Serological Detection of Abnormal Concentrations of Serum Lipoproteins In Dogs on Cholesterol-Thiouracil Atherogenic Diets.* (20040)

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Correlation between diet or serum cholesterol concentration and the incidence of atherosclerosis in man so far has been indecisive. Rabbits and chickens can be made to develop atherosclerosis readily by increasing the cholesterol content of their diet. Atherosclerosis can be produced also in dogs with the Steiner-Kendall cholesterol-thiouracil dietary regimen. The role played by cholesterol and metabolically related unsaturated fatty acids in the production of atherosclerosis is still obscure. In a previous paper(1) dealing with these dietary factors, a successful differentiation was reported, made by means of a simple serological test, between dogs developing atherosclerosis on a Steiner-Kendall regimen and normal dogs. In that paper(1) the authors discussed the presence in the sera of dogs on a cholesterol-thiouracil atherogenic diet of an antigen which will stimulate antibody production in rabbits. This immune serum was used in precipitin tests to detect the concentration of antigen in the sera of dogs on the atherogenic diet. Using the ultracentrifuge Gofman(2) has shown abnormal serum lipoproteins and lipoprotein concentrations in the sera of rabbits and man. He has correlated these lipoproteins with the development of atherosclerosis in rabbits and man. These ultracentrifugally-defined lipoprotein molecules offered a better and more physiologic basis than total serum cholesterol concentrations on which to study the development of atherosclerosis in the dog.

Recently the presence of abnormal lipoproteins and abnormal concentrations of lipoproteins in the sera of dogs on cholesterol-thiouracil atherogenic diets has been demonstrated by means of the ultracentrifuge(3). These lipoproteins presumably contain the antigen used previously(1) to stimulate production of immune sera in rabbits. The experiments reported here were designed to test this assumption.

Procedure. Two series of dogs were placed on experimental diets and their serum lipoprotein concentrations were followed serologically and ultracentrifugally. In the first series, 4 litter mate dogs, A, B, C, and D, 9 months old, weighing from 12 to 16 kg, received daily diets as follows in addition to their stock diets: Dog A—cholesterol (Eastman—30 g) and thiouracil (Lilly—1.2 g); Dog B—cottonseed oil (Procter & Gamble—60 ml) and thiouracil (1.2 g); Dog C—thiouracil (1.2 g); and Dog D—stock control diet only. After nineteen weeks their individual sera were fractionated and analyzed for S_f 2-30 lipoproteins in the following manner: Ten ml of serum were adjusted to a density of 1.062 $\pm$ 0.001 by the addition of 22% sodium chloride solution. Twelve ml of this serum preparation were placed in a Spinco Model L preparative ultracentrifuge for 10 hours at 40,000 rpm (105,000 x g) at a temperature of 15° $\pm$ 1°C. The top 1.2 ml of this preparation were then withdrawn by means of a curved capillary tube attached to a 2 ml glass hypodermic syringe. This fraction represented a lipoprotein concentration 6 times that of the unfractionated serum. Of this supernatant fraction, 0.8 ml was placed in the analytical Spinco Model E ultracentrifuge at 59,780 rpm (260,000 x g) for 30 minutes at a temperature of 27°C $\pm$ 1°C. Photographs were taken at intervals while the centrifuge was at speed. From these photographs lipoprotein peaks and the lipoprotein concentration of the fraction were determined.

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In the fraction from the serum of Dog A, 3 peaks (S_f 2-7, S_f 8-11 and S_f 13-17) were observed; sera of normal dogs contained only one peak (S_f 2-7). From the 1.2 ml supernatant obtained from the serum of Dog A on the cholesterol-thiouracil atherogenic diet, two 0.5 ml aliquots were taken. Each was made up to a volume of 5 ml by addition of sterile 0.9% sodium chloride solution. These preparations were then injected intravenously into two young adult white rabbits, a_1 and a_2, at 7 day intervals for a total of 4 injections. Seven days after the last injection, these rabbits were bled aseptically from the heart. The sera obained from these rabbits were used in precipitin tests against the unfractionated sera from Dogs A, B, C, and D.

These *precipitin tests* were performed as follows: with unfractionated dog serum diluted 1:10 in 0.9% sodium chloride solution, doubling dilutions were made to 1:640. Four-tenths ml of each dilution was placed in precipitin tubes and to each tube was added 0.05 ml of immune serum from rabbit a_1. Similar titrations were performed using the immune serum from rabbit a_2. Appropriate controls were included with each titration. Immediately after adding the immune rabbit serum, the precipitin tubes were incubated in a water bath at a temperature of 37° ± 1°C for one hour. The tests were placed in a refrigerator at 4°C and the titers were read the next day. A second series of 2 dogs, S and R, 2 to 3 years old, weighing 12 to 14 kg, were placed on daily diets of cholesterol (30 g) and thiouracil (1.2 g), and a stock control diet, respectively. After these dogs had been on their diets for 8 weeks, their unfractionated sera also were titered against immune rabbit sera a_1 and a_2. Precipitin tests were performed weekly for periods of 6 and 7 weeks on the first and second series of dogs, respectively. Random samples of serum from 2 other dogs on the stock diet were similarly examined.

