Folic Acid Analogs and the Growth of Embryo and Tumor Tissue

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Analogs of pteroylglutamic acid (folate acid) have been extensively investigated from both the standpoint of their preparation and their biological effects. Numerous compounds of this type have been synthesized and their effects tested with respect to the growth of various animals and tissues. The present status of this field of research has been summarized in some of the contributions to the recent series of papers on anti-metabolites given under the auspices of the New York Academy of Sciences (2-6, 9).

It is generally considered that these compounds, when introduced into an animal, serve as antagonists of folate acid and especially affect those tissues which are sensitive to an insufficiency of this vitamin. It has also been demonstrated that there is a wide variation in the dosage of the available analogs required to block out folate acid from the cells (4, 5, 7).

A number of folate acid analogs have been tested on malignant neoplasms (9, 10). It has been found that growth inhibition may result from the application of these compounds. 4-Aminopteroylglutamic acid (aminopterin) has proved especially effective in this regard. However, nontumor tissue is also adversely affected. Other less toxic folate acid analogs such as pteroylglutamic acid (teropterin) and pteroylglutamic acid (an-Fol-A) have been found to be less effective, even at comparatively large dosages. Stock (9) and associates found that teropterin was ineffective on egg-cultivated tumors when dosages as high as 20 mg. per injection were used. Such data as are available indicate that different types of tumors do not react similarly to such compounds. Sugiyama (10) and co-workers have reported that aminopterin had an inhibitory effect at toxic levels on transplants of a sarcoma in rats and on a carcinoma, mammary carcinoma, and melanoma in mice. Spontaneous breast tumors in mice were not affected.

The present investigation was undertaken to test representatives of folate acid analogs on the growth of embryo and yolk sac tumors of tumor-bearing eggs. Tumors cultivated in this manner grow in the embryonated egg, sharing a common blood supply with the embryo but without otherwise interfering with the embryonic course of development.

This method of growing tumor tissue has been used in this laboratory for more than 8 years (18-15). During that time hundreds of thousands of eggs have been inoculated with tumor tissue in association with various research projects. The technics involved in the process have been progressively improved and simplified. During the past 2 years yolk sac-cultivated tumors have been used in cancer chemotherapy studies (11, 12). Since the publication of the first report on this work, about 18,000 tumor-bearing eggs have been utilized in testing compounds. It has been shown that tumors grown by the yolk sac method give 100 per cent "takes" and grow as uniformly as implants of the tumor tissue in the natural host. Furthermore, the growth of the tumor used in these experiments is so rapid that 24-hour tests are possible.

Three folate acid analogs, aminopterin, teropterin, and an-Fol-A have been used in a series of tests using embryonated eggs implanted in the yolk sac with a C3H mouse mammary carcinoma. Tests were also made with folate acid, synthetic folinic acid (citrovorum factor), and these compounds in combination with aminopterin and teropterin.

The object of the investigation was to determine the comparable reactivity of tumor and embryo to the test compounds. Data obtained by the same approach with cortisone acetate are included for comparison.

MATERIALS AND METHODS

Embryonated eggs with yolk sac implants of a C3H mammary tumor were used in these studies. The eggs were inoculated with tumor tissue on about the fourth day of incubation, as previously

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1 Supplied by Lederle Laboratories.
2 Supplied by Dr. William Shive of the University of Texas Biochemical Institute.
The tumor grows rapidly and relatively uniformly by this method of cultivation in every inoculated egg.

On the twelfth day of egg incubation (8 days after tumor inoculation), the compound to be tested was introduced between the shell membranes and the blood vessels of the chorioallantoic membrane and area vasculosa. This was done by hypodermic injection through a dented area in the shell, the details of which have been described (11).

The compounds used in these experiments were injected, in most instances, as aqueous solutions or suspensions in combination with cottonseed oil. One part of the aqueous solution or suspension was thoroughly mixed with 2 parts of the oil, and 0.2 cc. of the emulsion was introduced into the experimental eggs. This was done to prevent too rapid absorption and excretion of the test compound into the allantoic fluid. The control eggs were injected with the suspending fluid.

The tumor weights averaged 0.21–0.40 gm. per egg on the twelfth day of incubation. In the next 24 hours these yolk sac tumors increased an average of 70 per cent in size. The embryos supporting the tumors averaged 2.9–3.4 gm. and gained in weight an average of 30 per cent for the same time interval. This 24-hour period was used for testing the effects on the growth of tumor and embryo of the injected compound.

