EFFECT OF THE DEOXYRIBOSIDE OF 6-AZATHYMINE (AZATHYMIDINE) ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID BY BONE MARROW AND NEOPLASTIC CELLS (*IN VITRO*)*

by

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Nucleic acids are involved not only in the biosynthetic activities of living cells, but also, in the case of deoxyribonucleic acid (DNA), in the transmission of inheritable characteristics and probably in the most fundamental aspects of the processes of reproduction. Since all known forms of DNA contain the base thymine, an effective and possibly specific antagonist of the utilization of this base theoretically could be uniquely capable of interfering with the biosynthesis of DNA and with cellular reproduction.

6-Azathymine (6-methyl-*as*-triazine-3,5-(2*H*, 4*H*)-dione, the 6-nitrogen analog of thymine), under certain circumstances, is a competitive antagonist of the growth of *Streptococcus faecalis* (8043) and several other strains of microorganisms in media supplemented appropriately with pteroylglutamic acid, vitamin B_{12}, thymine or thymidine[1]. Studies of the mechanism of action of azathymine revealed that *S. faecalis* can convert the analog to the corresponding deoxyriboside, azathymidine[2]. The formation of the latter does not represent a method of depriving the cell of a supply of deoxyribose necessary for the biosynthesis of DNA; rather, it signifies a metabolic conversion to a more active compound. That azathymidine is a more potent inhibitor than azathymine of bacterial growth has been demonstrated not only with *S. faecalis*, but also with *Lactobacillus leichmannii* (7830) and *Thermobacterium acidophilus* R-26[3]. Some of the possible mechanisms of inhibition by azathymidine have been discussed elsewhere[2,3].

The present report describes an extension of these studies to mammalian systems.

EXPERIMENTAL

Preparation of cell suspensions

Rabbit bone marrow was prepared by the procedure described by TOTTER[4]. The strain of Ehrlich ascites tumor cells employed was obtained from Dr. A. CONGER, Biology Division, Oak Ridge

* This investigation was supported, in part, by a grant (CH-7C) from the American Cancer Society, as recommended by the Committee on Growth, National Research Council, in part, by a grant (A-469) from the National Institutes of Health, Public Health Service, and, in part, by a grant from Lederle Laboratories Division, American Cyanamid Company. We are grateful also for the support of one of us (L. G. LAJTHA) by the American Cancer Society, as a British-American Exchange Fellow at Yale University.

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National Laboratories and carried in DBA/2 mice purchased from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine. Five or six days following the intraperitoneal inoculation of mice with tumor cells, ascitic fluid was collected from a suitable number of mice and pooled in a graduated centrifuge tube (40 ml) which contained 2–3 ml of Chambers' solution. After centrifugation, the residual packed cells were suspended in 4 volumes of TOTTER's modification[4] of Chambers' solution. Care was taken to minimize injury and to avoid clumping of the cells.

Preparation of solutions

Sodium formate-^{14}C, with a specific activity of 1.5 microcuries per micromole, was obtained from the California Foundation for Biochemical Research, as was uracil deoxyriboside (UDoR) and cytosine deoxyriboside (CDoR). Thymidine (TDoR) was purchased from the Schwarz Laboratories and azathymine deoxyriboside was prepared in this laboratory by a modification of the procedure described previously[2]. The deoxyriboside, prepared from azathymine by enzymic transdeoxyribosidation, was separated from the latter by a counter-current technique employing phosphate buffer (0.05 *M*, pH 5) and ethyl acetate, followed by column chromatography as employed previously.

Isolation of DNA bases

After incubation of the cellular suspensions at 37° for 2½–3 hours, the contents of identical beakers (see tables) were combined and the reaction was stopped by the addition of 2 volumes of ethanol (95%) or by cooling and the addition of a solution of trichloroacetic acid (TCA) (6 *M*) to a final concentration of 7%. The acid-soluble and phospholipid fractions were removed[5] and the residue was dissolved in 10 volumes of a solution of KOH (0.5 *N*) and kept at 37° overnight. An equal volume of ethanol (95%) was added following acidification with glacial acetic acid and the mixture was stored in an ice-bath for 6 hours. The hydrolysis of RNA and separation of DNA were repeated. The residue obtained was heated with TCA (5%) at 90° for 30 minutes[6], and then was separated by centrifugation. The TCA was removed from the supernatant solution by extraction with ethyl ether and the residual solution was evaporated to dryness on a steam bath. After hydrolysis with perchloric acid the bases were separated by paper chromatography, with an *iso*propanol-2 *N*-HCl system[7]. The individual bases were eluted with water and again subjected to paper chromatography, in a butanol-ammonia system[8]. The concentrations of the individual bases were determined in the Beckman UV-spectrophotometer; their radioactivity was measured, following the plating of appropriate aliquots in the center of a stainless steel planchet, by counting in a windowless flow counter.

