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Role of Base Damage in Aberration Formation:

Interaction of Aphidicolin and X-Rays<sup>1</sup>

Michael A Bender and R. Julian Preston

Medical Department, Brookhaven National Laboratory, Upton, NY, U.S.A.

and

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.

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Medical Department

Brookhaven National Laboratory

Upton, NY 11973 U.S.A.

(Ikegami, et al., 1978). Unlike CA, which has been shown to interfere with DNA synthesis through actual polymerization of several CA residues into the nascent chain (Hiss and Preston, 1977), it seems likely that APC inhibits by binding to and inactivating the DNA- $\alpha$  polymerase complex (Huberman, 1981).

Because both CA and APC are  $\alpha$  polymerase inhibitors and because both interact synergistically with UV in the production of SCE, we have asked whether APC also shares other cytogenetic properties of CA, e.g., clastogenicity and synergism with ionizing radiation for induction of chromosomal aberrations in  $G_0$  and in  $G_2$  human peripheral lymphocytes. Though we have as yet completed only a few experiments, the results at hand are already sufficient to answer the questions: like CA, APC is clastogenic in both  $G_0$  and  $G_2$ , and it also interacts synergistically with x-rays to increase chromosomal aberration production in both  $G_0$  and  $G_2$ .

## 2. MATERIALS AND METHODS

2.1. Aphidicolin. The aphidicolin used was obtained from Imperial Chemical Industries, Ltd., through the kindness of Dr. A.H. Todd. The compound was dissolved in dimethyl sulfoxide (DMSO) at  $5 \times 10^{-3}M$  and stored at about  $-20^\circ C$ . It was added to lymphocyte cultures to a level of  $5 \times 10^{-5}M$ ; DMSO concentration in the APC treated cultures was thus 1%, and solvent controls were run with DMSO at this level. The drug or solvent was added immediately after irradiations were completed.

2.2. X-ray. Cultures were irradiated with x-rays from a General Electric machine operated at 250 KVp and 30 mA with 1.0 mm Al and 0.5 mm Cu added filtration, giving an exposure rate of approximately 100 R/min. Irradiations were at room temperature ( $\sim 23^\circ C$ ). Exposures were 200 R for  $G_0$  irradiations and 100 R for  $G_2$  irradiations.

## 1. INTRODUCTION

The base analog cytosine arabinoside (CA) is an inhibitor of DNA synthesis that is able to induce chromosomal aberrations not only in the DNA synthetic (S) phase of the cell cycle but in cells in the pre- ( $G_0$  or  $G_1$ ) and in the post-DNA-synthetic ( $G_2$ ) phases of the cell cycle as well (Kihlman, et al., 1963; Brewen, 1965; Brewen and Christie, 1967). Incubation of human peripheral lymphocytes in CA following either  $G_0$  or  $G_2$  x-irradiation causes a synergistic increase in chromosomal aberration frequency (Preston, 1980). CA is believed to preferentially inhibit DNA polymerase  $\alpha$  (Loeb, et al., 1980). In addition to inhibiting normal semi-conservative DNA synthesis, CA inhibits unscheduled DNA synthesis induced by ultraviolet light (UV), and Hiss and Preston (1977) have shown that CA inhibits the polymerization step in the repair of DNA damage by x-rays as well as by alkylating agents. Preston (1980) has suggested that it is inhibition of the repair of x-ray-induced base damage that is responsible for the synergistic effect on chromosomal aberration production he observed with x-ray and CA treatment of human peripheral lymphocytes.

Ishii and Bender (1980) observed that CA induces sister chromatid exchanges (SCE) in mammalian cells when present during normal DNA replication and that it also interacts synergistically with UV in the induction of SCE. A number of other inhibitors of DNA synthesis were also tested, and though several failed to show a similar synergism with UV, one, aphidicolin (APC), did produce effects similar to CA at the same concentration.

