Suppression of Experimental Atherosclerosis in Dogs by Dietary Linoleic Acid.* (31900)

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The induction of atherosclerosis in rabbits by diets deficient in essential fatty acids and devoid of cholesterol(1), focused attention on essential fatty acid metabolism in atherogenesis(2). It has been shown that both cholesterol and saturated fat increase the requirement for essential fatty acids in the rat(3,4), which suggests an antagonism between dietary cholesterol and essential fatty acids.

Using the method of Steiner and Kendall (5) we have induced atherosclerosis in dogs in 4 months by feeding cholesterol and thiouracil simultaneously(6). These two agents were added to a diet in which linoleic acid comprised 4% of total calories, a level about 4 times greater than the minimum daily requirement for the rat(3). The proportion of cholesteryl linoleate and cholesteryl arachidonate in the serum cholesterol esters decreased markedly on this diet.

The purpose of this investigation was to determine the effects of diets deficient in linoleic acid and diets rich in linoleic acid upon serum total cholesterol and upon the lesions formed in dogs fed cholesterol and thiouracil for a period of 4 months.

Materials and methods. The synthetic basal diet used in this investigation is shown in Table I. For diets deficient in linoleic acid, commercial natural butter was used as the fat source. Diets high in linoleic acid contained corn oil (Distillation Products Industries) from which most of the plant sterols had been removed by molecular distillation. Linoleic acid comprised less than 0.5% total calories in the butter diets and 22% total calories in the corn oil diets as determined by gas-liquid chromatography. Arachidonic acid was not detected in the diets. The basal diet contained 4.67 cal/g and all dogs were fed at the rate of 60 cal/kg/day. Dogs fed cholesterol were given 0.5 g/kg body wt/day, and dogs fed thiouracil were given 200 mg/kg body wt/day, mixed in their basal diets.

TABLE I. Synthetic Basal Diet.

	% Total diet calories
Casein*	40
Sucrose	20
Fat (see text)	40
	g/100 g diet mixture
Vitamin mixture†	2.0
Hegsted salt mixture*	4.0
"alpha-cell"*	4.0

* Nutritional Biochemicals Corp.
† Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corp.

Sexually mature mongrel dogs of both sexes were used. A total of 18 dogs were divided into 5 groups and fed the diets shown in Table II.

Serum samples were obtained prior to feeding the synthetic diets but after the dogs had been maintained for at least 3 weeks on a commercial dog chow. The synthetic diets were then started and fed for 120 consecutive days. Fasting serum samples were obtained at 60 and 120 days. Immediately after obtaining the final serum sample, the dogs were killed by overdoses of pentobarbital-sodium and complete autopsies were performed.

Serum total cholesterol was determined by the method of Abell, Levy, Brodie & Kendall (7). Serum total lipids were extracted by a modification of the method of Folch *et al*(8) and the serum total lipids were determined by drying a known fraction of the extracts to constant weight at 100°C. The cholesterol ester fractions of the serum total lipids were separated on silica gel-rhodamine 6G coated thin layer chromatographic plates utilizing a solvent system of petroleum ether : diethyl ether : acetic acid (85:15:1 v/v/v). Areas of silica gel containing the cholesterol esters were visualized under ultraviolet light and these were scraped off into screw cap culture

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TABLE II. Serum Total Cholesterol During Feeding Period (Means and Standard Errors).

Group No.	No. of dogs	Fat source	Cholesterol	Thiouracil	Total cholesterol 0 days	60 days	120 days
I	4	Butter			140 ± 23.8	217 ± 17.2†	281 ± 28.8†
II	2	"	X		125 ± 10.0	318 ± 22.5†	304 ± 11.6†
III	4	"	X	X	160 ± 50.2	2990 ±1730 *	3000 ±1040 †
IV	4	Corn oil			164 ± 15.5	202 ± 48.3	215 ± 61.0
V	4	" "	X	X	130 ± 24.2	1413 ± 71.5†	2588 ± 342 †

* Difference between mean and that of the control significant to $p < .05$.
† Difference between mean and that of the control significant to $p < .02$.

tubes. The cholesterol ester fatty acids in the scraped silica gel particles were transesterified by using 6% conc. sulfuric acid in absolute methanol for 15 hours at 80°C. The methyl esters were extracted with hexane and the composition of the mixtures determined by isothermal gas-liquid chromatography (Barber Coleman Model 10 Gas Chromatograph). The columns were 9 feet long × 3 mm I.D. packed with 15% diethylene glycol succinate on 100-140 mesh Gas-chrom Z (Applied Science Laboratories). Column temperature was 180°C.

