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# Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits

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**1** Probucol was administered to rabbits fed a cholesterol-enriched (2% wt/wt) diet to determine potential anti-atherogenic effects in a preparation in which the disease process is due to elevated plasma concentrations of cholesterol ester-rich very low density lipoproteins (CER-VLDL).

**2** Probucol was supplemented to the diet at 1% wt/wt which resulted in plasma concentrations rising steadily to $53 \pm 8\ \mu g\ ml^{-1}$ after 14 days, with no significant changes during continued administration. Dietary consumption and body weight gains were comparable in the drug-treated and control groups during the observation period.

**3** Probucol treatment did not significantly affect plasma concentrations of total cholesterol, unesterified cholesterol, triglycerides or phospholipids.

**4** The concentration of CER-VLDL in plasma and its physicochemical characteristics were not significantly changed during administration of probucol. CER-VLDL from both control and probucol-treated animals was a potent stimulant of the augmentation of the intracellular incorporation of [$^3$H]-oleate into cholesteryl-[$^3$H]-oleate in cultured macrophages.

**5** Despite the lack of effect of probucol on concentrations of plasma lipids and the cell interaction characteristics of CER-VLDL, administration of the drug markedly decreased the extent of intimal aortic surface area covered by grossly discernible atherosclerotic lesions from $55.6 \pm 11.8\%$ to $11.6 \pm 1.9\%$ in thoracic sections, and from $49.1 \pm 10.2\%$ to $7.2 \pm 0.4\%$ in abdominal sections. Furthermore, probucol treatment significantly reduced the deposition of total cholesterol in vascular tissue.

**6** Probucol reduced the extent of aortic atherosclerosis produced by diet-induced hypercholesterolemia in rabbits. This reduction occurred in the absence of any significant change in the characteristics of plasma lipoproteins that were determined. These results indicate that either there is a role of oxidation in the disease process of this animal model of atherosclerosis or that probucol is acting via a presently undefined mechanism.

## Introduction

Probucol (4,4′-(isopropylenedithio)bis(2,6,-di,-butylphenol) is a moderately effective drug for reducing plasma concentrations of low density lipoproteins (LDL) in human subjects through a mechanism that has not been elucidated (Steinberg, 1986). Although probucol reduces plasma concentrations of LDL, the anti-atherogenic properties of this drug have been doubted due to the concomitant reduction in high density lipoprotein (HDL). Despite this caveat, anti-atherogenic properties of probucol have been demonstrated recently following administration of the drug to Watanabe heritable hyperlipidemic (WHHL) rabbits (Kita *et al.*, 1987). WHHL rabbits have elevated plasma concentrations of LDL and are thought to simulate the disease process of familial hypercholesterolemia. It has been hypothesized that the anti-atherogenic properties of probucol in WHHL rabbits may be independent of the mild reduction of plasma concentrations of cholesterol and, instead, related to the anti-oxidant properties of the drug (Carew *et al.*, 1987).

To further the understanding of the mechanism of the anti-atherogenic properties of probucol, in the present study the effects of the drug were characterized in rabbits fed cholesterol-enriched diets. In contrast to WHHL rabbits, rabbits fed a cholesterol-enriched diet transport excessive concentrations of cholesterol in cholesterol ester-rich very

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low density lipoproteins (CER-VLDL; Daugherty *et al.*, 1985). CER-VLDL is thought to be a highly atherogenic lipoprotein, that acts through promotion of uncontrolled cholesterol esterification in monocyte-derived macrophages that have been recruited to the arterial wall (Mahley, 1982). The results of this study demonstrate that administration of probucol produced a profound reduction in the extent of diet-induced atherosclerosis in the absence of any significant effects on plasma concentrations of lipids or the observed characteristics of CER-VLDL. A preliminary communication of these results has been presented (Daugherty *et al.*, 1988a).