Aphidicolin is a tetracyclic diterpinoid isolated from Cephalosporium aphidicola and other fungi. It inhibits the growth of eukaryotic cells by inhibition of DNA synthesis, and this action has been shown to result from specific inhibition of DNA polymerase  $\alpha$ , but not of polymerases  $\beta$  or  $\gamma$

2.3. Lymphocyte Culture. Sterile blood samples were obtained from normal healthy adult volunteers by venipuncture into heparinized Vacutainer tubes. Leukocyte counts were made and then aliquots added to individual 10 ml cultures to give an inoculum of  $5 \times 10^6$  leukocytes per culture. The medium was RPMI 1640 (GIBCO) with 15% foetal calf serum, penicillin and streptomycin. Sterile 15 ml conical plastic screw capped centrifuge tubes (Corning) were used for the cultures. After addition of 0.25 ml of phytohemagglutinin (PHA; GIBCO) the cultures were incubated at  $37^\circ \text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in air. Either 2 or 3 hours prior to fixation, depending on the experiment, colcemid was added to the cultures to a level of 0.1  $\mu\text{g/ml}$ .

2.4. G<sub>0</sub> Experiments. Experiments followed essentially the plan of the CA experiments reported earlier by Preston (1980). The first experiment actually included a set of CA treatments (at 50  $\mu\text{M}$ ) as a positive control, and as in the earlier CA experiments, deoxycytidine (100  $\mu\text{M}$ ) was added to the incubation medium along with the PHA prior to incubation. Fixation for this experiment was at 52 hr. In a second experiment without a CA positive control, no deoxycytidine was added. The culture medium, however, contained 5-bromo-deoxyuridine at 25  $\mu\text{M}$ , in order to allow differential staining of the fixed metaphases and scoring of unequivocal first in vitro mitoses (Bender, 1979). Fixations for this experiment were done at both 52 and 56 hours.

In both experiments the APC (or CA) treated cultures were incubated at  $37^\circ \text{C}$  without PHA for 1, 2 or 3 hr. Controls, solvent controls and cultures with only x-ray treatment were similarly incubated for 3 hr. Following incubation the cells were washed twice with Hanks balanced salt solution and finally resuspended in fresh culture medium with PHA.

2.5. G<sub>2</sub> Experiments. One G<sub>2</sub> experiment has been done. Cultures were set up and incubated for 72 hr with PHA before x-ray and/or APC (or solvent) treatment. Colcemid was added immediately, and the cultures incubated for an additional 2 or 3 hr prior to fixation.

2.6. Fixation, Staining, and Scoring. Cultures were processed by standard techniques. After 15 min hypotonic treatment in 75 mM KCl, the cells were fixed in 3:1 absolute methanol: glacial acetic acid. After washing, drops of suspension of fixed cells were dropped on clean, wet glass slides and air dried. Conventional staining was with 10% Giemsa. Bromodeoxyuridine-substituted cells were differentially stained with 4% Giemsa following staining with Hoechst #33258 and black light exposure (Ishii and Bender, 1980).

Scoring for chromosomal aberrations (including achromatic lesions) and all chromatid and chromosome types was done according to generally accepted criteria. Isolocus breaks without sister union were classified as isochromatid deletions in the G<sub>2</sub> experiment and as chromosome deletions in the G<sub>0</sub> experiments. Scoring of differentially stained metaphases was confined to those with unequivocal first division (uniform) staining pattern. Cells with less than 44 centromeres were excluded from the scored samples.

### 3. RESULTS

3.1. G<sub>2</sub> Experiment. Cultures from two different blood donors were treated simultaneously in a single experiment. One hundred cells were scored for each donor for each point. As there were no significant differences in the results for the two donors, the data have been pooled for presentation in Table 1. It is immediately evident from inspection of the data that APC alone produces a significant increase in the frequencies of chromatid aberrations, but that the aberrations induced do not include any chromatid exchanges.

It is also evident that post-treatment with APC synergistically increases the yield of x-ray-induced aberrations. The values are about 2.7 times the sums for the individual treatments for achromatic lesions and chromatid deletions at two hours, and average 5.5 times at three hours. The increases for isochromatid deletions, though not as great, are still substantial. It should also be noted that the increases for these three aberration categories are probably underascertained, as many cells given the combined treatments had in excess of 10 gaps and breaks, making accurate scoring difficult.

