THE USE OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

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SINCE Woods[1] described the interference of *p*-aminobenzoic acid on the action of sulfonamides, the antagonism between compounds promoting growth and those which inhibit growth has received increasing attention. Analogues of pteroylglutamic acid (folic acid) have been studied extensively.[2] The antagonistic activity of these compounds in the growth of *Streptococcus faecalis R* has been examined by Hutchings and Stokstad.* Experiments leading to the demonstration of a stimulatory effect of pteroylglutamic acid and an inhibitory effect of its antagonists on Rous chicken sarcoma have been reported by Little and co-workers[4] and by Woll.[5]

In the present report we wish to present more recent findings with regard to the effect of choice of antagonists and method of administration on the outcome of experiments with Rous chicken sarcoma. Table I contains a list of the antagonists of pteroylglutamic acid which we have tested for ability to control Rous chicken sarcoma. Both the abbreviated names of compounds (such as used in our first report on Rous sarcoma) and the complete chemical names are shown as an aid in referring to reports dealing with the synthesis of these compounds. The antagonists which we have found most useful, for reasons to be discussed, are the 4-amino-pteroylaspartic acid and the 4-amino-pteroyl d(-)glutamic acid which were synthesized by Mowat and others,[10]* and the 4-amino-pteroylglutamic acid synthesized by Seeger, Smith, and Hultquist.[7]†

PROCEDURE

In producing Rous chicken sarcoma we have employed homogeneous samples of frozen virus prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry ice chest until used. Preliminary suspensions containing 10 mg. in a 0.25 ml. dose were prepared by adding 50 ml. of 2 per cent peptone solution to 2 Gm. of tumor tissue. A Ten Brock grinder was employed, and further dilutions were made in peptone solution. The size of inoculum used in our experiments was determined by titration of the virus for even distribution of tumor responses over a period of time from the eighth to the sixteenth day of the test. The frozen virus was found to be stable for at least a month with respect to the effect of concentration of virus on the time distribution of tumor responses. Table II illustrates the method employed for standardizing the virus.

HANDLING OF CHICKS

New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report)[4] were injected in the right breast with 0.25 ml. of the suspension containing the dose required for the desired distribution of tumor responses. The feathers were removed from the breast area before inoculation. Groups of ten birds were compared with

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TABLE I. LIST OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID TESTED ON ROUS CHICKEN SARCOMA

ABBREVIATED NAME	CHEMICAL NAME	REFERENCE
Pteroylaspartic acids*	*N*-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-aspartic acid	6
4-Amino-folic acid* or 4-Amino-pteroylglutamic acid	*N*-[4-{[(2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid	3, 7
N-methyl-folic acid* or *N*-methyl-pteroylglutamic acid	*N*-[4-{*N*-[(2-amino-4-hydroxy-6-pteridyl)-methyl]-*N*-methylamino}-benzoyl]-glutamic acid	3, 8
N-methyl-pteroic acid*	4-{*N*-[(2-amino-4-hydroxy-6-pteridyl)-methyl]-*N*-methylamino}-benzoic acid	3, 8
4-Amino-*N*-methyl-folic acid* or 4-Amino-*N*-methyl-pteroylglutamic acid	*N*-[4-{*N*-[(2,4-diamino-6-pteridyl)-methyl]-*N*-methylamino}-benzoyl]-glutamic acid	3, 9
4-Amino-*N*-methyl-pteroic acid*	4-{*N*-[(2,4-diamino-6-pteridyl)methyl]-*N*-methylamino}-benzoic acid	9
4-Amino-pteroylaspartic acid*	*N*-[4-{[(2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-aspartic acid	10
4-Amino-folic acid with d(-)glutamic acid* or 4-Amino-pteroyl-d(-)glutamic acid	*N*-[4-{[2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-d(-)glutamic acid	10

*Designation used in our first report on the effect of folic acid and its antagonists on Rous chicken sarcoma.[4]