The *unfractionated sera* from Dogs A, B, C, D, S, and R also were titered against sera from rabbits injected with unfractionated sera from Dogs A, B, C, and D respectively. Other controls included precipitin tests with sera of normal rabbits against the unfractionated sera of Dogs A, B, C, D, S, and R. Both normal rabbit sera and a_1 and a_2 immune rabbit sera also were tested against a saturated solution of cholesterol in 0.9% sodium chloride solution, and cholesterol added in excess to normal dog serum.

Total serum cholesterol was determined by the Sperry-Schoenheimer method in Dogs A, B, C, and D at intervals while they were on the experimental diets. The serum of a 77-year-old man with diabetes mellitus and arteriosclerotic heart disease, as well as the sera of 3 other men, aged 26, 27, and 53, in whom no vascular disease had been detected, were tested by precipitin technic against immune rabbit sera a_1 and a_2. *Nitrogen* was determined (micro-Kjeldahl) in order to standardize the ultracentrifugal lipoprotein fraction used as antigen. After determining optimal proportions and equivalence zones by precipitin tests using the sera of Dogs S and R against the immune rabbit sera a_1 and a_2, nitrogen determinations were made on the precipitates.

Results. 1) The concentrations of S_f 2-30 lipoproteins in Dogs A and S on the cholesterol-thiouracil atherogenic regimen were consistently elevated above the concentrations found for Dogs B, C, D, and R; the precipitin titers were of the order of 1:100 or greater in Dogs A and S, usually ranging in the vicinity of 1:500. These titers occurred in the samples shown in Table I as having an S_f 2-30 lipoprotein level varying between 112 and 176 mg %. These lipoproteins are represented by 3 peaks, S_f 2-7, found in all dogs studied, and S_f 8-11 and S_f 13-17, found only in dogs on atherogenic dietary regimens.

2) The concentrations of S_f 2-30 lipoproteins in dogs on non-atherogenic diets: Dog B (on cottonseed oil and thiouracil), Dog C (on thiouracil), and Dogs D and R (on the stock control diet), were consistently low; the precipitin titers were 1:100 or less. These titers occurred with S_f 2-30 lipoprotein levels varying between 8 and 51 mg % as shown in Table I. Similar low titers were found when random samples of sera from two other normal dogs on stock diets were tested. This lipoprotein is represented only by the S_f 2-7 peak found in all dogs studied.

TABLE I. Comparison of Lipoprotein Precipitin Titers and S_f 2-30 Lipoprotein Concentrations in Dog Sera.

Date samples drawn	Titer/lipo. Dog A	Dog B	Dog C	Dog D	Dog R	Dog S
7/14	1:500	1:10	1:10	1:100	1:10 8	1:500 112
14	1: 80	1:20	1:20	1: 80	1:40	1:640
21	1:100 172	1:10	1:10	1: 10 17	1:10 11	1:500 123
28	1:500 171			1:100	1:10	1:500 126
8/ 4	1:320 125	20	29	10	1:40	1:400
11	1:160	1:20	1:40	1: 40	1:20 10	1:640 142
11	1:320	1:40	1:40	1: 80	1:40	1:640
11	1:640 A_f	1:80 B_f	1:80 C_f	1: 80 D_f	1:80 R_f	1:640 S_f
18	1:640 145	1:40 36	1:80 32	1: 80 11	1:20	1:640
18	1:640 A_f	1:80 B_f	1:80 C_f	1: 80 D_f		
21				10		
25		1:80 31	1:80 51		1:40 11	1:320 176
27			44			

Dog A—Cholesterol-thiouracil + stock diet. Dog B—Cottonseed oil-thiouracil + stock diet. Dog C—Thiouracil + stock diet. Dog D—Stock diet. Dog R—Stock diet. Dog S—Cholesterol-thiouracil + stock diet.

A_f, B_f, C_f, D_f, R_f, and S_f are the ultracentrifugal lipoprotein fractions S_f 2-30 from the respective dog sera used as antigen in precipitin tests.

Lipoprotein concentration expressed in mg %.

3) *Precipitin tests* using the unfractionated sera of Dogs A, B, C, D, S, and R against sera from rabbits previously injected with unfractionated sera from Dogs A, B, C, and D, respectively, showed no significant differences in titer.

