and fully sensitive to calcium-induced cell death. The age at which the heart becomes fully sensitive to calcium-induced cell death may be extended beyond 15 days by decreasing the time of calcium-free perfusion, but we think that maximum sensitivity would still be expressed at 3 to 4 weeks of age. At this age, the structural and functional complexity of the surface membrane has matured, plus the calcium binding and release properties of the sarcoplasmic reticulum and the calcium sensitivity of the myofilaments have both reached their adult levels (*3*).

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Atherosclerosis: Prevention by Agents Not Affecting Abnormal Levels of Blood Lipids

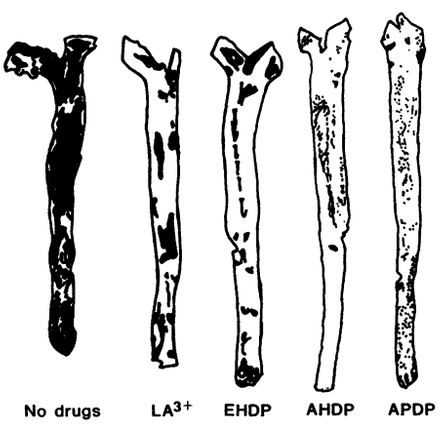
Abstract. *Diet-induced atherosclerosis in macaque monkeys was suppressed by anticalcifying agents without changing abnormal levels of blood cholesterol and lipoprotein. The agents included inhibitors of arterial calcium deposition (diphosphonates) and a calcium ion antagonist (lanthanum). The study suggests that regulation of calcium flux and extracellular deposition in arteries may offer new principles of treatment for cardiovascular disease.*

Cardiovascular disease is still the leading cause of death in industrial nations (*1*). The principal underlying disorder is atherosclerosis (*1*), especially the fibrotic atheromatous plaque (*2*). Studies involving the use of low-fat diets (*1*) or antilipemic drugs (*3*) have not provided convincing evidence that lowering of blood cholesterol concentrations prevents heart disease or protects against atherosclerosis (*4*). It therefore appears desirable to search for alternative methods of treatment.

Recent studies on rabbits (*5*) indicate that agents which inhibit excessive deposition of calcium into arterial walls also inhibit diet-induced atherosclerosis despite high levels of serum cholesterol. The agents include the anticalcifying agents ethane-1-hydroxy-1,1-diphosphonate (EHDP), azacycloheptane-2,2-diphosphonate (AHDP), amino-1-hydroxypropane-1,1-diphosphonate (APDP), and the specific calcium ion antagonist lanthanum (La^{3+}). In the present study these substances were tested for their ability to suppress atherosclerotic plaque formation in monkeys.

Adult male *Macaca fascicularis* monkeys were randomly assigned to one of four groups (eight monkeys per group). One group was placed on a normal diet and three groups on an atherogenic diet containing (by weight) 10 percent butter and 0.1 percent cholesterol. One of the latter three groups received the high-fat diet alone; the other two also received either $LaCl_3$ or EHDP. Two additional groups of three monkeys each received the same atherogenic diet with added AHDP or APDP. Daily dosages were as follows: EHDP and $LaCl_3$, 120 mg/kg for 6 months and 40 mg/kg thereafter; AHDP and APDP, 40 mg/kg. Blood samples were taken bimonthly and analyzed for total cholesterol in plasma, high-density lipoprotein, low-density plus very low density lipoprotein, and total and ionized calcium in serum (*5*). The various cholesterol lipoprotein fractions were separated by magnesium phosphotungstate precipitation (*6*). After 24 months the monkeys were killed by an overdose of pentobarbital. At autopsy, the aortas were overlaid with clear plastic foil, the aortic contours and lesions were outlined in ink, and the percentage of intima affected by atherosclerosis was determined by point counting (*7*). Cross sections were then removed from the left and right proximal coronary arteries and the opened aortas for histological examination. Whole aortic intima and media were analyzed biochemically for the content of collagen, elastin, cholesterol, and calcium by methods previously described (*5*). DNA content was measured by the method of Burton (*8*).