Means and standard errors were calculated from the data for each group. Values of t were calculated from the difference of the means and the standard errors and the P values determined from Fisher and Yates(9).

Results. The weights of all dogs at autopsy were within 5% of their pre-experiment weights.

Table II presents the results of the serum total cholesterol determinations. Serum total cholesterol levels of dogs fed the butter diet (Group 1) increased 100%, whereas that of dogs fed corn oil (Group 4) increased less than 50%. Addition of cholesterol to the butter diets (Group 2) caused an increase in serum total cholesterol levels only slightly higher than that of Group 1, but the maximum levels were reached much sooner. Serum total cholesterol levels of dogs fed the butter diet containing both thiouracil and cholesterol (Group 3) increased to very high levels within the first 2 months, then remained essentially constant during the remainder of the feeding period. The serum total cholesterol levels of dogs fed the corn oil diet containing both cholesterol and thiouracil (Group 5) increased more slowly, being only half that of Group 3 at 2 months and approximating that of Group 3 only after 4 months of feeding.

In all groups, the serum total lipid levels paralleled the serum total cholesterol levels.

At autopsy, all dogs fed the basal diet containing butter, butter and cholesterol, or corn oil (Groups 1, 2, & 4) were completely free of atherosclerotic lesions of the aorta. Those fed the basal diet containing butter, cholesterol, and thiouracil (Group 3) all had extensive and severe atherosclerotic lesions of the aorta and smaller arteries. Three of those fed the basal diet containing corn oil, cholesterol, and thiouracil were free of lesions of the aorta and the fourth dog had a small atherosclerotic lesion involving about one percent of the total surface of its aorta.

Table III presents the proportion of esterified serum cholesterol bound to linoleic and arachidonic acids. In all dogs fed butter diets, there was a significant decrease in the proportion of cholesterol esterified to linoleic acid, whereas the corn oil diets caused a significant increase. Both the butter and corn oil diets had a tendency to decrease the proportion of cholesterol bound to arachidonic acid.

Discussion. The less prevalent, less severe, and less extensive lesions in dogs of Group 5 (compared to Group 3) correlate with their slower rate of increase of serum total cholesterol (and total lipid) levels. The serum total cholesterol levels of Group 3 dogs reached maximal levels in less than 2 months and these dogs were exposed to cholesterol levels in excess of 2500 mg/100 ml serum for at least 2 months. The dogs in Group 5 were exposed to similar cholesterol levels for only a brief period of perhaps one or two weeks at

TABLE III. Essential Fatty Acids in Serum Cholesterol Esters (Means and Standard Errors).

Group	0			60			120		
	18:2*	20:4†	Total	18:2	20:4	Total	18:2	20:4	Total
I	44 ± 4.1	7 ± 2.1	51	27 ± 4.9§	4 ± 2.9	31	26 ± 3.5§	6 ± 1.9	32
II	50 ± 1.8	13 ± 1.3	63	28 ± 1.4§	4 ± 1.0‡	32	21 ± 7.8	3 ± 3.2	24
III	45 ± 6.0	6 ± 3.1	51	23 ± 2.9§	3 ± 1.1	26	17 ± 1.9§	4 ± 2.5	21
IV	50 ± 2.5	13 ± 3.9	66	62 ± 7.4‡	9 ± 1.2	71	63 ± 6.3‡	12 ± 1.3	75
V	46 ± 3.8	15 ± 2.3	61	65 ± 5.0§	5 ± 3.1§	70	70 ± 4.2§	5 ± 4.2‡	75

* 18:2 = linoleic acid (% of total cholesterol ester fatty acids).
† 20:4 = arachidonic acid (% of total cholesterol ester fatty acids).
‡ Difference between mean and that of the control significant to $p < .05$.
§ Difference between mean and that of the control significant to $p < .02$.

the end of the feeding period. Previous experience has shown(6) that atherosclerotic lesions develop in dogs only after exposure of the arteries to high serum cholesterol levels for an appreciable period of time. The rate of rise of serum total cholesterol levels in dogs of Group 5 strongly suggests that the prevalence, extent, and severity of lesions in this group would have approached that seen in Group 3 dogs had the experiment been extended to 6 or 7 months. Therefore, the diet rich in linoleic acid appears to have retarded the rate of rise of serum cholesterol in dogs of Group 5, although it did not completely overcome the accumulation of serum lipids resulting from thyroid inhibition and dietary cholesterol.