In notable contrast to the large increases for the achromatic lesion and deletion classes, the frequency of chromatid exchanges actually was reduced when the x-ray-treated cells are post-treated with APC: the effect is highly statistically significant. Thus, the "synergism" for this aberration class is a negative one.

3.2. G<sub>0</sub> Experiments. Two separate G<sub>0</sub> experiments were carried out; the first fixed after 56 hr incubation and conventionally stained, and another in which 5-bromodeoxyuridine was added to the culture medium, samples fixed at both 52 and 56 hr, and the slides differentially stained so that unequivocal first post-treatment mitoses could be scored.

3.2.1. Experiment 1. Cultures of blood from two different donors were treated simultaneously. As there were no significant differences in the results for the two donors, the data are pooled in Table 2. It may be seen that neither CA nor APC alone (there were no mitoses in the samples from either donor for 2 or 3 hr APC treatments, so only the 1 hr material could be scored) induced any significant increase in chromosome-type aberrations. Indeed, it seems not unlikely that the few deletions seen may actually have been isochromatid deletions without sister union which, of course, could not

be distinguished from chromosome deletions. As had been seen by Preston (1980), the CA post-treatment resulted in significantly higher chromosome-type aberration yields than did x rays alone; there is a suggestion of saturation in yields for the longer CA treatment times.

The APC post-treatment also appears to have synergistically increased the chromosome-type aberration yields; the increases for the 2 hr treatment are statistically significant. The increases are, however, neither so dramatic nor so prompt as those seen for CA post-treatments. But the APC post-treatment also produced another phenomenon, one not previously noted for CA: in the 1 hr post-treated sample there was a significant increase in chromatid-type aberrations. Notably, increases are evident in all of the chromatid aberration classes; furthermore, the chromatid aberrations were seen in the material from both donors, and often occurred in cells that also contained chromosome-type aberrations. Though less dramatic, and thus not noted in the earlier study with x rays and CA, the data in Table 2 also suggest a similar phenomenon for this inhibitor as well. Unlike the case for  $G_2$  treatment with either inhibitor, both APC and CA treatments, alone or following x ray, appear to have induced chromatid exchanges as well as achromatic lesions and chromatid deletions. Also evident for both inhibitors is a marked tendency for all these aberration categories for the frequencies to decrease with increasing post-treatment duration.

3.2.2. Experiment 2. Because of concern that despite the early (52 hr) fixation time, the results of our first  $G_0$  experiment might have been influenced by inclusion of some second in vitro mitoses among the metaphases scored, a second experiment was done in which 5-bromodeoxyuridine (25 mM) was added to the culture medium so that differential staining would allow the scoring of only unequivocal first divisions. In order to check whether this might have been the case, the

frequencies of first, second, and third or later mitoses were determined on samples from cultures from a single donor treated as before with x rays and with APC for one hour, alone and in combination, were determined for 200 mitosis samples from material fixed at 52 (solvent control and x ray) and at 56 hr (APC-treated cultures). The results, shown in Table 3, suggest that the frequencies of post-first mitoses may indeed have been of the order of about 10% for the control culture in the first experiment, but it appears unlikely that there was significant contamination of any of the samples from treated cultures.

Because of the lack of adequate numbers of mitoses in the APC-treated 52 hr samples, scoring for aberrations was done on the 56 hr samples for comparison with the aberration yields in the 52 hr solvent control and x-irradiated samples. The results are shown in Table 4. Though the numbers of cells scored are less, it is nevertheless evident that this experiment yielded qualitatively the same result as the first. Thus, it appears that neither the early increase in chromatid-type aberrations nor the increase in chromosome-type aberration yields for the combined treatment could be an artifact of inclusion of post-first in vitro mitoses. It is also evident that neither the inclusion of deoxycytodine in the culture medium in the first experiment, nor the inclusion of 5-bromodeoxyuridine in the second could have influenced the results (except, of course, for the unlikely possibility that they both had precisely the same effect).