In monkeys on the atherogenic diet the concentration of total cholesterol in plasma rose from the control level (132 ± 43 mg/dl, mean ± standard deviation) to atherogenic levels regardless of whether or not anticalcifying drugs were given: for untreated monkeys 468 ± 134 mg/dl; for monkeys treated with La^{3+}, 425 ± 123 mg/dl; with EHDP, 434 ± 125 mg/dl; with AHDP, 448 ± 135 mg/dl; and with APDP, 511 ± 208 mg/dl. These increases were due to increases in low-density plus very low density lipoprotein. The mean concentration of high-density lipoprotein in each of the experimental groups was not significantly different from the control value (52 ± 15 mg/dl). Likewise, concentrations of total and ionized calcium in serum were about



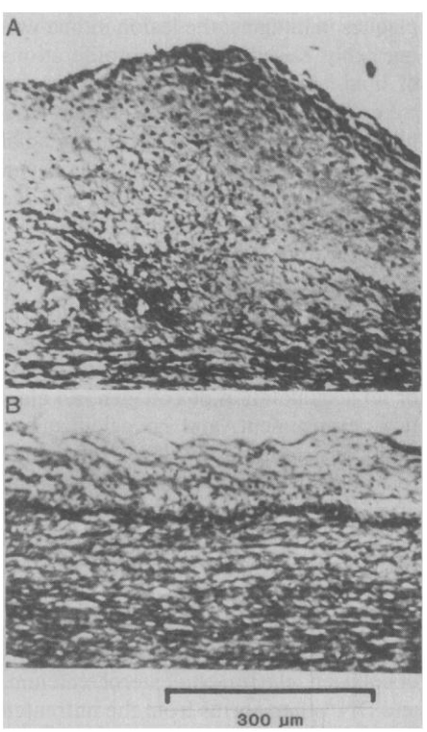


Fig. 1 (left). Tracings of aortas from monkeys that received the atherogenic diet with or without one of several anticalcifying agents. Black areas represent intimal atherosclerotic lesions. Fig. 2 (right). Micrographs of atherosclerotic lesions. (A) Plaque from aorta of untreated monkey on atherogenic diet. The intima is markedly raised (top half of micrograph) by proliferated lipid-laden foam cells (large clear cells) surrounded by a capsule of collagen accumulations (gray area). There is destruction of intimomedial elastica (black bands), and collagen and elastica changes reach into the inner aortic media (bottom half). (B) Aortic lesion from La^{3+}-treated monkey on atherogenic diet. The intima is only slightly raised by a few layers of foam cells; there is no collagen accumulation or elastica derangement in the intima or media. Aortic lesions from monkeys treated with the other anticalcifying agents showed similar morphology. Some were even more superficial.

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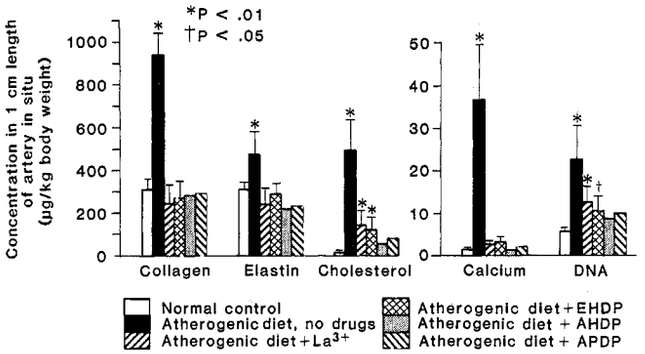

Fig. 3. Biochemical composition of intimal and medial layers of aorta from monkeys in the various groups.

the same in all groups on the atherogenic diet.

Figure 1 shows tracings of one representative whole aorta from each group. There was remarkable suppression of atherosclerotic lesion formation in all drug-treated animals. The percentage of intimal surface affected by lesions was 75 ± 22 in untreated animals, 21 ± 10 in animals given La^{3+}, 14 ± 7 (EHDP), 12 ± 8 (AHDP), and 18 ± 11 (APDP). Between-group differences are significant at $P < .001$ (one-way analysis of variance), and each treated group differs from the untreated group at $P < .01$ (Dunnet's *t*-test).

