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Synergistic Effect of Herpes Simplex Virus and Cytosine Arabinoside on Human Chromosomes

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The combined treatment of cultures of human embryonic lung cells with herpes simplex virus type 2 and cytosine arabinoside produced a significantly increased number of cells containing multiple chromatid and chromosome breaks. The incidence of such cells was found to be approximately two and one half times greater than the additive effects of virus and cytosine arabinoside induced separately and is therefore synergistic.

After the initial report of Hampar and Ellison (6), the capacity of herpes simplex virus (HSV) to produce chromosome breakage has been well documented (1, 11, 23). Recently, HSV type 1 and HSV type 2 (HSV-2) have been shown to produce chromosome abnormalities in human cultured cells as early as 4 hr after virus inoculation (17). These abnormalities include chromatid breaks, accentuation of the secondary constrictions on chromosomes no. 1, 9, and 16, and chromosome pulverization (17). Cytosine arabinoside (ara-C), an effective inhibitor of deoxyribonucleic acid (DNA) synthesis, has also been shown to produce chromosome breakage in human cells as early as 1 to 3 hr after inoculation (8, 14). It has been noted that the chromosomal effects of ara-C (8, 14) and other chemicals (15) are similar to those produced by Rous sarcoma virus (8, 14). Ara-C is of especial interest since it is sometimes used in the treatment of herpes simplex keratitis (7, 25). Ara-C chemotherapy may have some clinical advantages over the more commonly employed iododeoxyuridine (IDU), since drug-resistant mutants of HSV arise less frequently to ara-C than to IDU (2, 26).

Since ara-C has been shown to inhibit viral DNA synthesis (10) and to produce virus-like chromosome abnormalities, we undertook a study of the combined effects of HSV and ara-C on human chromosomes. Stocks of HSV-2 were prepared by propagating the virus in cell cultures derived from human embryonic kidneys (HEK) or human embryonic lungs (HEL). The virus was titrated in primary rabbit kidney cell cultures growing in 60-mm petri dishes under a methylcellulose overlay as previously described (19). The virus titer was expressed in plaque-forming units (PFU) per ml. Chromosome preparations were made by the technique described by Miles and O'Neill (12).

To test the effect of ara-C on DNA synthesis in HEL cultures infected with HSV-2, monolayers on cover glasses (22 by 40 mm) were infected with the virus at an input multiplicity of 5 PFU per cell. The virus was allowed to adsorb at 37 C for 1 hr, and the cultures were then exposed to 10 μg of ara-C per ml in Eagle's basal medium containing 10% fetal bovine serum. Virus-inoculated controls received medium without the antimetabolite. All cultures were then incubated for an additional 3 hr at 37 C. During the final hour of this incubation, the cultures were treated with 0.5 μCi of ^{3}H-thymidine per ml (20 Ci/mmole) or 0.5 μCi of ^{3}H-deoxycytidine per ml (10 Ci/mmole). The cultures were then washed two times in tris(hydroxymethyl)aminomethane buffer (*p*H 7.4) and fixed in Carnoy's fixative. After staining with acetic orcein, the cultures were dipped in Kodak NTB-3 liquid nuclear track emulsion and exposed for 7 to 10 days. After fixation and development of the autoradiograph, the nuclei were examined for incorporation of ^{3}H-thymidine.

To study the combined cytogenetic effects of ara-C and HSV-2, two flasks containing logarithmically growing HEL cells were inoculated with HSV-2 at an input of 5 PFU per cell, and the virus was adsorbed at 37 C for 1 hr. A third flask remained uninoculated. After adsorption, medium containing 10 μg of ara-C per ml was added to the uninoculated and to one of the virus-inoculated flasks. The third flask received medium without ara-C. All cultures were returned to the incubator for 3 hr and then harvested for chromosome preparations. The total virus exposure was

4 hr. Three slides from each of the three different treatment schedules were coded and analyzed for cells containing one, two, and three or more chromosome or chromatid breaks and for cells containing accentuation of secondary constrictions on chromosomes no. 1, 9, or 16. The constrictions were considered accentuated if they extended beyond the width of a metaphase chromosome.

The results from these experiments performed with two different strains of HSV-2 (332 and 333) show that ara-C did not prevent breaks or accentuated secondary constrictions induced by HSV-2 but acted synergistically with the virus to produce a significant increase ($P < 0.01$) in the number of cells containing three or more breaks (Tables 1 and 2). The number of such cells was calculated to be approximately 2.5 times greater than the additive effects induced separately by the virus and antimetabolite. In many instances, the appearance of such cells was striking with chromosomes exhibiting a large number of breaks (Fig. 1). Similar results were also obtained in experiments employing a plaque-purified strain of 333 virus.

