Modification of Radiation Damage of Bacteria by Folic Acid Antagonists

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Received for publication 10 July 1965

ABSTRACT

PITTILO, ROBERT F. (Southern Research Institute, Birmingham, Ala.), MARY LUCAS, ROBERT T. BLACKWELL, AND CAROLYN WOOLLEY. Modification of radiation damage of bacteria by folic acid antagonists. *J. Bacteriol.* 90:1548-1551. 1965.—The folic acid analogues, 2,4-diamino-6-methylpteridine, amethopterin, and aminopterin, have been found to sensitize certain bacteria, especially *Escherichia coli*, to the lethal action of ionizing irradiation. Data are presented which indicate that (i) the compounds must be present during the irradiation period for maximal sensitization to be observed, (ii) the sensitizing effect can be nullified by cysteine or cysteamine, (iii) the sensitizing effect occurs in a number of diverse bacterial genera, and (iv) folic acid neither sensitizes bacteria to irradiation nor prevents the sensitization caused by these antifolic agents.

2,4-Diamino-6-methylpteridine (DAMP), a compound with modest antifolate activity in *Streptococcus faecalis* (Seeger et al., 1949), was found to potentiate the lethal action of ionizing irradiation in nonproliferating bacteria, especially *Escherichia coli*. The degree of sensitization is less than that exhibited by 2-fluoro-adenosine (Pittillo and Lucas, 1965), sparsomycin (Pittillo et al., 1965), or numerous other compounds. However, radiation sensitization by an antifolate related structurally to the clinically effective antileukemic agents amethopterin (*N* - {[2,4 - diamino - 6 - pteridinyl] - methyl} - ethylamino}benzoyl} - glutamic acid) (Seeger, Smith, and Hultquist, 1947) and aminopterin (*N* - {[2,4 - diamino - 6 - pteridinyl] - methyl} amino}benzoyl} - glutamic acid) (Seeger et al., 1949) was of considerable interest to us.

MATERIALS AND METHODS

The procedures used to determine radiation effects and modification by chemicals have been described (Pittillo and Lucas, 1965; Pittillo et al., 1965). In essence, the procedure used for measuring effects on nongrowing cells consists of exposing washed cells (grown on simple glucose-salts medium and suspended in 0.067 M phosphate buffer) to irradiation from a cobalt-60 source (~ 1,000 c, dose rate of 2.0 kr/min) with and without test compound. In experiments with growing cells, the

test compound was added to the glucose-salts medium at the time of inoculation, and samples of cell suspensions were irradiated at various times during the growth cycle. In most experiments, all treatments were in duplicate.

RESULTS

A typical experiment which shows the potentiating action of DAMP on Co⁶⁰-irradiation damage of nonproliferating cell suspensions of *E. coli* ATCC 9637 is presented in Fig. 1; the similar activities of aminopterin and amethopterin are presented in Fig. 2 and 3, respectively. It may be noted that in all instances the effects appear to be dose-related, i.e., high concentrations of these antifolics result in greater sensitization to radiation than do low concentrations. Also, it appears that a moderately damaging dose of radiation is required before the antifolics may exert their potentiating effect.

The effect of these three compounds at non-toxic concentrations in combination with irradiation (Co⁶⁰) on a variety of nonproliferating wild-type bacteria and selected strains of *E. coli* resistant to various inhibitors has been determined in replicate experiments (Table 1). These compounds had no effect on the sensitivity of *Sarcina lutea*, *Streptococcus faecalis*, or *Pseudomonas aeruginosa* to radiation. Certain differences in the potentiating activity of these compounds on these bacteria are noteworthy. DAMP was significantly more effective in causing radiation sensitivity of cultures that were resistant to the

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FIG. 1. 2,4-Diamino-6-methylpteridine modification of the damaging action of ionizing irradiation (Co^{60}) on *Escherichia coli* ATCC 9637 (nonproliferating). Symbols: ●, 1.25 kr; ○, 2.5 kr; ▲, 5.0 kr; △, 10 kr; ■, 20 kr.