## Methods

### *Animals*

New Zealand rabbits (2 to 3 kg body weight) were obtained from the Boswell Rabbit Farm (Pacific, MO). Animals were initially fed a standard laboratory diet (Ralston Purina, St. Louis, MO) for at least 7 days after delivery to Washington University School of Medicine. Diet and water were available *ad libitum*. All protocols concerning animals were approved by the Washington University Committee for the Humane Care of Laboratory Animals.

### *Diet and drug regimens*

Diet enriched in cholesterol (2% wt/wt) was obtained from Ralston Purina Test Diets (Richmond, IN). Probucol (a gift from Merrell Dow Research Institute, Cincinnati, OH) was dissolved in chloroform and sprayed evenly over the diet in the proportion of 1% wt/wt. Diet for the control group was sprayed with chloroform alone. Treated diets were kept under a fume hood until the odour of the solvent had dissipated. Food intake was monitored daily for both control and drug-treated groups.

### *Isolation of CER-VLDL*

CER-VLDL was isolated from the plasma of cholesterol-fed rabbits by ultracentrifugation as described previously (Daugherty *et al.*, 1987).

### *Characterization of plasma and lipoproteins*

Concentrations of triglycerides, cholesterol esters, unesterified cholesterol and phospholipids in plasma and in isolated lipoprotein fractions were determined with commercially available enzyme kits (Wako Chemical Company, Dallas, TX). Concentrations of phospholipids and triglycerides were calculated based on mean molecular weights of 722 and 866 respectively. Mass of protein in lipoprotein fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Pierce Chemical Company, Rockford, IL) as standard. Electrophoretic mobility of lipoproteins was assessed with agarose gels (0.5% wt/vol) stained with Fat Red 0.

Plasma concentrations of probucol were determined by reverse phase high performance liquid chromatography by the method of Satonin & Coutant (1986). Concentrations of probucol in plasma were determined relative to an internal standard supplied by the Merrell Dow Research Institute (MDL 27,272).

### *Determination of cholesteryl-[$^3H$]-oleate deposition in cultured macrophages*

Pentobarbitone-anesthetized New Zealand rabbits of either sex were exsanguinated via the abdominal aortae. Saline, containing heparin ($5\,u\,ml^{-1}$), was introduced into the alveoli through tracheal cannulae. Lungs were lavaged five times with 50 ml fluid washes. Cells were plated in Dulbecco's minimum essential medium containing newborn calf serum (20% vol/vol) at a density of $3–5 \times 10^6$ cells per 35 mm well. Cells were incubated overnight at 37°C in a 5% $CO_2$ incubator. Cellular deposition of cholesteryl-[$^3$H]-oleate was determined as described previously (Daugherty *et al.*, 1987).

### *Determination of percentage of intimal area covered by atherosclerotic lesions*

After 60 days of study, animals were killed by an overdose of sodium pentobarbitone ($120\,mg\,kg^{-1}$ administered intravenously). Aortae were rapidly dissected free from the ascending arch to the ileal bifurcation. Extraneous tissue was removed and full length incisions exposed intimal areas. Intimal areas were photographed with a Polaroid camera. The areas of grossly discernible normal and atherosclerotic intima were digitized from these photographs using a Numonics model 2210 tablet (Numonics Corporation, Lansdale, PA) and SigmaScan (Jandel Scientific, Corte Madera, CA) run on a XT computer.

### *Determination of total cholesterol and unesterified cholesterol content of aortae*

Cholesterol esters and unesterified cholesterol content of vascular tissue were determined by gas chromatography as described by Ishikawa *et al.* (1974) with 5-α-cholestane as a standard.

*Statistical analyses*

Comparisons were made with Student's $t$ test (two-tailed) performed with Stats-2 (Statsoft, Tulsa, OK). A $P$ value of less than 0.05 was considered significant. Values are presented as means with s.e.means, where applicable.