RESULTS

Rabbit bone marrow studies

In Table I are shown the effects of azathymidine and other nucleosides on the incorporation of the carbon of radioactive formate into DNA-thymine. Azathymidine, but not azathymine, markedly depressed (61–84%) the uptake of formate. An equimolar concentration of unlabeled thymidine produced an even more marked depression

TABLE I

THE EFFECT OF THE DEOXYRIBOSIDES OF AZATHYMINE, THYMINE AND URACIL ON THE INCORPORATION *in vitro* OF FORMATE-^{14}C INTO THE DNA-THYMINE OF RABBIT BONE MARROW*

Nucleoside added	*Specific radioactivity of thymine (c.p.m. per μM)*		
	Expt. 1	*Expt. 2*	*Expt. 3*
None (control)	7850	1670	4430
Azathymine deoxyriboside	1240	657	988
Thymine deoxyriboside	25	56	187**
Uracil deoxyriboside		3640	6090

* The reaction mixtures consisted of 0.25 ml of packed cells, 5 μc of formate-^{14}C, 10 μ*M* of nucleoside; a modified Chambers' solution was added to give a final volume of 2.1–2.5 ml. The incubations were conducted in 20 ml beakers, in quadruplicate, in a Dubnoff metabolic shaker at 37° under air, and agitated at 90 cycles per minute for 2.5–3.0 hours.

** Thymidine concentration reduced to 1.0, rather than 10 μ*M*.

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(97–99%), a finding in agreement with radioautographic studies of human bone marrow[9]. The inclusion of uracil deoxyriboside, in lieu of thymidine or its analog, produced a very significant increase in the extent of the incorporation of formate-carbon into DNA thymine (40 to 120% greater than that of the control).

Mouse Ehrlich ascites tumor studies

As in the experiment with rabbit bone marrow, thymidine and azathymidine produced a marked depression of the uptake of formate-carbon into DNA-thymine (91 and 78%, respectively; Table II). Aminopterin, an antagonist of the formation of functional derivatives of pteroylglutamic acid, even in the relatively massive concentration of 0.4 μM per ml, exerted no greater inhibitory effect than azathymidine, in fact, its effect was slightly less.

TABLE II

THE EFFECT OF AMINOPTERIN AND THE DEOXYRIBOSIDES OF 6-AZATHYMINE, THYMINE AND URACIL IN THE INCORPORATION *in vitro* OF FORMATE-^{14}C INTO DNA-THYMINE OF EHRLICH ASCITES TUMOR CELLS

Nucleoside added	*Specific activity of DNA-thymine c.p.m. per μM*
None (control)	431
Azathymine deoxyriboside	95
Thymine deoxyriboside	38
Uracil deoxyriboside	1050
Cytosine deoxyriboside	1700
Aminopterin*	132

The composition of the reaction mixture is described in Table I. In addition, there was included 0.1 ml of human serum (final concentration, 4–5%).

* 1.0 μM.

DISCUSSION

Exogenous thymine is an exceedingly poor precursor of DNA in mammalian systems[10,11,12], at least when administered in tracer doses. In view of the rapid rate of degradation of thymine, both *in vivo* and *in vitro*[12], this is not surprising. On the other hand, thymidine appears to be utilized readily[13,14]. It is evident from these observations that the deoxyriboside of azathymine could have an opportunity to exert an inhibitory effect on the biosynthesis of DNA, either by competing with endogenous thymidine or as a result of competition between comparable metabolites of the two deoxyribosides. Exertion of such a competitive action would not be anticipated with azathymine, the analog of thymine. These expectations were confirmed experimentally, since azathymidine markedly inhibited the biosynthesis of DNA in two mammalian systems, the one composed of normal cells (rabbit bone marrow) and the other of neoplastic cells (Ehrlich ascites tumor). Because of the very limited amount of azathymidine available these studies, of necessity, were performed *in vitro*. Preparation of relatively large amounts of azathymidine for investigations of a possibly greater activity on various neoplasms than on normal tissues *in vivo* is suggested by the findings.

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Formate, serine and methionine each can serve as the donor of the carbon of the methyl group of DNA-thymine[16,17,18], as well as of the 2- and 8-carbons of nucleic acid-purines, *in vivo*[19]. The utilization of formate for the biosynthesis of DNA *in vitro* has been studied previously[4,20,21]; in most instances the uptake of formate-carbon under these conditions has been primarily into the methyl group of thymine[4] with relatively little isotope appearing in the purines. Our studies with the mouse Ehrlich ascites tumor cells also indicate that, *in vitro*, there is relatively little incorporation of formate-carbon into the DNA-purines; in fact, over 90% of the total radioactivity of DNA was found in thymine. The comparative ineffectiveness of formate for the biosynthesis of purines presumably reflects a relatively inefficient mechanism for the synthesis *de novo* of purines in rabbit bone marrow and Ehrlich ascites tumor cells, and a dependence on preformed purines; that these can be utilized readily has been shown previously[22,23,24].