#### 4. DISCUSSION

The experiments we have done, though admittedly limited in scope, appear to us to answer unequivocally the questions we have posed; like CA, APC is clearly clastogenic in both the  $G_0$  and the  $G_2$  phases of the cell cycle, and, though the effect appears less marked in the case of  $G_0$  than that of the same

molar concentration of CA, APC post-treatment clearly interacts synergistically with both  $G_0$  and  $G_2$  x irradiation.

Brewen and Christie (1967<sup>7</sup>) found that CA induced chromosome-type aberrations when administered in the  $G_0$  phase of the cell cycle; in our experiments neither CA nor APC unequivocally increased the frequencies of chromosome-type aberrations. However, the earlier experiments with CA of Preston (1980) also failed to demonstrate an increase in chromosome-type aberrations, though they clearly did show an increase in the chromatid-type aberrations induced by most chemical clastogens administered in  $G_0$  or  $G_1$  (Bender et al., 1974). We are unable to explain this difference. In any case, the mechanism by which APC, or for that matter CA, causes aberrations in non-S phase cells is unclear. One possibility is that the inhibitors interfere with a maintenance repair function that normally corrects spontaneously arising DNA lesions. If so, then one might consider that the presence of the inhibitor simply increases the spontaneous aberration frequency, rather than actually inducing aberrations in the strict sense of the word.

An interpretation of the  $G_2$  x-ray-APC synergism comes readily to mind: as with CA, it seems clear that the presence of APC following irradiation interferes with some sort of repair process that normally removes induced lesions which, when their repair is incomplete, can be manifest at metaphase as achromatic lesions and chromatid deletions. In view of the arguments we have offered previously for the roles of both double and single polynucleotide strand breaks in aberration production (Bender et al., 1973a; 1973b; 1974; Bender, 1980), the argument of Preston (1980) regarding the effect of CA post-treatment following x rays appears cogent; we feel it most likely that the effect of APC is on the repair of some form of radiation-induced base damage. If this interference were with the polymerization of the missing

bases left following excision and incision of a series of bases including the damaged one, then single polynucleotide strand gaps would result. Those near enough each other in opposite polynucleotide chains would give double strand breaks directly. Furthermore, some might well be converted to double polynucleotide strand breaks by single strand nuclease attack as postulated earlier (Bender et al., 1973a). Since APC, at least, clearly affects DNA polymerase  $\alpha$ , though not polymerases  $\beta$  and  $\gamma$ , it seems reasonable to speculate that polymerase  $\alpha$  is the enzyme involved in this type of base damage repair.

Both the failures of CA and APC to induce chromatid exchanges and their ability to reduce the yields of these aberrations following  $G_2$  x irradiation also appear to implicate DNA polymerase  $\alpha$  in the formation of chromatid-type exchanges. If the model of exchange formation suggested earlier (Taylor, 1963; Bender et al., 1974) is correct, it seems reasonable to infer that failure of the polymerization step during resynthesis following annealing of sticky ends is the step affected, and, consequently, that polymerase  $\alpha$  may be the enzyme involved. This implies, of course, that annealing, by itself, is insufficient to result in an exchange manifest at metaphase.

The  $G_2$  results reported here for APC, and earlier for CA (Brewen, 1965; Brewen and Christie, 1967; Preston, 1980), are in at least some ways parallel to the findings that x irradiation of cells from persons affected with both Fanconi's anemia (FA) and ataxia telangiectasia (AT) show striking increases in the yields of chromatid aberrations following  $G_2$  irradiation (Rary et al., 1974; Bigelow et al., 1979; Bender et al., 1981). This seems to imply that the genetic defects in both diseases may involve base damage repair; such a defect has been reported for at least some individuals affected with AT (Paterson et al., 1976).

