Inhibition of Experimental Hypercholesterolemia and Atherogenesis by 4-Amino Pteroylglutamic Acid (Aminopterin)

By Carl Muschenheim, M.D., Seymour Advocate, M.D., and Donald W. Hoskins, M.D.

Atherosclerosis has been found to be characteristically absent or slight in pernicious anemia. This observation, though not to our knowledge documented in medical literature, was communicated to one of us by pathologists with large human autopsy experience prior to the era of effective therapy in this disease. The blood cholesterol was long ago reported to be low in pernicious anemia, and it was also reported that the level is raised by liver feeding in normal dogs and in normal humans, as well as in those with pernicious anemia. Furthermore, the increase of blood cholesterol in treated pernicious anemia occurs at the onset of remission, i.e., before any increase in the red blood cell count.

Atherosclerosis has also been noted to be inconspicuous in human cases of sprue. This negative correlation has been attributed to malabsorption of fats. However, malabsorption of vitamin B₁₂ is also known to occur in sprue. Moreover, this vitamin is reported to influence lipid metabolism. Folic acid also may affect lipid metabolism by its relationship to coenzyme A.

These considerations have suggested the possibility that these vitamins may play a role in atherogenesis, and that antimetabolites of vitamin B₁₂ or of folic acid may exert an inhibitory effect.

The folic acid antagonist Aminopterin* (4-amino pteroylglutamic acid) is known to produce a megaloblastic anemia in certain species of laboratory animals. In preliminary trials of the present studies, a similar effect was found in the rabbit indicating metabolic activity of the substance. This animal, in which atherosclerosis is readily produced experimentally by cholesterol feeding, was selected for the initial experiments to test whether any inhibition of atherogenesis might result from simultaneous administration of the folic acid antagonist.

Methods

Animals

Normal rabbits, weighing 2 to 4 Kg. at the start of the experiments, were divided equally between treated and control groups. The animals were of mixed breeds. Two separate experiments were necessitated by a limitation of cage space. Experiment A was performed in the fall of the year and experiment B in the winter and spring.

Diet

A diet containing 3/4 per cent cholesterol was prepared by dissolving 7.5 Gm. of cholesterol in 400 ml. of ether. The resultant solution was then thoroughly mixed with 1,000 Gm. of stock rabbit pellets, which were then allowed to dry for 24 hours, leaving a cholesterol coating on each pellet. The batches for experiment A and for experiment B were separately prepared.

Aminopterin

A 0.1 per cent solution of Aminopterin for subcutaneous injection was prepared each day immediately prior to its use. The dry powder was pre-

*The Aminopterin used in this study was generously supplied by the Lederle Laboratories Division, American Cyanamid Co.
Previously weighed out in 120 mg. quantities and kept in tightly stoppered brown glass bottles. Each 120 mg. of the powder required the addition of 5.4 ml. of 0.1 normal sodium hydroxide for complete solubility, and the final volume was brought to 120 ml. by the addition of distilled water. The pH of the solution was 8.3.

**Buffer solution**

A solution of sodium bicarbonate adjusted to pH 8.3, the same as of the Aminopterin solution, was prepared for subcutaneous injection of the control animals.

**Serum cholesterol determinations**

The serum cholesterol levels were determined by the method of Abell, Levy, Brodie and Kendall on blood drawn from the marginal ear vein.

**Hematologic studies**

Complete blood counts and hemoglobin determinations were performed by the standard hematologic technics. Hematocrit determinations were made by the capillary tube technic on an Adams microhematocrit centrifuge at 12,000 r.p.m. for 3 minutes.

**Anatomic studies**

At the end of the experimental period, all surviving animals were sacrificed by air embolism and examined immediately. The heart and aorta of each animal were removed en bloc and preserved in 10 per cent formalin. After fixation, the extent of atherosclerosis of the aorta was observed grossly and graded on a scale of 0 to 4+; where 0 represents absence of visible lesions, 1+ a few barely visible plaques, 4+ extensive coverage with numerous and confluent raised intimal lesions and grades 2+ and 3+ intermediate states. The viscera were examined grossly, and blocks of tissue for microscopic studies were taken from the liver, spleen, jejunum and kidney. The blocks of tissue were fixed in 10 per cent formalin and stained with hemotoxylin and eosin. Liver sections were stained also with fat stain, oil red "0".

**Experimental Procedure**

Aminopterin administered in the dose of 10 mg./day subcutaneously produced a profound and sometimes fatal anemia in the rabbits. It was observed, however, that if a 2-day rest period was given each week, the anemia could usually be maintained in an hematocrit range of 20 to 30 per cent, and the animals appeared healthy and maintained good nutrition. Each animal was weighed weekly, but during the cholesterol feeding period the total weekly food consumption of each animal was measured, and the mean daily food consumption was calculated. The total cholesterol ingestion during the 8 weeks was thus also determined for each animal (table I).