The marked inhibition of the incorporation of formate-^{14}C exerted by thymidine in both the rabbit bone marrow and the Ehrlich ascites tumor cells is attributable to its dilution of the radioactive thymidine (or a metabolic equivalent) formed from the formate. That a great excess of thymidine had been added in relation to the normal size of the "thymidine pool" is indicated by the inability of the cells to increase greatly the formate-^{14}C uptake into DNA-thymine when the concentration of the exogenous thymidine was reduced by a factor of 10 (Table I). It must emphasized, however, that it is difficult to estimate "pool" sizes in these experiments, using tissues in which active nucleosidase cleavage of added thymidine almost certainly was occurring. The remarkable resistance of azathymidine to all catabolic influences, including those catalyzed by nucleosidases, has been commented on previously[25].

The marked increase in formate-^{14}C uptake which resulted from the addition of deoxyuridine is in agreement with the report of FRIEDKIN AND ROBERTS that the deoxyriboside of uracil-2-^{14}C is utilized for the biosynthesis of DNA-thymine in the chick embryo or in rabbit and chicken bone marrow[15]. The addition of serum (human or goat) to the Ehrlich ascites cells resulted in a marked increase in the uptake of formate-carbon into DNA-thymine, whether in the presence or absence of an added nucleoside acceptor. This may be a result of serum maintaining the integrity of the cell for a longer period of time during the incubation; however, other possibilities must be considered. Preliminary evidence has been obtained that Ehrlich ascites tumor cells *in vitro*, in the presence of serum, utilize cytosine deoxyriboside more efficiently than deoxyuridine as a precursor of DNA-thymine. Whether cytosine deoxyriboside penetrates to the site of formate utilization at a greater rate than uracil deoxyriboside, and then is deaminated prior to the acceptance of a precursor of the methyl group, or whether cytosine deoxyriboside is the primary acceptor is under investigation. Partial deamination of deoxycytidine to form deoxyuridine can be demonstrated.

In Fig. 1 certain possible sites of interference by Aminopterin and azathymidine are considered. Whereas Aminopterin interferes with either the formation or condensation of the single carbon component "C", azathymidine probably interferes with the utilization of thymidine or a metabolic derivative thereof. Whether azathymidine exerts its effect as an antagonist of the utilization of thymidine formed *de novo* at an enzyme level or whether it replaces thymidine in the DNA of these mammalian tissues is under investigation. In a bacterial system (*Streptococcus faecalis*) azathymine-^{14}C

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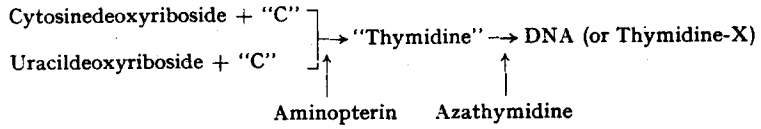

Fig. 1. Mechanism of inhibition of formate utilization for DNA biosynthesis.

was incorporated into DNA to an extent equivalent to 12–18% of the complement of thymine[25,26]. However, other data strongly suggest that profound inhibition of bacterial growth, and subsequent death of the bacterial cells[3], can be accomplished with only relatively insignificant incorporation into DNA[26]. The possibility that a derivative of thymidine is involved critically in other ways than as a precursor of DNA (*e.g.*, a hypothetical coenzyme), or that reproductive failure and death of cells can result from interference with the formation or function of a very specific, but quantitatively insignificant type of DNA, must be considered.

Of particular pertinence to the attainment of possibly greater inhibition of DNA-synthesis, via sequential blockade, will be studies of the combined use of the deoxyribosides of azauracil[27,28,29], azacytosine, and comparable analogs of one-carbon substituted pyrimidines, such as 5-methylcytosine, 5-hydroxymethylcytosine[30], and compounds observed in this laboratory by PRUSOFF AND LAJTHA[31].

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Doctor T. H. JUKES and his associates of the Lederle Laboratories Division, American Cyanamid Company, and to Doctor MAIRE HAKALA, for their help in the preparation and isolation of azathymidine, and to Miss ANGELA SCIMONE for her proficient technical assistance.

SUMMARY

Incorporation *in vitro* of radioactive formate into the bases of the DNA of rabbit bone marrow and Ehrlich ascites tumor cells was related predominantly to the formation of thymine. Thymine deoxyriboside by dilution, and 6-azathymine deoxyriboside and Aminopterin by inhibition, caused a marked decrease in the utilization of formate for the biosynthesis of DNA-thymine. Deoxyuridine and deoxycytidine each may serve as an acceptor of formate-carbon for the biosynthesis (of the methyl group) of DNA-thymine. Certain implications of these findings are discussed.

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Received November 29th, 1955