The butter diet contained an appreciable amount of short chain fatty acids whereas the corn oil diet did not. The severe lesions found in dogs fed butter (Group 3) are not attributed to the dietary short chain fatty acids because in earlier experiments(6) lesions of equal prevalence, extent, and severity were induced in dogs fed diets containing a moderate quantity of linoleic acid (4% total calories) and no detectable quantity of short chain fatty acids.

The mechanism by which dietary linoleic acid retarded the rise of serum total cholesterol in dogs in Group 5 appears to involve the composition of the serum cholesterol esters. At least two-thirds of the serum esterified cholesterol of dogs fed corn oil diets was bound to linoleic or arachidonic acids. Less than one-third of the serum esterified cholesterol was bound to these acids in dogs fed the butter diet. Pinter, Hamilton, and Miller(10,11) have demonstrated that cholesterol esterified to linoleic and arachidonic acids is excreted from rat serum at a higher rate than that esterified to other fatty acids. The retardation of the rise of serum total cholesterol by dietary linoleic acid in this experiment suggests that this same mechanism is also operative in the dog.

Summary. Severe atherosclerotic lesions were induced in 4 months in dogs fed a diet restricted in linoleic acid content and supplemented with cholesterol and thiouracil. Only one minor lesion was found in a group of dogs fed a similar diet supplemented with cholesterol and thiouracil which was isocaloric in fat but contained a high concentration of linoleic acid. In the low linoleic acid group, serum total cholesterol increased to high levels within 2 months and remained constant for the remainder of the feeding period. In the high linoleic acid group, serum total cholesterol levels increased more slowly reaching levels compatible with the group fed low linoleic acid diets at the end of 4 months. Less than 25% of the serum esterified cholesterol was bound to linoleic and arachidonic acids in dogs fed diets low in linoleic acid, whereas this value was 75% in dogs fed high linoleic acid diets. It is concluded that dietary linoleic acid retards lesions in dogs by promoting cholesterol excretion.

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Effects of Multiplicity of Poliovirus Infection of HeLa Cells Upon Regulation of DNA Synthesis.* (31901)

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Infection of HeLa cells with poliovirus can either stimulate or inhibit the rate of DNA synthesis(1,2). The effects of virus are determined in part by environmental factors (2), in part by an intrinsic programming of viral genetic information(3), and in part by the multiplicity of infection. These are interrelated variables.

It has been suggested that poliovirus inhibition of DNA synthesis is a two-part process: First, a development, after the third hour of infection, of a potential for inhibition (possibly a messenger RNA) and second, the expression of this potential in the form of protein synthesis. The latter is sensitive to the concentration of certain amino acids in the medium whereas the former is not(3). Thus the amino acid composition of the culture medium which affects the rate of DNA synthesis in the uninfected cell(2), influences as well the viral effects upon this synthesis in infected cells. The concentrations of arginine or histidine in the medium required for viral multiplication, for viral inhibition of DNA synthesis and for normal rates of DNA synthesis in uninfected cells, increase in the order listed(2).

This paper is concerned with how the multiplicity of infection interrelates with the stage of infection and amino acid composition of the culture medium in the regulation of DNA synthesis in poliovirus infected HeLa cells.

Materials and methods. Cells. HeLa cells were grown in monolayers at 37°C with Eagle's basal medium(4), twice concentrated, and 10% calf serum and passaged every 7 days. The cells were grown in Roux bottles for experiments which involved isolation of DNA for chemical analysis and in 2 ounce prescription bottles for studies of viral replication. During the experimental period, the medium was replaced with one modified with regard to serum and amino acid content as described in specific experiments. Periodically, cells and fluid were inoculated into special media to ensure they were free of mycoplasma and bacteria(5).

Virus. The Mahoney strain of type 1 poliovirus was passaged routinely in HeLa cells, washed and concentrated by centrifugation and used as a suspension in phosphate buffered saline. The virus was assayed, using a plaque assay, on monolayers of HeLa cells in 2 ounce prescription bottles and concentration expressed in plaque forming units.

Deoxyribonucleic acid. DNA was extracted by the method of Colter and associates(6), isolated as described by Martinez-Segovia *et al*(7), and quantitatively determined by the method of Burton(8).

Radioactivity. For measurement of radioactivity, 0.1 to 0.5 ml of sample was added

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