## Results

Nine rabbits in each group were initiated on the experimental protocol. One death occurred in both the control and drug-treated groups. Both deaths were attributable to intestinal obstruction and not as a result of either diet or drug effects. The acceptance of the diet supplemented with probucol was rapid and no significant differences were observed between the groups in the daily consumption of the diet (Figure 1a). No significant difference in body weights was observed during the experimental period (Figure 1b)

Plasma concentrations of probucol were monitored continuously throughout the study. Plasma concentrations increased progressively during the initial 20 days of administration of the drug. Thereafter, plasma concentrations of probucol were not significantly altered from a value of approximately 55 $\mu g\,ml^{-1}$, although a statistically insignificant trend toward increased concentrations of the drug was observed (Figure 2). All plasma from the probucol-treated rabbits had a distinctive green colour. This colouration was apparently due to the occurrence of an oxidized degradation product of probucol (Barnhart *et al.*, 1989). While no quantification of this colouration was performed, it appeared that a large percentage was present in the lipoproteins of density less than 1.006 $g\,ml^{-1}$. As expected, no probucol was detectable in the plasma of control rabbits.

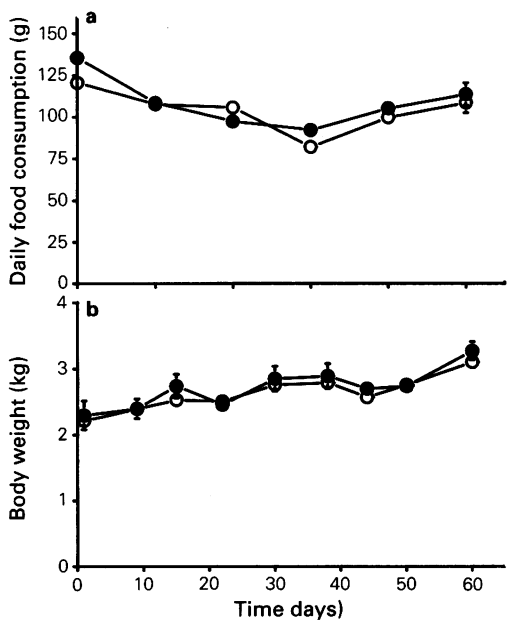


**Figure 1** (a) Daily consumption of cholesterol-rich diet in control (○) and probucol-treated rabbits (●). Values are expressed as the mean daily consumption for each 10 day interval. (b) Body weight determined weekly in the same control (○) and probucol-treated (●) animals ($n$ = 8). Vertical lines show s.e.mean.

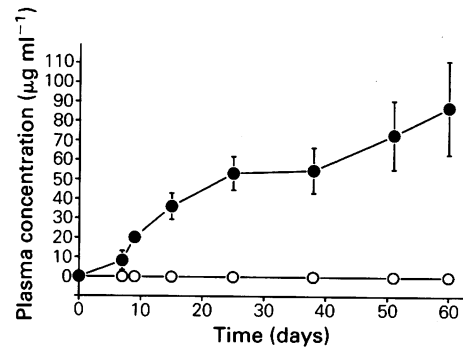


**Figure 2** Plasma concentrations of probucol in control (○) and probucol-treated (●) rabbits during the study ($n$ = 8). Vertical lines show s.e.mean.

Plasma concentrations of total cholesterol, unesterified cholesterol and phospholipids in control rabbits increased progressively for the initial 30 days, followed by no further significant increases as described previously (Daugherty *et al.*, 1988b). The highly variable plasma concentrations of triglycerides was probably due to the non-fasted conditions under which plasma was collected. The cholesterol-enriched diet did not consistently affect the plasma concentrations of trigylcerides. Probucol administration did not significantly affect any of the plasma lipid concentrations. At the end of the study, the respective plasma concentrations of controls compared to probucol-treated animals were $56.6 \pm 8.6$ vs $47.6 \pm 40\,mmol\,l^{-1}$ for total cholesterol, $14.1 \pm 1.2$ vs $13.4 \pm 1.6\,mmol\,l^{-1}$ for unesterified cholesterol, $8.8 \pm 1.3$ vs $8.0 \pm 0.6\,mmol\,l^{-1}$ for phospholipids, and $1.6 \pm 0.4$ vs $3.2 \pm 1.3$ $mmol\,l^{-1}$ for triglycerides (Figure 3). Although there was a trend for plasma concentrations of total and unesterified cholesterol to be reduced during the administration of probucol, none of these values was statistically significant.