Each experiment was carried out as follows:

Twelve-day tumor-bearing eggs were divided into comparable groups of about 15 eggs each. One group was harvested at once to obtain the average tumor and embryo weight at the beginning of the experiment. The other eggs were divided into experimental and control groups and injected with the appropriate material. The eggs were incubated at 37° C. for 24 hours. The embryos and tumors were then harvested and weighed individually.

Representative samples of control and experimental tumors were selected for histological examination.

The effect on growth was evaluated on the basis of the increase in size of embryo and tumor which occurred in the control eggs during the period of the experiment.

Teropterin was used in sixteen experiments involving a total of 248 tumor-bearing eggs, aminopterin in ten experiments for a total of 240 eggs, and An-Fol-A was the test compound in nine experiments for a total of 240 eggs. These experiments were performed with aminopterin and teropterin. Aminopterin was tested in six experiments for a total of 192 tumor-bearing eggs. Comparable nontumor eggs were used in a number of experiments to check the effect of the compounds on the embryo uncomplicated by the presence of a tumorous growth in the yolk sac.

RESULTS

The principal data are summarized in Tables 1 and 2. The statistical validity of these results is evident.

It will be noted in Table 1 that teropterin was a more effective inhibitor of tumor growth under the conditions of the experiments than aminopterin. Furthermore, the effect on the tumor was obtained with very little disturbance to the embryo. It is also evident that dosage was an important factor in the experiment with teropterin. Tumor growth showed a maximum inhibition when 0.1 mg. of the compound per injection was used. Lower dosages not recorded in the table were used in a few experiments but were less effective. A number of experiments were carried out with teropterin at various levels in solution without the admixture of cottonseed oil. Under those circumstances the effect on tumor and embryo growth was negative. Apparently, the injected material was taken up by the blood and passed into the allantoic fluid too rapidly to be effective. Tests were also made with nontumor embryonated eggs. These embryos were not affected by dosages used for the tumor-bearing egg injection.

The effect of teropterin on tumor growth was almost entirely eliminated when administered in association with either folic acid or folinic acid (Table 2). The data indicate, however, that there was still more inhibition of tumor growth than was true for either folic acid or folinic acid alone.

An-Fol-A resembled teropterin at the 1.0-mg. per injection level. Lower levels were ineffective. This compound was also relatively nontoxic to the chick embryo.

Aminopterin at subtoxic dosages had little effect on tumor or embryo growth. At a dosage which gave evidence of disturbance to the embryo, tumor growth was significantly inhibited.

Folic acid in combination with aminopterin reduced the inhibitory effect on tumor growth with possibly some protective action on the embryo. The data indicate that folinic acid, when administered with aminopterin, accentuated the inhibitory effect on tumor growth with about the same effect on the embryo as the folic acid-aminopterin combination.

Cortisone acetate, as the table shows, was used at levels varying from 0.1 to 1.5 mg. per injection. At all these dosages the growth of tumor and embryo was inhibited and to about the same degree.
Histological examination of the tumor tissue showed no necrosis or other evidence of toxicity. The tumor cells appeared to be unaffected in association with almost complete inhibition of growth. As further evidence of this, when eggs treated with the folic acid analogs were allowed to continue their development with no further injections, the yolk sac tumors resumed their usual rapid growth.

DISCUSSION

The use of yolk sac cultivated tumors makes it possible to compare the reactions of neoplastic and rapidly proliferating non-neoplastic tissue to a particular compound. The relationship of the yolk sac tumors and the host embryo are such that, in the early stages, they maintain vigorous and independent growth and at the same time share a common blood stream and a common environment.

The method of introducing the test compound into the egg which was used in these experiments ensures absorption into the chick blood system. In a recent paper Galinsky (1) demonstrated that radioactive phosphorus injected by this technic over the chorioallantoic membranes and area vasculosa was taken up by the blood stream and distributed to the chick embryo, yolk sac tumor, and other parts of the egg.

The rate of absorption naturally varies with the type of material injected. In the present investigation the best results were obtained when absorption of the inoculum was made to proceed more slowly by the use of cottonseed oil in the suspending medium.

