Nucleic Acid Changes in Sarcoma 180 Following Administration of Aminopterin.* (19690)

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It has been noted that aminopterin (4-amino-pteroylglutamic acid) retarded the growth of transplantable sarcoma 180 in mice and altered the histology of the tumor(1). The histologic changes observed in treated tumors included a marked decrease in the number of mitoses, the appearance of many cells with very large altered nuclei, and either diminution or vacuolization of the cytoplasm. As a result of these histologic observations, chemical and histochemical studies were initiated to determine whether demonstrable changes in the amounts of pentose- and desoxypentose nucleic acids (PNA and DNA) had occurred after administration of the folic acid antagonist.

It was recognized that chemical analysis of homogenized tumor tissue could only furnish evidence of gross changes induced by chemotherapy. Concomitant histochemical studies were carried out to measure alterations occurring in individual nuclei(2). Tissue homogenates of tumors usually contain a mixture of several types of cells. The sarcoma 180 was harvested in the pre-necrotic stage (within 10 days after implantation) and the cellular composition was predominantly tumor cells. It is believed that the changes observed in the PNA and DNA contents are referable to the tumor cells.

The general procedures employed, such as the care of the mice, implantation of tumor and administration of aminopterin, were similar to those previously described(1). At the termination of each experiment the mice were sacrificed by cervical dislocation, the tumors were dissected free from adhering tissue and blotted with filter paper to remove tissue fluids. The tumors were placed in tightly stoppered flasks immersed in a CO_2 ice-acetone bath, and stored in a CO_2 ice cabinet until analyzed.

The tissues were analyzed for both types of nucleic acid according to the method of Schneider(3). Nitrogen was determined by the micro-Kjeldahl method. A model DU Beckman spectrophotometer, employing one cm cuvettes, was used for all the colorimetric measurements.

Results. The results of 4 separate chemotherapeutic experiments are presented in Table I. There are 15 individual analyses of control and 15 of aminopterin-treated tumors. The number of replicate determinations within each experiment was determined by the number and size of the tumors available. It has often been noted that the growth of sarcoma 180 is subject to variation which could be attributed to such factors as host response and the size of the initial tumor inoculum. Because of this biological variability the treated tumors of each experiment were compared with concomitant controls. Another variable factor considered was the range of nucleic acid values for individual samples found by Schneider(3). Therefore, each analysis was performed on a pool of tumor tissue consisting of 10 to 40 tumor samples obtained from control or treated animals. Thus, within each experiment the control and treated material represented a fair sampling of the population of that particular trial.

One may employ the nitrogen content per gram of tissue as a rough index of the cellularity and degree of hydration of the tumors.

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TABLE I. Summary of Nucleic Acid Determinations on Sarcoma 180.

Exp. (1)	Age of tumors days (2)	Saline control: Sample No. (3)	N, g* (4)	PNA, mg (5)	DNA, mg* (6)	PNA/DNA (7)	Aminopterin treated: Sample No. (8)	N, g* (9)	PNA, mg* (10)	DNA, mg (11)	PNA/DNA (12)	Mean % change from control value: DNA (13)	PNA (14)
	6	1	1.81	522	473	1.10	2	2.40	482	368	1.31	−24.6	−10.2
		1A	"	533	499	1.11							
	10	3	2.20	608	548	1.11	4	2.12	597	497	1.20		
EA 181		3A	"	684	563	1.22	4A	"	626	536	1.17	− 6.5	− 7.4
		3B	"	688	544	1.27							
	10	5	2.22	681	592	1.15	6	2.06	709	503	1.41	− 7.4	+ 3.6
		5A	"	701	512	1.37	6A	"	724	518	1.40		
EA	9	7	1.90	655	546	1.20	8	1.81	648	473	1.37	−14.3	− 4.1
		7A	"	655	560	1.17	8A	"	608	465	1.28		
	10	9	2.52	962	655	1.47	10 †	2.38	919	553	1.66	−14.3	− 6.7
WC		9A	"	990	646	1.53	10A †	"	903	563	1.60		
	10	"	"	"	"	"	11 ‡	2.43	1009	624	1.76	− 6.9	+11.3
							11A ‡	"	1072	588	1.82		
	10	12 §	2.30	762	609	1.25	13 §	2.18	854	617	1.38	0	+14
BG				730	603	1.21			846	592	1.43		
	10	14 ‖	2.46	722	624	1.24	15 ‖	2.46	845	601	1.41	− 4.3	+11.1
				753	616	1.22			851	584	1.46		
Mean ± S.D.						1.24 ± .13					1.44 ± .21		