alkylating agents [2,2'-dichloro-*N*-methyl-diethylamine, hydrochloride (HN_2); 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)] (Schabel and Pittillo, 1961; Pittillo, Narkates, and Burns, 1964) than in sensitizing the parent cultures from which they were derived. Both aminopterin and amethopterin sensitized *E. coli* ATCC 9637 to radiation but were not effective in the nitrogen mustard-resistant strain. Amethopterin sensitized *E. coli* B (Hill) to radiation but was not effective in the derived BCNU-resistant strain; aminopterin, however, failed to potentiate radiation action in either of these two cultures. *E. coli* B_s (Hill) was reported (Hill, 1958) to lack radiation-damage repair mechanisms, and the ineffectiveness of amethopterin in increasing radiation kill in this strain *vis-à-vis* the parent culture suggests this metabolic area as one possible site of amethopterin action. However, *E.*

FIG. 2. Aminopterin modification of the damaging action of ionizing irradiation (Co^{60}) on *Escherichia coli* ATCC 9637 (nonproliferating). Symbols: ●, 1.25 kr; ○, 2.5 kr; ▲, 5 kr; △, 10 kr; ■, 20 kr.

coli B_s (Hill) is four times more sensitive to DAMP action than is the parent culture.

Potentiation of irradiation damage of nonproliferating *E. coli* ATCC 9637 by DAMP, aminopterin, or amethopterin may be prevented by the previously reported radiation protectors cysteine or cysteamine (see Table 2).

It has been observed that the compounds must be present during the irradiation period if potentiation is to be obtained. *E. coli* ATCC 9637 cells were exposed in buffer containing 100 μg/ml of aminopterin, amethopterin, or DAMP, washed by replicate centrifugation, and exposed to irradiation. No potentiation was noted.

Folic acid, at concentrations as high as 1 mg/ml, does not sensitize nonproliferating *E. coli* ATCC 9637 to radiation, nor does folic acid (1 mg/ml) protect against the radiation-sensitizing effect of amethopterin.

In studies with proliferating *E. coli* ATCC 9637, it was found that no potentiation was

TABLE 1. Effect of 2,4-diamino-6-methylpteridine (DAMP), aminopterin, and amethopterin on the damaging effects of ionizing irradiation (Co^{60}) on a spectrum of bacteria and derived resistant strains of *Escherichia coli*

Bacterium*	DAMP (100 μ g/ml)			Aminopterin (100 μ g/ml)			Amethopterin (1,000 μ g/ml)		
	Per cent survivors		Ratio†	Per cent survivors		Ratio†	Per cent survivors		Ratio†
	Radiation (20 kr)	Radiation + DAMP		Radiation (20 kr)	Radiation + aminopterin		Radiation (20 kr)	Radiation + amethopterin	
<i>Escherichia coli</i> ATCC 9637.....	0.14	0.03	4.62	0.35	0.01	26.90	0.71	0.06	12.5
<i>E. coli</i> ATCC 9637/FAS†.....	0.83	0.19	4.35	0.34	0.08	4.20	0.66	0.44	15.0
<i>E. coli</i> ATCC 9637/AZA.....	18.90	3.12	6.06	26.60	0.52	51.10	23.05	10.80	2.1
<i>E. coli</i> ATCC 9637/DON.....	0.91	0.02	45.50	0.19	0.008	24.30	0.80	0.15	54.0
<i>E. coli</i> ATCC 9637/HN ₂	2.67	0.33	8.02	6.85	4.16	1.65	8.46	5.45	1.55
<i>E. coli</i> B (ORNL).....	0.36	0.04	9.0	0.07	0.05	1.33	0.12	0.03	3.51
<i>E. coli</i> B/r.....	15.50	4.65	3.33	33.80	14.60	2.30	14.2	5.79	2.46
<i>E. coli</i> B (Hill).....	0.12	0.07	1.71	0.20	0.22	0.90	0.19	0.01	10.50
<i>E. coli</i> B (Hill)/BCNU.....	5.60	0.79	7.09	9.38	5.38	1.74	1.18	2.0	0.59
<i>E. coli</i> B _s (Hill).....	0.06	0.007	8.60	0.05	0.03	1.78	0.04	0.03	1.41
<i>E. coli</i> B ATCC 11303.....	4.45	0.70	6.35	3.34	1.06	3.15	5.62	2.0	2.81
<i>E. coli</i> B ATCC 11303/AMP.....	1.62	0.43	3.78	1.62	0.48	3.38	3.28	0.47	7.0
<i>Pseudomonas aeruginosa</i> ATCC 10145.....	0.11	0.09	1.23	0.30	0.48	0.64	0.13	0.66	0.20
<i>Serratia marcescens</i> ATCC 274.....	0.52	0.17	3.10	0.86	1.00	0.86	0.83	0.83	1.0
<i>Streptococcus faecalis</i> ATCC 8043.....	8.13	9.36	0.87	24.20	22.60	1.07	55.0	95.0	0.58
<i>Sarcina lutea</i> ATCC 9341.....	85.60	57.20	1.50	55.20	55.20	1.00	52.1	57.3	0.91
<i>Salmonella typhimurium</i> SR-11.....	2.36	0.65	3.63	2.67	1.93	1.39	1.67	1.60	1.05