The results of the  $G_0$  experiments reported here also seem to implicate base damage repair as already suggested by Preston (1980) for CA. Though we cannot rule out the possible involvement of single polynucleotide strand breaks, we speculate that the presence of APC (or CA) following x irradiation of  $G_0$  cells results in the conversion of some form of DNA damage, most likely base damage, that would ordinarily not give rise to chromosome-type aberrations, into one that does. Because the increase in aberration yield is greater than that predicted by additivity alone, because it involves exchange-type aberrations as well as simple breaks (deletions), and in view of the arguments already presented, it seems reasonable to infer that the molecular mechanism involved may well be: 1) the conversion of base damage into single polynucleotide gaps through inhibition of DNA polymerase  $\alpha$ , 2) their conversion to double strand breaks by DNA single strand nuclease, and 3) the interaction of these "extra" double strand breaks in both deletion and exchange production. Though we favor such an interpretation at the moment, however, we are very much aware that the experiments here reported do not rule out the possibility that some other form of DNA lesion such as the single polynucleotide break may be involved; experiments on the biochemical and biophysical level will settle this question.

The curious early increase in chromatid-type aberrations following x ray and both APC and CA post-treatments does seem compatible with our interpretation, though. If the primary effect of either inhibitor is to interrupt the normally efficient repair of some form of base damage to yield single polynucleotide gaps, then this implies that if the inhibition of repair is released early enough, conversion to double strand breaks does not occur. Instead, we infer, some form of repair is completed so that no double strand breaks result, thus explaining the minimal synergism for chromosome-type aberration production.

Such repair cannot, however, be perfect, because of the increases observed in chromatid-type aberrations. Instead, some lesion must be left behind which, like those induced by UV or most alkylating agents, results in later S-dependent chromatid-type aberration production (Bender et al., 1973a; 1973b; 1974). In fact, the observation of Hiss and Preston (1977) that CA residues are actually incorporated into the repairing region before strand elongation is completely terminated is compatible with this idea. If CA residues are left in the repaired region following resumption of repair synthesis, then these could be responsible for S-dependent chromatid-aberration production later on. However, since insertion of APC residues into DNA seems extremely unlikely (Huberman, 1981), a mechanism in the case of APC must be different.

From the data presented in Tables 2 and 4, it is evident that  $G_0$  treatment with APC, either alone or following x ray, can, unlike the case for  $G_2$  treatment (Table 1), increase the frequency of chromatid exchanges. This is, however, not incompatible with the hypotheses presented here; in the  $G_2$  experiment reported, as in the similar one of Preston (1980) with CA, the inhibitor was present for the entire time before the cells were fixed. In the  $G_0$  experiment, on the other hand, the inhibitor was removed long before the beginning of the S phase when, presumably, the chromatid exchanges must have arisen. Indeed, data reported by Preston (1980) support this view: when CA was added to a  $G_2$ -irradiated culture an hour after the irradiation, instead of immediately, several chromatid exchanges were observed.

We also note that there is at least a partial parallel between the present  $G_0$  results and those for  $G_0$ -irradiated lymphocytes from persons affected with AT. Taylor et al. (1976,1978) reported that  $G_0$  irradiation results in increases in chromatid-type aberrations, an observation confirmed by later experiments of Bender, Rary, and Kale (Bender, 1980). Again, it is possible to infer that

a defect in the polymerization step of base damage repair is involved.

Because lesions produced by both APC and CA post-treatments following  $G_0$  x irradiation of human lymphocytes appear able to induce chromatid-type aberrations, and because most agents capable of inducing S-dependent production of chromatid aberrations are also very efficient inducers of SCE (Perry and Evans, 1975), we might anticipate that APC or CA post-treatment of x-irradiated lymphocytes might also result in a synergistic increase in SCE frequency. However, experiments with both CA and APC have failed to detect such an effect (Bender, Kale and Harris, in preparation). Thus, the lesions we hypothesize are responsible for chromatid aberration production following  $G_0$  irradiation and CA or APC post-treatment must be unlike those induced by UV or most chemical agents in this respect.

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Table 1. Chromatid aberration frequencies in peripheral lymphocytes treated with 100R of x-rays and  $5 \times 10^{-5}$ M aphidicolin. Two subjects were used, and 100 cells scored per point; the results for the two subjects, which did not differ significantly, are pooled, so each point represents 200 cells scored. See text for experimental details. Percentages plus or minus Poisson errors.