TABLE II. TITRATION OF ROUS SARCOMA VIRUS FOR EVEN DISTRIBUTION OF TUMOR RESPONSES

AMOUNT OF TISSUE IN INOCULUM (0.25 ML.)	TOTAL CHICKS USED	TOTAL TESTS MADE	PERCENTAGE OF CHICKS WITH TUMORS				
			DAY				
			8	10	12	14	16
10 mg.	37	4	64	83	90	90	97
1 mg.*	140	14	14	40	58	71	91
100 μg	140	14	0	6	26	45	71
10 μg	140	14	0	1	3	14	31

*This amount of virus gave the desired distribution of tumor responses.

as many untreated controls. The chicks were maintained in electric brooders at 90° F.; water and food were supplied ad libitum. Baby chicks varying from 1 to 8 days old were tested for ability to resist the toxic effect of different antagonists of folic acid.

A daily record of observations was made from the eighth day of the test to the twentieth day. The growth of tumors from the time of first appearance was recorded in the manner previously described. This information enabled us to demonstrate retardation of tumor growth when inadequate doses of antagonists were used. In the tables presented in this paper, chicks showing the slightest evidence of tumor have been counted in with those showing the usual tumor response characteristic of untreated groups.

EXPERIMENTAL RESULTS

Table III shows the results of experiments with 4-amino-pteroylaspartic acid. This antagonist of folic acid was administered (1) by daily intraperitoneal injection to chicks which were 2 days old at the start, (2) by daily intraperitoneal injection to chicks which were 8 days old at the start, and (3) by feeding to chicks which were only 1 day old at the start. It is apparent from Table III that under the conditions in (1) the toxicity of the chemical for the chicks interfered with its value as an inhibitor of tumor growth. Under the conditions in (2) the chicks were more resistant to the toxic effect of doses inhibitory to tumor growth. Under the conditions in (3), chicks only 1 day old at the start could

TABLE III. USE OF 4-AMINO-PTEROYLASPARTIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2	0.02	10	0	44	0
2	0.1	10	40	50	10
2	0.2	20	75	80	5
2	0.2	10	80	50	0
2	0.2	10	60	50	0
(2) Antagonist Administered by Daily Intraperitoneal Injection to Week-Old Chicks					
8	0.2	10	0	60	10
8	0.4	10	10	44	10
(3) Antagonist Administered in Diet of Baby Chicks					
1	20/kg.	15	0	0	9
1	80/kg.	20	10	75	5

TABLE IV. USE OF 4-AMINO-PTEROYL-D(-)GLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
3	0.01	10	0	22	0
2	0.02	10	20	42	0
3	0.1	10	30	75	0
2	0.2	20	95	66	5
2	0.2	10	80	50	0
(2) Antagonist Administered by Daily Intraperitoneal Injection to Week-Old Chicks					
8	0.1	10	20	37	10
8	0.2	10	30	42	10
8	0.4	10	60	100	10
(3) Antagonist Administered in Diet of Baby Chicks					
1	20/kg.	15	0	0	9
1	80/kg.	20	0	55	5

TABLE V. USE OF 4-AMINO-PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2 days	0.01	10	100	—	0
3 days	0.1	10	100	—	0
(2) Antagonist Administered in Diet of Baby Chicks					
1 day	20/kg.	20	100	—	9
(3) Antagonist Administered by Daily Intraperitoneal Injection to Adult Birds					
6 weeks	1.0	10	10	62	0
7 weeks	1.0	10	0	60	0
8 weeks	1.0	10	0	40	0

be treated successfully with 80 mg. of 4-amino-pteroylaspartic acid per kilogram of diet without toxic effect. The diet was the regular commercial chick ration.

Table IV shows that similar results were obtained with 4-amino-pteroyl-d(-)glutamic acid. Daily injections of the doses of chemical required to inhibit tumor growth resulted in impairment of health and eventual loss of birds when the chicks were 2 to 3 days old at the start. Chicks 8 days old at the start showed greater resistance to the toxic effects of doses inhibitory to tumor growth. Chicks only 1 day old at the start showed no toxic effect when fed 80 mg. of 4-amino-pteroyl-d(-)glutamic acid per kilogram of diet.