The results unexpectedly also show far fewer

TABLE 1. *Effect of cytosine arabinoside (ara-C) and herpes simplex virus (HSV) type 2 (332) on human chromosomes*

Treatment	Breaks per cell			Secondary constrictions (total)	Total no. of cells
	One	Two	Three or more		
Ara-C + HSV-2...	4	11	66	48	150
HSV-2 alone.....	6	4	21	50	150
Ara-C alone.....	19	2	3	2	138
Control.....	4	0	0	0	225

TABLE 2. *Effects of cytosine arabinoside (ara-C) and herpes simplex virus (HSV) type 2 (333) on human chromosomes*

Treatment	Breaks per cell			Secondary constrictions (total)	Total cells
	One	Two	Three or more		
Ara-C + HSV-2...	9	7	78	27	225
HSV-2 alone.....	15	4	18	33	225
Ara-C alone.....	37	13	13	3	225
Control.....	4	1	0	2	225

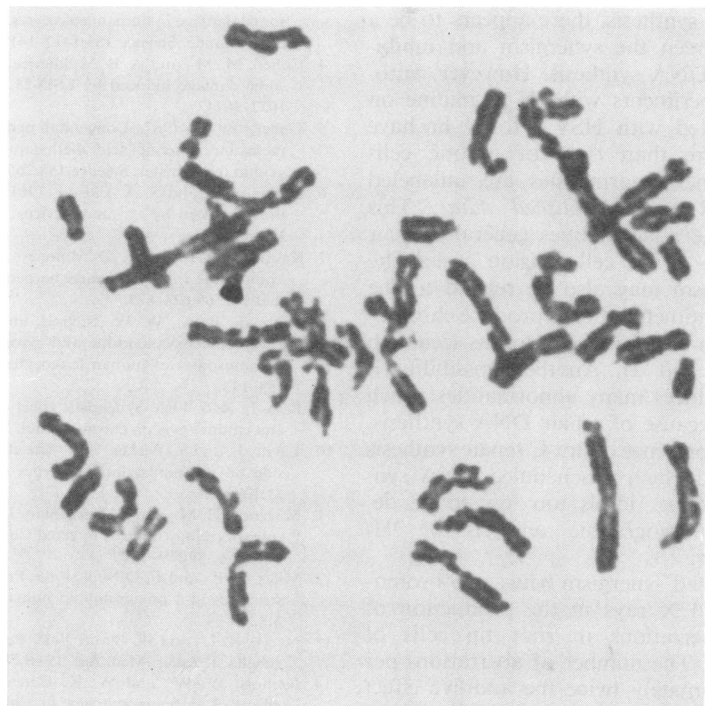

FIG. 1. *Metaphase of human embryonic lung cell treated with herpes simplex virus type 2 for 4 hr and cytosine arabinoside (10 μg/ml) for the final 3 hr of incubation. Note extensive chromatid breakage.*

cells with chromosomes exhibiting one break in cultures receiving both virus and ara-C than in cultures receiving only ara-C. This suggests that HSV acts on many of the cells which contain only one ara-C-induced break to produce cells containing multiple chromatid breaks. The results of the autoradiography experiments show that ara-C completely inhibited DNA synthesis in HSV-2-infected cells as measured by the failure to detect uptake of ^{3}H-thymidine. HSV-2-infected control cultures showed many nuclei with extensive labeling. Ara-C also inhibited virus multiplication to 99.9% of controls when added 1 hr after adsorption of HSV-2.

In experiments performed with 25 μg of IDU per ml in place of ara-C, no synergism was observed. It was also found that IDU alone did not produce chromosome breakage when inoculated into cultures for the last 3 hr of incubation. In this respect, it differed from ara-C which by itself produced breakage (Tables 1 and 2). However, IDU was not found to inhibit DNA synthesis in HEL cells infected with HSV-2 for 4 hr, since there was no detectable inhibition in ^{3}H-deoxycytidine uptake. Like ara-C, IDU inhibited virus multiplication to 99.9% of controls.

The mechanism by which ara-C and HSV-2 act to produce the synergistic effect is unknown. Since only ara-C produced a marked inhibition of cellular DNA synthesis, there appears to be a relationship between the synergism and inhibition of cellular DNA synthesis. However, autoradiographic experiments with ^{3}H-thymidine on HEL cells infected with HSV-2 for 4 hr have shown that more than 80% of mitotic cells with chromosome abnormalities are unlabeled (O'Neill and Rapp, *unpublished data*). This indicates that the abnormalities generally occur in the G2 phase of the cell division cycle. The observed synergism may also be related to the capacity of the antimetabolite to produce chromosome breakage in uninfected cultures as seen with ara-C (Tables 1 and 2). Another possibility is that HSV-2 produces many abnormalities which go undetected because of repair DNA synthesis. However, in the presence of ara-C repair synthesis may be inhibited. Such unscheduled DNA synthesis may occur at levels too low to be detected by autoradiographic analysis of ^{3}H-thymidine uptake.

Koo (9) reported synergism between 5-bromodeoxyuridine and X rays in the production of chromosome aberrations in root tip cells of *Zebrina pendula*. The number of aberrations per cell was approximately twice the additive effect of X-ray and 5-bromodeoxyuridine treatment. Nichols et al. (15) observed synergism between cytidine triphosphate (CTP) and Rous sarcoma virus in the production of chromosome breakage. Since they observed that both virus and CTP produced similar types of chromosome abnormalities, they concluded that the synergistic effect bears some relationship to these similarities. In our studies, we have observed no striking similarities in the separate effects of HSV and ara-C.

The possible teratogenic and carcinogenic effects of environmental mutagens such as lysergic acid diethylamide (3–5, 22), cyclamate (21, 24), and some viruses (13, 16, 18, 20) is a topic of widespread concern. The possibility that chemical and viral chromsome mutagens act synergistically to produce chromosome abnormalities obviously warrants further investigation.

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