### TABLE 1

<table>
<thead>
<tr>
<th>No. Doses</th>
<th>No. Exps.</th>
<th>Survival control</th>
<th>Av. Initial weight (gm.)</th>
<th>Av. Tumor weight, end of test period (gm.)</th>
<th>Av. Embryo weight, end of test period (gm.)</th>
<th>Growth inhibition (per cent)</th>
<th>Tumor Embryo</th>
<th>Embryo</th>
<th>Tumor Embryo</th>
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<tr>
<td>Teropterin</td>
<td>92 3 97</td>
<td>1.0</td>
<td>0.54±0.06 3.07±0.21</td>
<td>4.6±0.07 4.88±0.19 9.09±0.27</td>
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<td>Aminopterin</td>
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<td>0.59±0.02 3.39±0.15</td>
<td>4.7±0.05 4.66±0.09 4.09±0.17</td>
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<td>An-Fol-A</td>
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<td>0.1</td>
<td>0.39±0.04 3.15±0.12</td>
<td>4.44±0.05 4.06±0.06</td>
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<td>Cortisone acetate</td>
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<td>1.0</td>
<td>0.55±0.07 3.13±0.22</td>
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<td>Folinic acid</td>
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<td>0.43±0.08 3.26±0.15</td>
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**TABLE 2**

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<tr>
<th>Material injected</th>
<th>Dose (mg.)</th>
<th>No. Doses</th>
<th>No. Exps.</th>
<th>Tumor Embryo (gm.)</th>
<th>Av. Initial weight (gm.)</th>
<th>Av. Tumor weight, end of test period (gm.)</th>
<th>Av. Embryo weight, end of test period (gm.)</th>
<th>Growth inhibition (per cent)</th>
<th>Tumor Embryo</th>
<th>Embryo</th>
<th>Tumor Embryo</th>
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<td>Folic acid</td>
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<td>66</td>
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<td>27 3</td>
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* Indicates per cent growth acceleration.
The data indicate that an egg-cultivated mammary carcinoma was more sensitive to certain folic acid analogs than the associated chick embryo. This was particularly true with respect to teropterin and An-Fol-A. Such a difference in the reaction of embryo and tumor of tumor-bearing eggs to an introduced compound is very uncommon. Several hundred compounds have been tested to date, and in most instances there is either no effect, or the tumor and embryo react similarly. The usual outcome of such experiments is exemplified in the data on cortisone acetate. This compound inhibited both types of tissue to about the same degree.

The results obtained when folic acid and folinic acid were used in combination with either aminopterin or teropterin are of some interest since they indicate, as did the reaction of the tumor to teropterin, a possible difference in the biochemistry of the tumor, as compared to the embryo. This is particularly true with respect to the effect on tumor growth obtained by a combination of aminopterin and folic acid.

Snell and Cravens (8) reported that concentrates of the citrovorum factor did not lessen the inhibitory effect of aminopterin on chick embryos. They also obtain negative results in this respect with folic acid. In the present investigation folic acid not only failed to reverse the effect of aminopterin but increased the inhibitory effect on tumor growth to the point where it equaled that of teropterin.

A more extended investigation of the role of folic acid on tumor and embryo biochemistry is contemplated.

**SUMMARY**

Folic acid analogs were introduced over the chorioallantoic membrane and area vasculosa of 12-day embryonated eggs having yolk sac implanted mouse mammary carcinoma. A total of 35 experiments involving 758 tumor-bearing eggs were utilized.

Teropterin inhibited tumor growth an average of 50, 78, and 83 per cent, at dosages of 1.0, 0.5, and 0.1 mg. per injection. Growth of the associated chick embryos was only slightly affected.

Aminopterin inhibited tumor growth an average of 67 and 12 per cent at levels of 0.15 and 0.1 mg. per injection. The growth of the chick embryos was inhibited 11 and 15 per cent, with evidence of some toxicity at the higher level.

An-Fol-A at 1.0 mg. per injection inhibited tumor growth an average of 56 per cent, with the chick embryos very slightly affected. Lower dosages were ineffective.

The effect of folic acid and folinic acid alone and in combination with teropterin and aminopterin was tested. Each of these compounds in 0.1-mg. doses slightly inhibited tumor and embryo growth. Both folic acid and folinic acid counteracted the tumor growth inhibitory effect of teropterin. Folic acid had little or no effect on the tumor growth inhibitory action of aminopterin. Folinic acid in combination with aminopterin was more inhibitory of tumor growth than aminopterin alone.

Microscopic examinations of tumor tissues disclosed no evidence of histological or cytological disturbance.
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Alfred Taylor and Nell Carmichael


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