Table V shows that 4-amino-pteroylglutamic acid (4-amino-folic acid)[4] is suitable for use in the treatment of adult birds only. In a series of experiments we succeeded in neutralizing the toxicity of 4-amino-pteroylglutamic acid for baby chicks by giving simultaneous injections of pteroylglutamic, pteroyldiglutamic, or pteroyltriglutamic acid. It was found that 0.25 mg. doses of pteroylglutamic acid protected 60 per cent of chicks, 0.25 mg. doses of pteroyltriglutamic acid protected 50 per cent of chicks, and 0.25 mg. doses of pteroyldiglutamic acid protected 10 per cent of chicks against the toxic effect of 0.01 mg. doses of 4-amino-pteroylglutamic acid. This antagonist did not prevent tumor growth in baby chicks thus protected from its toxic effect.

TABLE VI. USE OF 4-AMINO-*N*-METHYL-PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2	0.02	10	0	22	0
2	0.1	10	80	50	10
2	0.2	10	70	33	0
2	0.2	20	55	77	5

Table VI shows the results of experiments with 4-amino-N-methyl-pteroylglutamic acid. When administered to baby chicks by daily intraperitoneal injection, this antagonist demonstrated toxicity and also inhibited tumor growth. It was not administered in the diet because of lack of material. The toxicity of 4-amino-*N*-methyl-pteroylglutamic acid appears to be similar to that of 4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid. These antagonists, when injected, produce symptoms resembling those caused by maintaining chicks on folic acid free diets. We have found that chicks showing severe symptoms as a result of injection with these antagonists quickly revive when treatment is discontinued.

DISCUSSION

It is evident from our experiments that Rous chicken sarcoma may be controlled by regulating the amount of pteroylglutamic acid in the tissues of the chicken, either by use of synthetic folic acid free diets[4] or by use of suitable

chemical antagonists. Severe deficiencies of the vitamin, whether caused by lack of folic acid in the diet or by the injection of powerful antagonists such as 4-amino-pteroylglutamic acid, regularly result in death of birds. It is well known that the amount of folic acid required to maintain the normal health of chickens is relatively small. According to Oleson[11] 0.5 mg. per kilogram of diet is suboptimum, 1 to 2 mg. are optimum, and 2 to 5 mg. are superoptimum.

Our experiments indicate that at least two of the chemical antagonists of folic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) and possibly a third (4-amino-*N*-methyl-pteroylglutamic acid) may be appropriate tools for controlling the amount of folic acid available in the body for tumor growth without depriving the body of the amounts of vitamin required for normal health. When the treated animal is a rapidly growing chick, these chemicals appear to be more serviceable than 4-amino-pteroylglutamic acid. When the treated animal is an adult chicken, 4-amino-pteroylglutamic acid is not only serviceable, but may well be the chemical of choice inasmuch as it is effective in small doses.

The toxicity of these chemicals appears to be due solely to the severity of the vitamin deficiency which may result from too intensive treatment. Our experiments indicate that treatment by mouth is the method of choice, since tumor growth may be inhibited by this method in a greater percentage of animals without toxic effect. Evidently the tumors of Rous Chicken sarcoma are more directly dependent for their growth on the folic acid furnished by the diet than are the normal tissues of the chick. Since chicks do not develop severe deficiencies of the vitamin in the time required to demonstrate inhibition of tumor growth by use of folic acid free diets (as shown in our previous report),[4] it is possible that the health of such chicks is protected by stores of the vitamin which are not available to tumors of this type. If such is the case, the most desirable method of treatment would be the administration in the diet of chemicals which would antagonize the vitamin present in food. We have shown that 4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid can produce this result.

SUMMARY

Two chemical antagonists of pteroylglutamic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) have been found capable of inhibiting tumor growth in baby chicks inoculated with Rous sarcoma virus when the chemicals were fed ad libitum at a concentration of 80 mg. per kilogram of diet. These chemicals and also 4-amino-*N*-methyl-pteroylglutamic acid were active in inhibiting tumor growth in baby chicks when injected. Toxic effects resembling the effect of severe vitamin deficiency occurred when the chemicals were injected, but not when the chemicals were fed.

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