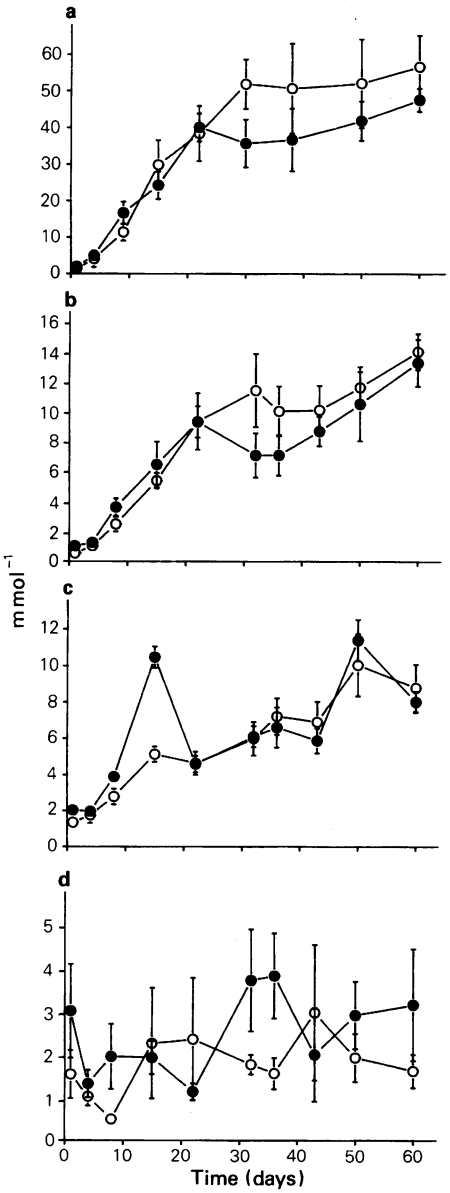


**Figure 3** Plasma concentrations of total cholesterol (a), unesterified cholesterol (b), phospholipids (c), and triglycerides (d) in rabbits fed the cholesterol-enriched diet (2% wt/wt). Controls are represented by (○) and probucol-treated by (●) ($n$ = 5–8). Vertical lines show s.e.mean.

Concentrations of CER-VLDL in plasma were unaltered by probucol treatment. In addition, the chemical composition of CER-VLDL was also unchanged (Figure 4). Furthermore, there was no

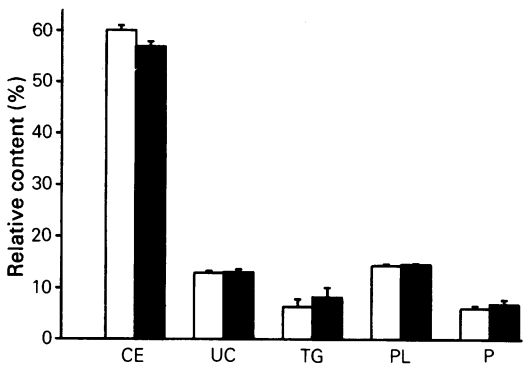


**Figure 4** Chemical composition of cholesterol ester-rich very low density lipoproteins (CER-VLDL) from control (open columns) and probucol-treated (solid columns) animals. CER-VLDL was harvested at the time of excision of the aortae. The relative content of cholesterol esters (CE), unesterified cholesterol (UC), triglycerides (TG), phospholipids (PL), and protein (P) were determined as described in the Methods section ($n$ = 8). Vertical bars show s.e.mean.

change in the electrophoretic mobility of the intact lipoprotein or in the apolipoprotein content of CER-VLDL (data not shown).