No measurements were made of the food intake in experiment A. In experiment B only spot checks of daily food intake were made, but during the cholesterol feeding period the total weekly food consumption of each animal was measured, and the mean daily food consumption was calculated. The total cholesterol ingestion during the 8 weeks was thus also determined for each animal (table I). It is apparent from the standard deviations listed in the table that individual variations in average daily food consumption were not significantly different between the controls and the Aminopterin-treated animals. The average total cholesterol ingestion for each animal was 38.3 Gm. for the treated survivors and 40.2 Gm. for the controls, a negligible difference.
Table 1

Mean Changes in Hematocrit, Total Serum Cholesterol, and Body Weight*†

<table>
<thead>
<tr>
<th></th>
<th>Hematocrit per cent</th>
<th>Total serum cholesterol mg. per cent</th>
<th>Change in body weight Gm. 0-8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial level 0 weeks</td>
<td>Change 0-4 weeks</td>
<td>Change 0-8 weeks</td>
</tr>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopterin group</td>
<td>Mean 32</td>
<td>-4.8</td>
<td>-7.5</td>
</tr>
<tr>
<td>(6 animals)</td>
<td>S.D. 6.4</td>
<td>5.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Control group</td>
<td>Mean 38</td>
<td>-6.3</td>
<td>-6.9</td>
</tr>
<tr>
<td>(8 animals)</td>
<td>S.D. 2.4</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopterin group</td>
<td>Mean 33</td>
<td>-1.9</td>
<td>-7.3</td>
</tr>
<tr>
<td>(7 animals)</td>
<td>S.D. 7.9</td>
<td>7.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Control group</td>
<td>Mean 41</td>
<td>-4.9</td>
<td>-9.2</td>
</tr>
<tr>
<td>(13 animals)</td>
<td>S.D. 3.6</td>
<td>2.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*Experiments A and B were performed at separate times, each with concurrent controls. Data are listed for 6 surviving treated animals in experiment A and 7 surviving treated animals in experiment B. (For times and causes of deaths see the text.) Observations were tabulated from start (0 weeks) of cholesterol feeding period. Injections of Aminopterin and of control buffer were begun 4 weeks prior to start of cholesterol feeding and were maintained during the ensuing 8 weeks. Animals were sacrificed at 8 weeks after the start of injections of Aminopterin and of control buffer.

†A more detailed form of tables 1, 2 and 3 has been deposited as Document Number 6245 with the ADI auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting $1.25 for photoprints, or $1.25 for 35 mm. microfilm. Advance payment is required. Make checks or money order payable to: Chief, Photoduplication Service, Library of Congress.

Results

As may be seen in table 1, the rise of total serum cholesterol levels at the midpoint (4 weeks) of the atherogenic diet was substantially less in the Aminopterin-treated than in the control animals. In experiment A, the hypercholesterolemia at this point in time attained a mean rise of 529 mg. per cent in 6 treated animals, as compared with a mean rise of 839 mg. per cent in 8 controls. In experiment B, the mean rises at the corresponding time were 136 mg. per cent in 7 surviving treated animals and 371 mg. per cent in the 13 controls. At the termination (8 weeks), there was in experiment A little further change in the level of hypercholesterolemia in either the treated or the control group, while in experiment B there was a further rise in each group, but the levels retained a similar relative difference, with a mean rise of 354 mg. per cent in the treated and 666 mg. per cent in the controls. The hypercholesterolemia was thus approximately 30 to 50 per cent less in the treated group, both at the midpoint and at the termination, in each experiment. These differences are statistically significant.

No proportional relation was found between the weight gains of individual treated animals and the cholesterol levels. No apparent correlation was observed among the treated animals between the degree of anemia and the level of hypercholesterolemia or of the extent of atheromatosis. As a consequence of the prolonged cholesterol feeding, the control animals also developed an appreciable anemia, so that during each experiment there was an approximately equal decline in the mean hematocrit levels in the treated and control groups (table 1). This factor of anemia in the untreated animals thus served as a partial control for
Table 2

Average Daily Food Consumption and Total Cholesterol Feeding Calculated from Total Weekly Food Consumption of Each Animal, Experiment B

<table>
<thead>
<tr>
<th>Experiment B</th>
<th>Average daily food consumption 0-8 weeks</th>
<th>Total cholesterol ingested 0-8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopterin group</td>
<td>91.3 Gm. 38.3 Gm.</td>
<td>20.77 8.72</td>
</tr>
<tr>
<td>Control group</td>
<td>95.1 Gm. 40.2 Gm.</td>
<td>14.99 6.58</td>
</tr>
</tbody>
</table>

any possible hemodynamic influence which anemia might exert per se in impeding cholesterol deposition in the arteries. However, the treated animals were more anemic and were anemic longer because at the start (0 weeks) of the cholesterol feeding period, these had already received 4 weeks of pretreatment with Aminopterin and had significantly lower initial hematocrit levels (table 1).

When sacrificed, the control animals all had grossly demonstrable 1+ to 4+ atheromatosis of the aorta. Of the 13 surviving Aminopterin-treated animals, 9 had no discernible atheromatosis or had doubtful (±) lesions, less clearly defined than the smallest deposits in the controls. Only 4 of the treated animals had atheromatosis which was definitely demonstrable on macroscopic examination and was comparable to that of the controls. In 3 of the 4 it was of small (1+) extent, equal only to the least amount of atheromatosis observed in the controls. The gradings were performed without knowledge of which were treated and which were control animals, and these results are listed in table 3. The difference is statistically significant.