* Per 100 g tissue (wet wt). † Selected large tumors (see text). ‡ Selected medium tumors (see text). § Stock tumors implanted (see text). ‖ 6th generation of aminopterin-treated tumors implanted.

The ungrouped nitrogen values (col. 4 and 9) for all the observations are distributed about a mean of 2.20 g N per 100 g tissue (wet weight) with a standard deviation of 0.23. If the values are separated into control and treated groups, the means are 2.18 and 2.23 respectively. These values are not significantly different from each other and are within the range observed with liver or kidney tissue. From the individual analyses presented in Table I it may be noted that there is no consistent change in the nitrogen content of the treated tumors as compared to the controls. The total nucleic acid content (PNA and DNA) per 100 g of tumor tissue is approximately 1.2 g, equivalent to 0.2 g nitrogen. The detection of significant deviation in the nucleic acid content by means of nitrogen determinations is impossible because the standard deviation (0.23) for the total nitrogen content of tumor tissue noted with the ungrouped data is equal in magnitude to the total nucleic acid nitrogen.

The nucleic acid determinations and the alterations observed after antagonist administration are summarized in Table I. The observations may best be discussed under each experiment.

EA 181. Two hundred female mice were implanted bilaterally with tumor. One day after implantation and on 4 consecutive days, 120 mice received subcutaneous injections of aminopterin in a dosage of 0.3 mg per kg daily. The remaining 80 mice served as controls and received only injections of normal saline solution. On the sixth day after implantation, approximately one-half of each group was sacrificed. The remaining mice in the experimental group received 0.2 mg per kg of drug on the sixth and eighth days, while the controls received saline. All these mice were sacrificed on the tenth day after implantation. The material obtained from treated mice bearing 6-day-old tumors was sufficient for only a single nucleic acid determination (Table I, Col. 8, Sample 2). It

can be seen that the content of both PNA and DNA (Col. 10 and 11) in the tumors decreased compared to the 6-day control tumors (Col. 5 and 6, Sample 1). The decrease in DNA was greater than that of PNA (Col. 13 and 14). The 10-day tumors were analyzed on 2 separate occasions. The first set of analyses (Samples 3 and 4) showed a diminution of both PNA and DNA among the treated cells (Col. 13 and 14). A comparable decrease of both PNA and DNA in the tumors after aminopterin administration was only observed in this analysis and was not encountered again in any other experiments. The second analysis (Samples 5 and 6) showed a decrease in the DNA content of the treated tumors equal to that observed in the previous analysis but the PNA content was somewhat increased (Col. 13 and 14).

EA 191. The animals in this experiment received 5 daily injections of 0.3 mg of aminopterin per kg and were sacrificed on the ninth day after tumor implantation. The PNA and DNA in the tumors were both reduced after treatment, but the reduction was disproportionately great for DNA (Col. 13 and 14).

WC III. It had been repeatedly observed that treated tumors varied appreciably in size and therefore tumors were grouped for analysis at autopsy into 3 categories: large, medium and small. Control tumors in all experiments fell into the "large" class and these were not separated at this time. The tumors classified in the "small" group did not provide enough material for chemical analysis.

The results (Table I, Samples 9, 10, 11) show that in both the large and medium groups the DNA content decreased from that in the controls (Col. 13 and 14). The PNA content was either somewhat diminished or increased when compared to the controls (Col. 14). The ratio of PNA to DNA content of the grouped tumors varied in the following order: "medium" > "large" > control (Col. 12 and 7). These results are consistent with the observation that those tumors which were most affected by aminopterin would be smallest in size. The smaller tumors from treated mice appear to show the largest decrease in DNA relative to PNA.