It has been observed previously that fractions of CER-VLDL that have similar physicochemical properties can differ dramatically in their ability to augment the incorporation of [$^3$H]-oleate into cholesteryl-[$^3$H]-oleate in cultured macrophages (Daugherty *et al.*, 1988b). This assay is commonly considered as one criterion to quantify the atherogenic potential of specific lipoprotein fractions.

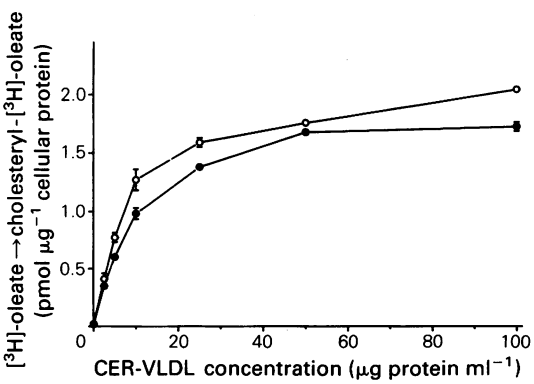


**Figure 5** Ability of cholesterol ester-rich very low density lipoproteins (CER-VLDL) from control aminals (○) and probucol-treated animals (●) to augment the incorporation of [$^3$H]-oleate into cholesteryl-[$^3$H]-oleate of cultured rabbit alveolar macrophages. Points represent the mean of six values from 2 separate assays. Vertical lines show s.e.mean.

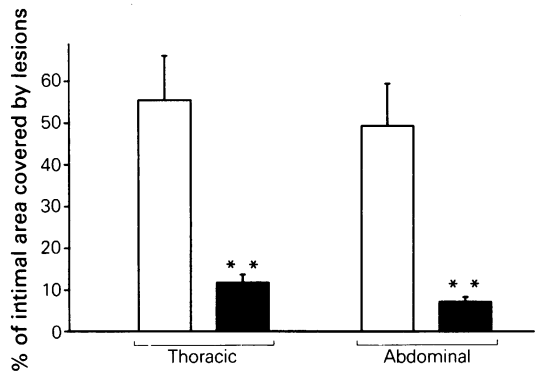


**Figure 6** Percentage of intimal surface area covered by grossly discernable atherosclerotic lesions, as determined by computer-assisted planimetry, in thoracic and abdominal aortae from control (open columns) and probucol-treated (solid columns) rabbits ($n = 8$). Vertical bars show s.e.mean. ** Difference significant at $P < 0.01$.

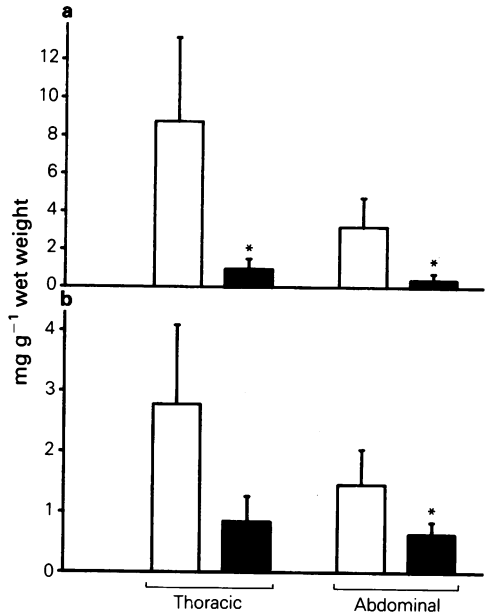


**Figure 7** Total cholesterol (a) and unesterified cholesterol (b) content of thoracic and abdominal aortae of control (open columns) and probucol-treated (solid columns) animals. $n = 8$; * difference significant at $P < 0.05$.