Fixation Time	Treatment	Achromatic Lesions	Chromatid Deletions	Isochromatid Deletions	Chromatid Exchanges
2 hr	Solvent Control	12 $\pm$ 2	0	1 $\pm$ 1	0
	X-ray	261 $\pm$ 11	232 $\pm$ 11	23 $\pm$ 3	20 $\pm$ 3
	APC	27 $\pm$ 4	20 $\pm$ 3	4 $\pm$ 1	0
	X-ray + APC	713 $\pm$ 19	743 $\pm$ 19	39 $\pm$ 4	1 $\pm$ 1
3 hr	Solvent Control	10 $\pm$ 2	1 $\pm$ 1	1 $\pm$ 1	0
	X-ray	121 $\pm$ 8	96 $\pm$ 7	8 $\pm$ 2	13 $\pm$ 2
	APC	44 $\pm$ 5	47 $\pm$ 5	10 $\pm$ 2	0
	X-ray + APC	685 $\pm$ 18	964 $\pm$ 22	45 $\pm$ 5	4 $\pm$ 1

**Table 2.** Pooled chromosomal aberration yields in human peripheral lymphocytes treated with 200R of X-rays,  $5 \times 10^{-5}$ M aphidicolin, and  $5 \times 10^{-5}$ M cytosine arabinoside, alone and in combination, prior to PHA stimulation and incubation for 52 hr. Two donors per point; 150 cells per donor per point except as noted. Percentages plus or minus Poisson errors. See text for experimental details.

Treatment	<u>Chromatid Type Aberrations</u>			<u>Chromosome Type Aberrations</u>	
	Achromatic Lesions	Deletions (incl. SU isos)	Exchanges	Deletions	Rings and Decentrics
Control	7 ± 2	1 ± 1	0	0	0
X-rays	11 ± 2	1 ± 1	0	40 ± 4	40 ± 4
APC (1 hr) <sup>1</sup>	54 ± 4	16 ± 2	1 ± 1	1 ± 1	0
X-rays + APC (1 hr)	144 ± 7	79 ± 5	5 ± 1	48 ± 4	44 ± 4
X-rays + APC (2 hr)	40 ± 4	18 ± 3	0.3 ± 1	62 ± 5	71 ± 5
CA (3 hr)	27 ± 3	7 ± 2	0.3 ± 1	1 ± 1	0
X-rays + CA (1 hr)	33 ± 3	33 ± 3	2 ± 1	61 ± 5	81 ± 5
X-rays + CA (2 hr)	40 ± 4	20 ± 3	1 ± 1	55 ± 4	102 ± 6
X-rays + CA (3 hr)	9 ± 2	10 ± 2	1 ± 1	71 ± 5	101 ± 6

<sup>1</sup>No mitoses were seen in material treated for either 2 or 3 hr.

<sup>2</sup>Only 131 cells could be scored from one donor, for a total of 281 for this point; no mitoses were seen in samples from either donor treated with X-ray and APC for 3 hr.

Table 3. Frequencies of first, second and later post-treatment mitoses in 200 metaphase samples from human lymphocytes given 200R exposures to X-rays, one hour treatment with  $5 \times 10^{-5}$ M APC, or both, allowed to incorporate 5-bromodeoxyuridine and differentially stained following fixation at either 52 or 56 hr after treatment.

Treatment	Fixation (hr)	Mitoses (%)		
		First	Second	Third and Later
Solvent Control	52	89	11	0
X-ray	52	(no mitoses)		
	56	98	2	0
APC	52	96	4	0
X-ray + APC	52	(very few mitoses; all firsts)		
	56	100	0	0

Table 4. Chromosomal aberration yields in cultured lymphocytes given 200R exposures to X-rays, one hour treatment with  $5 \times 10^{-5}$ M APC, or both (same experiment as Table 3: only unequivocal first in vitro mitoses scored). One hundred cells per point.

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Treatment	Fixation (hr)	Chromatid Aberrations			Chromosome Aberrations	
		Achromatic Lesions	Deletions (incl. SU isos)	Exchanges	Deletions	Rings and Dicentrics
Solvent Control	52	13	3	0	1	0
X-ray	52	13	0	0	21	46
APC	56	55	17	1	1	0
X-rays + APC	56	153	118	26	35	55