Discussion

The contrast in atheroma formation between the controls and the surviving treated animals is not accounted for by unequal ingestion of cholesterol. The differences in mean weight gains of the animals in both experiments and the differences in food and cholesterol consumption which were measured in experiment B were so slight as to be negligible. It is concluded, therefore, that a true inhibitory effect on hypercholesterolemia and atherogenesis in the cholesterol-fed rabbits resulted from the administration of Aminopterin. If this inhibition were attributable merely to the nonspecific effect of a toxic substance, there should have been manifested an appreciable impairment of general nutrition and food consumption in all of the treated animals, i.e., in those which recovered from the acute toxicity as well as in those succumbing during the experiment.

The inhibition of hypercholesterolemia and of atherogenesis which has been demonstrated is thought to be due to a specific effect of Aminopterin, either by an interference with cholesterol absorption, an interference with cholesterol metabolism, or by a combination of both mechanisms. Fatty infiltration of the liver was less conspicuous, both macroscopically and microscopically, in the treated animals. This might result, together with the lesser development of atheroma, merely from a defect in absorption. However, no abnormality in the mucosa of the first part of the jejunum was noted microscopically, as might be expected if an absorptive defect secondary to mucosal damage were the principal factor. It might be reasoned that interference with acetylation of coenzyme A and a consequent blocking of cholesterol synthesis may be the
fundamental mechanism, at least insofar as the atherogenesis is concerned. Folic acid antagonists have been shown capable of blocking other biologic acetylations, as in the case of isoniazid inactivation. In this connection, it is of interest that isoniazid itself, and its congeners, may affect cholesterol metabolism.

A combination of factors interfering with both absorptive and metabolic mechanisms appears more probable than either influence alone. According to experimentally confirmed concepts of the metabolism of cholesterol, it is unlikely that extensive degradation and resynthesis of exogenous cholesterol can take place. The major metabolic pathway of cholesterol catabolism is transformation to bile acids and excretion in the bile. By interference with certain enzyme systems concerned in this transformation, such as those involving coenzyme A, Aminopterin might play an inhibitory role. More cholesterol might consequently be lost in the stool and less be reabsorbed from the intestine. Experimental confirmation of such a possible mechanism would require the use of isotope-labeled cholesterol.

Further studies are projected with other antagonists of folic acid, with antagonists of vitamin B12 and of pantothenic acid, and with other substances which may act as antimetabolites in the enzyme systems related to the metabolism of cholesterol and other lipids concerned in atheroma formation.

Summary

Rabbits pretreated with Aminopterin (4-amino pteroylglutamic acid) for 4 weeks were fed an atherogenic diet for 8 weeks, during which Aminopterin injections also were continued. The atherogenic diet consisted of a stock diet with 3/4 per cent cholesterol added. As a result of some manipulation of the Aminopterin dosage during the period before cholesterol feeding was begun, some of the Aminopterin-treated animals lost weight from overdosage, and 7 of the original 20 died. The surviving animals upon reduction of dosage made good weight gains, comparable to the controls, during the cholesterol feeding period. When sacrificed after 8 weeks of cholesterol feeding, the control animals, 21 in number, all had grossly demonstrable 1+ to 4+ atheromatosis of the aorta. Of the 13 surviving Aminopterin-treated animals, 9 had no atheromatosis or doubtful deposits of minimal extent. Only 4 had definite atheromatosis comparable in extent to the controls. Serum cholesterol levels were 30 to 50 per cent lower in the treated animals than in the controls.

Summario in Interlingua

Conilios, pretractate con Aminopterina (acido 4-amino-pteroylglutaminica) durante 4 septimanas, recipiesva durante 8 septimanas un dieta atherogene sin que le injectiones de Aminopterina esseva suspendite. Le dieta esseva un dieta standard con le addition de 3/4 pro cento de cholesterol. Como resulto de un certe manipulation del dosage de Aminopterina durante le periodo ante le addition de cholesterol al dieta, certes del animates tractate con Aminopterina perdeva peso in consequentia de doses excessive, e 7 del 20 in le gruppo original moriva. Le superviventes—post quod le dosage habeva esseva reducida—gananva peso durante le periodo a cholesterol in un grado comparabile a illo del animates de controlo. Quando le animates esseva sacrificato post 8 septimanas de cholesterol, omn 21 controlos habeva macroscopicamente evidente atheromatosis nortia de grados 1+ a 4+. Inter le 13 superviventes del gruppo tractate con Aminopterin, 9 habeva nulle atheromatosis o solmente questionabile depositos de minime extension. Solmente 4 habeva definite atheromatosis, de grados comparabile al animales de controlo. Lo nivellos soral de cholesterol esseva plus basso per 30 a 50 pro cento in le animales tractate in comparation con le animales de controlo.

References


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