BG. It is well known that bacteria can develop or acquire resistance to chemotherapeutic agents(4). Burchenal *et al.* have also shown that a mouse leukemia could develop resistance to the folic acid antagonist, amethopterin(5). It seemed worthwhile to see whether sarcoma 180 would show changes in its properties upon serial passage through mice treated with aminopterin. The tumor was transplanted every 10 days for 6 transfers in "A" strain mice. They received 0.5 mg of aminopterin per kg body weight daily for a 6-day period. Tumors which had been exposed through 6 passages in treated mice were then implanted into CF1 mice. One group received aminopterin treatment (Sample 15), the other received saline injections (Sample 14). Tumors which had not been exposed to aminopterin therapy were also implanted into CF1 mice and these were similarly divided into a treated (Sample 13) and control (Sample 12) group.

From the results presented in the table, it can be seen that previous exposure to aminopterin had no effect on the tumor response to further treatment. In both groups the PNA:DNA ratio of the treated tumors increased in the same manner over the controls (Col. 12 and 7). These experiments and additional transfers in the presence of aminopterin did not indicate that tolerance to the antagonist developed when tumor size and weight were measured.

Discussion. An examination of the PNA:DNA ratios of all the experiments indicates that a consistent difference exists between treated and control tumors. The means and standard deviations of these ratios are presented in Table I. The coefficient of variation for the control groups is 10% while for the aminopterin-treated groups it is 15%. These coefficients are not very large considering the factors of variability previously mentioned. The difference of the means, 0.20, is more than 3 times 0.065, the standard error of the difference(6).

The deviations from the control values may be examined to note the factors responsible for the change in the ratio of PNA to DNA (Col. 13 and 14). Following aminopterin therapy the reduction observed in DNA varied

from 0 to 25%. The PNA content of treated tumors was lower than that of the controls in 4 instances (−4 to −10%) and higher in 4 (+3.6 to 14%). With the possible exception of analysis No. 4, DNA was reduced disproportionately more than PNA in each of the experiments. Since the DNA analyses in Samples 4 and 6 agree (Exp. EA 181, Col. 11) there is a possibility that the PNA analysis in Samples 3 or 4 (Exp. EA 181, Col. 5 and 10) was in error. From these data it appears that aminopterin administration interferes with the synthesis of desoxypentose nucleic acid in the tumor cells. The synthesis of pentose nucleic acid was not affected to the same degree as DNA and in several experiments no inhibition of PNA synthesis was noted. These results supplement the findings of Prusoff, Teply and King, who observed that *L. casei* grown on folic acid-deficient media had a lowered DNA but unaltered PNA content(7). Histochemical studies are in progress in which the relative nuclear volume and the DNA content of the individual nuclei are being measured. It is hoped that these latter studies will more clearly define the mechanisms within the cell responsible for the reduced DNA content in aminopterin-treated tumors.

Summary. The pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) content of sarcoma 180 tumors has been determined in mice receiving aminopterin and in controls. The chief alteration associated with aminopterin administration was an increase in the ratio of PNA to DNA due to a reduction in the relative DNA content. Tumors which had been serially transplanted into aminopterin-treated mice, when used as inocula, showed no difference when compared to stock tumors in their subsequent response to aminopterin.

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A Method for the Biological Preparation of Deuterium-Labeled Cholesterol From the Hen's Egg.*† (19691)

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It is well known that both deuterium-labeled acetate and deuterium oxide can be used by animals in the synthesis of cholesterol in tissues(1,2). Investigators have shown that the deuterium is present both in the ring and in the side chain(1) and that the incorporation of deuterium into the cholesterol is not simply an exchange reaction(2).

In work reported by Kritchevsky *et al.* in which sodium-acetate 1-C^{14} was fed to laying hens, it was found that there was a rapid incorporation of the acetate into various fractions of the egg with the specific activity higher in the cholesterol than in any other fraction(3,4). In this laboratory, experiments

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