Microscopic examination revealed that atherosclerotic plaques in the untreated monkeys (Fig. 2A) had several features in common with certain fibrous plaques in humans: the lesion intima was markedly raised by large proliferations of lipid-laden "foam" cells surrounded by collagen accumulations, including a fibrous cap; destruction of intimomedial elastica was extensive, with the collagen and elastin alterations reaching into the inner arterial media. Some of these lesions showed calcification. By contrast, in monkeys treated with anticalcifying agents the few lesions were quite superficial (Fig. 2B) and more akin to fatty streaks in human arteries. Showing little or no accumulation of collagen, no elastica derangement, and no calcification, they consisted of a few layers of intimal foam cells overlying an otherwise normal arterial wall. The characteristics of coronary artery lesions from untreated and treated monkeys were similar to those of their aortic counterparts.

Aortas from the drug-treated monkeys (Fig. 3) contained much smaller amounts of collagen, elastin, cholesterol, calcium, and DNA than aortas from the untreated animals on the same atherogenic diet. In treated monkeys the aortic content of collagen, elastin, and calcium was not significantly different from that of monkeys on the normal diet. The aortic cholesterol content in drug-treated animals was significantly higher than in monkeys on a normal diet, but significantly lower than in untreated monkeys on an atherogenic diet. The slightly increased number of intimal foam cells in the treated animals may have accounted for the nominal increases in aortic cholesterol and DNA.

Food intake, digestion, body weight, and the general well-being of the monkeys did not appear to be adversely affected by any of the drugs. Blood pressure, cardiac output, blood glucose, hemoglobin, hematocrit, complete blood count, and clotting time were within normal limits in all animals, as were bone density and the calcium, phosphorus, and collagen content of skeletal muscle and bone. At this writing, some of our lanthanum-treated monkeys have been exercising vigorously in a treadmill for $2\frac{1}{2}$ years.

The inhibitory effects of these drugs are not entirely surprising in view of the present understanding of atherogenic processes (*9*), which include an increase in endothelial permeability, migration of arterial smooth muscle cells and macrophages into the intima, intimal cell proliferation, and connective tissue secretion and lipoprotein endocytosis. Most of these processes require calcium-dependent energy from high-energy phosphates. In addition, as reviewed elsewhere (*5*), Ca^{2+} is required for the contraction-relaxation cycle in smooth muscle cells and endothelium; directional cell movement, as in chemotaxis; mitosis; secretion of proteins, including collagen (*10*) and presumably elastin; binding of lipoproteins to cell membrane receptors (*11*); and adhesion of platelets to arterial connective tissue, their aggregation, and their release. Focal increases in the concentration of arterial Ca^{2+} may facilitate these cellular responses to atherogenic stimuli. This may explain the inhibition of excessive cell responses by agents that inhibit the deposition of excess calcium. Although the mechanism by which diphosphonates act on cells is not known, they may exert part of their inhibitory effect in a manner similar to that of La^{3+}, which displaces and replaces Ca^{2+} on selected cell membrane loci, blocking the channels for influx and efflux of Ca^{2+} (*12*).

Elastin destruction and lesion calcification were also suppressed by the anticalcifying agents. Mechanisms that require or are influenced by calcium include elastolysis by macrophage elastase (*13*); increased adsorption of cholesterol by elastin due to Ca^{2+}-induced changes in elastin configuration (*14*); complexing of lipoproteins to glycosaminoglycans (*15*); and calcium mineralization of connective tissue. The effects of the anticalcifying agents used in the present study support the concept that arterial tissue calcium plays a key role in atherogenesis and that inhibition of calcium flux and deposition is essential to its prevention. This study and the previous studies on rabbits (*5*) suggest that agents capable of regulating calcium flux and extracellular accumulation in arteries may prevent atherosclerosis notwithstanding abnormal conditions in the bloodstream, such as hypercholesterolemia and unfavorable concentrations of lipoprotein.

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