates are unknown.

We speculate that the target of AraC action is a dC-dependent process associated in some way with trophic factor signal transduction. It has recently been shown that deoxycytidine triphosphate exists in 2 distinct intracellular pools, one used preferentially for DNA synthesis (Spyrou and Reichard, 1987) and the other for the synthesis of dC adducts such as dCDP-diglyceride (Ter Schegget et al., 1971), dCMP-phosphatidic acid (Medrano and Mordoh, 1979), dCDP-choline, and dCDP-ethanolamine (Sugino, 1957; Potter and Buettner-Janusch, 1958; Spyrou and Reichard, 1987). These dC adducts have been found to substitute effectively for cytidine adducts (Lehninger, 1982) as intermediates in the formation of phospholipids (Kennedy et

al., 1959; Ter Schegget et al., 1971). Also, the analogous AraC lipids have been shown to be produced by cells (Lauzon et al., 1978). It is possible that deoxyliponucleosides are distinguished from riboliponucleosides as a different set of lipid molecules, which serve some as yet uncharacterized function in neurons or other cells. Hence, by analogy to DNA and RNA, the deoxy-ribonucleolipids might be reserved for a different class of biochemical interactions than the ribonucleolipids. This class of deoxylipids might be intimately involved with signal transduction through membrane-associated, cell-surface receptors. This explanation is, of course, speculation because the issues of dC metabolism have not been examined in neurons or other non-replicating cells.

Model of neuronal reactions to AraC and NGF deprivation

Figure 12 illustrates our working hypothesis in schematic form. This model is largely based on the biology of NGF, but the principles outlined may apply generally to other trophic factors. This is valid because AraC was toxic not only to cells maintained with NGF, but also to those sustained with insulin or a factor isolated from pig lung (Wallace and Johnson, 1989).

The response to trophic factor begins with its binding to specific receptors located in the neuronal membrane. The trophic factor and receptor are both internalized and retrogradely transported to the cell body. The second messenger that carries the NGF signal to the nucleus is unknown. Nonetheless, the information reflecting the presence of NGF becomes available within the nucleus, where it regulates gene expression. We propose that the messenger of trophic factor signal regulates 2 genetic programs in a complementary fashion. When trophic factor is present, it stimulates the "trophic program" and represses the "death program."

The trophic program consists of a cascade of RNA and protein synthesis that results in the classic trophic responses to NGF, including neurotransmitter-synthesizing enzymes and proteins involved in the production and elongation of neurites. Evidence of a cascade comes from studies on PC12 cells, which show that NGF stimulates an early set of genes, e.g., *c-fos*, that is followed by the synthesis of other proteins, which make up the phenotype of the trophic-supported state.

We hypothesize that the death program is normally repressed by NGF, such that upon NGF deprivation it becomes derepressed. The death program may consist of a cascade of RNA and protein synthesis, analogous to events such as differentiation and mitosis (Buttayan et al., 1988), resulting in new mRNA that codes for proteins that proximately kill the cell. Although cycloheximide or actinomycin-D would be expected to block the trophic and death programs simultaneously, in practice we observed that blockade of protein synthesis did not kill neurons for many days. Neurons treated with inhibitors of protein or RNA synthesis (in the presence of NGF) atrophy, but die only several days later. Thus, inhibition of the trophic program is not lethal in the short term. In the absence of NGF, however, these neurons die rapidly. This predicts that inhibiting RNA or protein synthesis should block the death caused by NGF deprivation, which is, in fact, what is observed (Martin et al., 1988).

AraC kills neurons in a fashion that closely resembles the death resulting from trophic factor deprivation. This raises the possibility that these treatments may ultimately induce expression of the endogenous death program. AraC might trigger the death program by either of 2 mechanisms. First, AraC might

Figure 12. Model of NGF and AraC effects in neurons: Trophic and survival-promoting actions. Neurotrophic factor is released from target tissue, whereupon it binds to specific neuronal receptors and is retrogradely transported to the cell body. Trophic factor is degraded without entering the nucleus, although information representing its presence somehow regulates gene expression. A dC-dependent process that is blocked by AraC is necessary, but not sufficient, for transduction of the trophic signal. This transduction, represented by a "black box," is drawn schematically and is not meant to imply that it is necessarily located in the cytoplasm of the cell body. The dC-dependent intermediate is degraded such that if its synthesis is blocked by AraC, signal transduction fails sometime later. The presence of trophic factor stimulates the synthesis of proteins necessary for neurotransmitter production and neuritic outgrowth, i.e., the "trophic program." Trophic factor deprivation, or blockade of its signal transduction by AraC, unleashes a cascade of new mRNA and protein synthesis that ultimately results in killer protein(s) that destroy the neuron, i.e., the "death program." The death program is executed when neuronal death is physiologically appropriate, as when competition for target during development deprives neurons of trophic factor. Different cell types may govern the same death program via other physiological regulators. AMD, Actinomycin-D; CHX, cycloheximide; AraC, cytosine arabinoside; dC, deoxycytidine.

directly stimulate the death program at a site similar to that where the NGF signal represses death. This would involve a dC-dependent process that inhibits the death program much like NGF does. This possibility is unlikely, however, because dC cannot prevent death if NGF is absent. Furthermore, because AraC takes much longer to kill the cells than NGF deprivation itself, it is unlikely that the dC-sensitive site is at the genetic level.

A second possibility is that AraC blocks the ability of NGF to suppress the death program. In this scheme AraC would block a dC-dependent process necessary for trophic factor signal transduction. In effect, AraC would cause NGF deprivation by depleting the neuron of a component necessary for the response to NGF. Consistent with this hypothesis, we found that elevating NGF concentrations (up to 1 $\mu\text{g/ml}$) can partially overcome the lethal effects of AraC (Wallace and Johnson, 1989). If one assumes that this intermediate is slowly degraded such that its half-life is about 1 or 2 d, then the neuron would experience NGF deprivation about 2 d after AraC treatment. This may explain the longer lag period preceding morphological changes that was observed with AraC as compared to NGF deprivation. Both treatments, though, would ultimately produce the same neuronal response, i.e., activation of the death program.

Because the second scenario is more consistent with our data, we speculate that AraC blocks a dC-dependent process that is required for NGF signal transduction and, thereby, prevents the cell from responding to trophic factor. In the schematic (Fig. 12), the location of this dC-dependent process is not meant to be specific. An intermediate could be required anywhere along the path of the NGF signal from membrane-related events associated with receptor-ligand binding to the translocation of a second messenger into the nucleus.

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