CER-VLDL from both control and probucol-treated animals markedly stimulated the incorporation of [$^3$H]-oleate into cholesteryl-[$^3$H]-oleate in macrophages in culture, with no significant differences between the fractions (Figure 5).

Despite the lack of effect of probucol on concentrations of plasma lipids, administration of the drug profoundly reduced the severity of aortic atherosclerosis. The extent of aortic atherosclerosis was decreased significantly from $55.6 \pm 11.8\%$ to $11.6 \pm 1.9\%$ ($P < 0.01$) of surface area in the thoracic section, and from $49.1 \pm 10.2\%$ to $7.2 \pm 0.4\%$ ($P < 0.01$) in the abdominal section (Figure 6). Total cholesterol in thoracic and abdominal segments were reduced from $8.7 \pm 4.4\,\mu g\,mg^{-1}$ wet weight to $1.0 \pm 0.4$ ($P < 0.05$) and from $3.2 \pm 1.6$ to $0.4 \pm 0.3$ ($P < 0.05$) respectively. Probucol treatment reduced the deposition of unesterified cholesterol in thoracic and abdominal segments from $2.8 \pm 1.3\,\mu g\,mg^{-1}$ wet weight to $0.8 \pm 0.4$ (NS) and from $1.5 \pm 0.6$ to $0.6 \pm 0.2$ ($P < 0.05$) respectively (Figure 7).

Sections of aorta from both groups were subjected to histological analysis. Although the extent of atherosclerosis was markedly reduced by the administration of probucol, the morphology of the lesions that were present appeared similar. In both groups, the lesions consisted of lipid-engorged foam cells which are characteristic in this preparation of dietary-induced atherosclerosis (data not shown).

## Discussion

The purpose of the present study was to define the effects of probucol on the severity of diet-induced aortic atherosclerosis in rabbits. The atherosclerosis produced during the feeding of cholesterol-enriched diets to rabbits is thought to be due to the presence of elevated plasma concentrations of CER-VLDL.

Probucol was administered to rabbits in doses that produced clinically relevant plasma concentrations. However, probucol treatment of rabbits fed a cholesterol-enriched diet did not significantly reduce plasma concentrations of any of the lipids that were determined, in contrast with the hypolipidemic effect of probucol in man. The mechanism of the hypolipidemic effect in man has been attributed to a variety of mechanisms. These include decreased *de novo* cholesterol biosynthesis, increased activity of hepatic LDL receptors, and increased section of bile acids (Steinberg, 1986).

The mechanism of the hyperlipidemia produced during the feeding of cholesterol-enriched diets to rabbits has been attributed to both a reduction in the catabolism (Kovanen *et al.*, 1981) and an increase in synthesis (Daugherty *et al.*, 1986) of CER-VLDL. The elevated concentrations of CER-VLDL in plasma are primarily of hepatic origin (Daugherty *et al.*, 1988b). However, plasma concentrations of CER-VLDL were not influenced by the administration of probucol in rabbits fed cholesterol-enriched diets. In WHHL rabbits, an earlier study

demonstrated the effects of probucol in reducing plasma concentrations of cholesterol. The mechanism of this effect was ascribed to the production of a modified form of LDL that was more rapidly cleared from plasma (Naruszewicz *et al.*, 1984). However, the potency of the hypolipidemic effects of probucol in WHHL rabbits was not confirmed in a more recent study (Carew *et al.*, 1987).

Although probucol reduces plasma concentrations of total and LDL-cholesterol in human subjects, its effect on the atherogenic process has been questioned since the drug also produces a concomitant reduction in plasma concentrations of HDL-cholesterol. Despite this reservation two recent studies have demonstrated that the drug may prevent the progression of atherosclerosis in WHHL rabbits. Probucol was administered to WHHL rabbits within the first two months of parturition and resulted in marked reductions in the severity of grossly discernible atherosclerotic lesions on intimal surfaces of aortae (Kita *et al.*, 1987; Carew *et al.*, 1987). The atherosclerosis that develops in WHHL rabbits is thought to be attributable primarily to the elevated plasma concentrations of LDL.