* All strains of *E. coli*, *P. aeruginosa*, *S. marcescens*, *S. lutea*, and *S. typhimurium* were grown in a simple glucose-salts medium (Seeger et al., 1949); *S. faecalis* was grown in Flynn's medium with folic acid and no purines and pyrimidines (Flynn et al., 1951). All cultures were plated in Trypticase Soy Agar (BBL) after treatment.

† Ratio of per cent survivors with radiation alone to per cent survivors with radiation plus anti-folate.

‡ Resistance to a growth inhibitor indicated thus: name of parent culture/name of inhibitor to which strain is resistant: FAS = 2-fluoroadenosine; AZA = azaserine; DON = 6-diazo-5-oxo-L-norleucine; HN₂ = 2,2'-dichloro-N-methyl-diethylamine, hydrochloride (nitrogen mustard); r = radiation; BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; AMP = amethopterin.

TABLE 2. Effect of cysteine and cysteamine on the potentiation by certain folic acid analogues of irradiation damage in *Escherichia coli* ATCC 9637

Potentiator	Amt*	Per cent survivors to indicated treatment*		
		20 kr	20 kr + cysteine, 1.2 mg/ml	20 kr + cysteamine, 1.2 mg/ml
None.....	mg/ml	0.8	47.2	24.0
DAMP.....	0.1	0.05	13.6	2.3
Aminopterin...	0.1	0.03	47.2	22.0
Amethopterin..	1.0	0.16	47.2	24.0

* All compounds were nontoxic to unirradiated cells at the concentrations used.

obtained with any of the three compounds at concentrations which failed to inhibit cell division. Cultures grown in the presence of any one of the three compounds (100 μ g/ml) for 3 hr or longer contained approximately 20% of the number of viable, colony-forming cells in uninhibited cultures. Such compound-inhibited cultures when irradiated (30 kr) yielded approximately the same number of survivors as did control cultures.

DISCUSSION

Previous reports have suggested that amethopterin has radio-sensitizing activity in humans afflicted with advanced neoplasms (Burgheim, 1961; Bagshaw, 1963; Maisin, 1963), although

FIG. 3. Amethopterin modification of the damaging action of ionizing irradiation (^{60}Co) on *Escherichia coli* ATCC 9637 (nonproliferating). Symbols: ●, 1.25 kr; ○, 2.5 kr; ▲, 5 kr; △, 10 kr; ■, 20 kr.

definitive studies seem to be lacking. The demonstration that certain antifolate agents have radiosensitizing activity in some bacteria not only confirms earlier observations in other systems but also affords opportunity for further studies to elucidate the mechanism(s) involved.

The data presented here suggest that the sensitizing activity is not necessarily related to the antifolate activity of these compounds because (i) the strain of *E. coli* resistant to amethopterin is still sensitive to the amethopterin-sen-

sitizing action and (ii) folic acid fails to protect against the sensitizing action of amethopterin.

The differences between amethopterin, aminopterin, and DAMP in observed sensitization of the various bacteria tested cannot be explained.

ACKNOWLEDGMENTS

This investigation was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under National Institutes of Health contract PH43-62-485.

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