4) Sera of normal rabbits, when tested against the unfractionated sera of Dogs A, B, C, D, S, and R produced no precipitate, even with dilutions of dog serum as low as 1:5.

5) Neither normal rabbit sera, nor a_1 and a_2 immune rabbit sera, when tested against a saturated solution of cholesterol in 0.9% sodium chloride solution, or against cholesterol added in excess to normal dog serum produced any precipitate, even with dilutions of cholesterol solution as low as 1:10.

6) Total serum cholesterol concentrations, determined at intervals by the Sperry-Schoenheimer method in Dogs A, B, C, and D while on the experimental diet, were not related to precipitin titers or to lipoprotein concentrations.

7) Immune rabbit sera a_1 and a_2, when tested against the serum of a 77-year-old man with diabetes mellitus and arteriosclerotic heart disease, gave precipitin titers of 1:500, whereas the sera of 3 other men, 26, 27, and 53 years old, respectively, when tested in the same manner, produced no reactions, even with a dilution of 1:10.

8) Nitrogen determinations demonstrated a concentration of antigenic protein nitrogen in the serum of Dog S of 3750 μg of nitrogen per ml which was 7 times the concentration found in the serum of Dog R (550 micrograms of nitrogen per ml).

Discussion. The results presented here confirm and extend those previously reported (1) and describe a simple serological method for differentiating dogs on cholesterol-thiouracil atherogenic diets from other dogs by detecting their abnormally increased serum lipoprotein concentrations. Studies now in progress have given additional confirmation to the work discussed here. Since the rabbits were specifically immunized against the ultracentrifugally prepared fraction of dog serum which was photographically defined as containing a high concentration of lipoproteins S_f 2-30; it is presumed that the immune sera produced in these rabbits were a response to the antigenic properties of these lipoproteins. No better way of purifying and isolating these lipoprotein fractions from dog serum has yet been described.

No attempt has been made in this paper to quantitate the precipitin titers obtained with either total or abnormal lipoprotein concentration, since finite quantities of albumin in the S_f 2-30 fraction used as antigen may influence the titer to some degree. Further studies are

now in progress to quantitate this serological test.

Summary. By immunizing rabbits with a lipoprotein fraction obtained from the serum of a dog on a cholesterol-thiouracil atherogenic diet, it has been possible to produce immune sera which will detect the abnormally increased concentration of serum lipoproteins in dogs on cholesterol-thiouracil atherogenic diets and which will react with the serum from an atherosclerotic man. Sera of normal dogs tested with this immune serum by the precipitin technic, consistently show a lipoprotein concentration significantly less than that observed in the case of dogs on the atherogenic dietary regimen. Sera from three men in whom no vascular disease had been detected, did not react with the immune rabbit sera.

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In vitro Studies with 1-hydroxy-2(1H)pyridinethione. (20041)

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Shaw *et al.*(1) in their work on analogs of aspergillic acid reported the preparation and properties of 1-hydroxy-2(1H)pyridinethione, the sodium salt of which is designated MC 3277.* Because of its striking activity against a very wide group of microorganisms further studies were undertaken. The present report will discuss certain antibacterial and antifungal characteristics of this compound.

Assay of MC 3277. A turbidimetric tube assay has been developed using as an assay organism, *Saccharomyces cerevisiae* Squibb strain, and Difco penassay broth modified to contain 1% dextrose concentration as a test medium. Otherwise the assay procedure of Donovick *et al.*(3) is used unchanged. Statistical analysis† of data obtained with the assay shows that for a single assay on any one day, the assay will be within 8.5% of the true value 95% of the time.

In vitro spectrum. A. *Antibacterial.* The sensitivities of a group of microorganisms to MC 3277 were determined as follows: A 2-fold dilution procedure was used with incubation for 17 hours at 37°C for most organisms. Unless otherwise stated, Difco penassay broth was used as a test medium. Cultures in the logarithmic phase of growth with approximately 1,000 organisms per ml were used where possible. The amount of MC 3277 required to inhibit completely the growth of the organism as measured by an absence of turbidity is reported as the Minimal Inhibiting Concentration (M.I.C.). In Table I are listed the M.I.C.'s for a representative group of microorganisms. The M.I.C.'s range from 0.006 μg/ml for *M. tuberculosis* var. *bovis* Strain BCG to 4.0 μg/ml for *Pseudomonas aeruginosa.* The gram-positive organisms are most sensitive but the range of activity for the gram-negative group, from 1.5 μg/ml for *Klebsiella pneumoniae* and *Salmonella typhosa* to 4.0 μg/ml for *Ps. aeruginosa* is of a high order.

B. *Antifungal.* The action of MC 3277 against a group of molds, yeasts and yeast-like fungi was determined by the streak-plate method using a malt-yeast extracts dextrose agar. In Table II are reported the M.I.C.'s found for these fungi. The activity

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