The anti-atherogenic effects of probucol in WHHL rabbits have been attributed to the anti-oxidant actions of probucol. Indeed, probucol has potent anti-oxidant properties, being a derivative of butylated hydroxytoluene. In addition, probucol is highly soluble in lipid phases and a high concentration of drug will be transported in lipoproteins. The rationale for the involvement of the anti-oxidant activity is also based on the equivalent reductions in plasma concentrations of cholesterol that were produced by both the HMG-CoA reductase inhibitor, lovastatin, and probucol, but only the latter exerted anti-atherogenic actions (Carew *et al.*, 1987). Further support for the role of oxidation of LDL in the atherogenic process in WHHL rabbits comes from recent identification (Haberland *et al.*, 1988) and isolation (Daugherty *et al.*, 1988c) of oxidized lipoprotein products in atherosclerotic aortic tissue. However, in contrast to studies with WHHL rabbits, the present study has demonstrated the anti-atherogenic effects of probucol when administered to rabbits fed cholesterol-enriched diet. At present a role for oxidation has not been established in the initiation and propagation of aortic atherosclerosis in the rabbits fed cholesterol-enriched diets. Instead, under these conditions the development of atherosclerosis is thought to be due to elevated plasma concentrations of CER-VLDL.

The interaction of lipoproteins with macrophages has been implicated as a primary influence on the initiation and progression of atherosclerosis. In the case of WHHL rabbits, it is assumed that the involvement of LDL and its oxidized products are responsible for the transformation of macrophages to foam cells in the subintimal space. In corroboration with *in vivo* findings (Kita *et al.*, 1987; Carew *et al.*, 1987), probucol both reduces the oxidized modification of LDL (Parthasarathy *et al.*, 1986) and prevents the formation of foam cells from macrophages *in vitro* (Yamamoto *et al.*, 1986). This effect of probucol may be due to a reduction in the modification of surface properties of LDL produced by oxidation (McLean & Hagaman, 1989). In contrast, during feeding of cholesterol-enriched diets to rabbits, the evolution of atherosclerosis is generally thought to occur due to the interaction of CER-VLDL with macrophages that have been recruited to the arterial tissue (Fowler *et al.*, 1979; Schaffner *et al.*, 1980). Numerous studies have demonstrated the ability of CER-VLDL to interact with macrophages leading to unregulated intracellular cholesterol esterification and storage with the subsequent transformation into foam cells (Mahley *et al.*, 1980). This property is particularly prominent in the intestinally-derived fraction of CER-VLDL (Daugherty *et al.*, 1988b). However, in the present study probucol did not influence the interaction of CER-VLDL with macrophages in the incorporation of [$^3$H]-oleate into cholesteryl-[$^3$H]-oleate. Probucol was present in high concentrations in CER-VLDL in the plasma and consequently a high concentration of the drug would have been carried in the CER-VLDL that was transported into macrophages.

The marked anti-atherogenic effect of probucol that was demonstrated in the present study is in contradiction to the lack of effect observed by Hallermayer & Schierok (1987). The reason for this discrepancy is not apparent, although plasma concentrations of probucol were not stated in this study and it is possible that sufficiently high concentrations of drug were not attained.

In summary, the present study has demonstrated that the administration of probucol to rabbits fed a cholesterol-enriched diet markedly decreases the extent of aortic atherosclerosis through a mechanism that remains to be elucidated. These anti-atherogenic effects of probucol in cholesterol-fed rabbits may be related to a currently uncharacterized effect on lipid metabolism within arterial tissue. An additional possible anti-atherogenic action of probucol in this model could be to hinder the recruitment of macrophages to arterial tissue, although there are at present no data to support such an effect.

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