Biochemical Pharmacology, Vol. 33, No. 2, pp. 219–228, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HYPERALPHALIPOPROTEINAEMIC ACTIVITY OF BRL 26314—II

INHIBITION OF ATHEROSCLEROSIS IN RABBITS

R. FEARS,* A. ESMAIL, P. WALKER, W. R. RUSH and H. FERRES
Beecham Pharmaceuticals Research Division, Great Burgh, Epsom, Surrey KT18 5XQ, U.K.

(*Received* 6 *May* 1983; *accepted* 18 *August* 1983)

Abstract—Optimization of a combination of balloon catheter-induced aortic de-endothelialization with provision of a palatable atherogenic diet to rabbits leads to hyperbetalipoproteinaemia and atherosclerosis rather than to the cholesterol-storage disease which characterized earlier models. Administration of BRL 26314 [*N*-(4-chlorobenzyl)-L-phenylalanine] during the induction of atherosclerosis specifically raised high-density lipoprotein (HDL) and decreased the arterial content of cholesterol and collagen in association with reduction in severity of thoracic sudanophilic lesions and intimal-thickening. This anti-atherosclerotic activity was superior to that observed for various standard compounds, and the present studies, using BRL 26314 as a pharmacological tool, provide evidence *in vivo* for an association between the elevation of HDL and reduction of arterial disease.

BRL 26314 [*N*-(4-chlorobenzyl)-L-phenylalanine] (Fig. 1), a representative compound from a novel series [1], raised the serum concentration of high-density lipoprotein (HDL) cholesterol in rats in association with evidence for enhanced cholesterol centripetal transport and increased faecal excretion of sterols and bile acids [2]. The concomitant net reduction of arterial cholesterol concentration by BRL 26314 is consistent with the claim from epidemiological evidence [3] that circulating HDL is a negative risk factor for atherosclerosis and coronary heart disease, but because it is difficult to study arterial disease in rats it was necessary also to examine BRL 26314 in a species where atherosclerosis could be induced. We now describe the development of a model in rabbits where the combination of physical damage to the arterial endothelium with the provision of a palatable semi-synthetic diet leads to relatively advanced arteriosclerotic lesions in contrast with the high incidence of foam cells noted for earlier rabbit models. The present work uses BRL 26314 as a pharmacological tool in this model to demonstrate that raising the circulating concentration of HDL cholesterol in rabbits leads to a reduction in the severity of arterial disease. This anti-atherosclerotic activity was superior to the effects of certain hypolipidaemic agents.

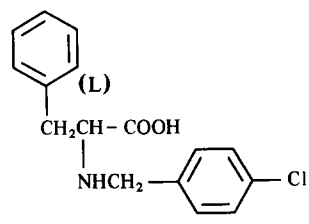

Fig. 1. Structure of BRL 26314.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Experimental model. The method for induction of atherosclerosis was adapted from the protocol of Katocs *et al.* [4] combining an atherogenic diet with de-endothelialization of the arterial wall using a balloon catheter. For routine purposes, the diet was adapted from Wilson *et al.* [5, 6], containing (g/kg dry wt): casein, 220; sucrose, 406; salt-free butter, 140; cellulose, 139; corn oil, 10; DL-methionine, 2; sorbitol, 0.5; vitamin mixture, 8; mineral mixture, 73; cholesterol, 1.5. To each kg of dry mixture was added 1 l. of 2% agar (dissolved by heating in H_2O to 90° and then cooled to 50°). The gelatinous mixture was stored at 5°. The cholesterol (obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.) was >99.9% pure 5-cholesten-3β-ol as evaluated by thin-layer chromatography (TLC) using the solvent system ethyl acetate–heptane (1:1, by vol.) and spraying with H_2SO_4. By comparison, a sample of aged cholesterol was only approximately 70% pure and six other, more polar, sterols could be detected. In view of the possible angiotoxicity of oxygenated sterols [7], we considered it necessary to standardize the model using pure cholesterol. New Zealand-White male rabbits, approximately 2.5 kg body wt (obtained from Ranch Rabbits Ltd., Crawley Down, Sussex, U.K.) were adapted to the experimental diet on arrival. After 5–7 days, rabbits were assigned to the experimental groups on the basis of serum total cholesterol, triglyceride and HDL cholesterol. Where applicable, compounds were administered in 1% methyl cellulose (2.0 ml/kg body wt) by daily oral dosing at 0900–1100 hr for 28 days; control rabbits received vehicle alone. For the standardized protocol, arterial de-endothelialization was per-

formed 7 days after the start of the dosing regimen, i.e. 12–14 days after initiation of experimental diet. Rabbits were anaesthetized with Hypnorm (Fentanyl, 0.2 mg/ml; Fluanisone, 10 mg/ml) 0.5 ml/kg intramuscularly and the right femoral artery was exposed. A 4F arterial embolectomy catheter (American Hospital Supply, Didcot, Oxon, U.K.) was inserted into the artery and advanced to the aortic arch [8]. The balloon was inflated with air (1.5 ml) and rapidly withdrawn through the aorta to the iliac bifurcation. It was necessary to deflate the balloon partially (0.5 ml) for withdrawal via the iliac and femoral artery. The blood vessel was sutured with a fine silk thread (0.7 mm Mersilk, Ethicon Ltd.) and the patency checked before closing the skin incision. Preliminary experiments showed that this procedure achieved extensive (>80%) de-endothelialization as detected by Evans blue uptake, and a second passage of the catheter was not found to be useful. The internal elastic lamina was intact in all sections examined microscopically, indicating that medial damage had not occurred. After 21 days, rabbits were stunned 1–2 hr after the final dose, killed by cervical dislocation and blood taken from the heart. Indices of arteriosclerosis were measured in both elastic (aorta) and muscular (femoral) arteries.

Preparation of arterial samples. The thoracic aorta was dissected free from periaortic adipose and connective tissue, fixed in formalin, stained using Sudan IV and the degree of atherosclerosis graded on a scale of 0–5 adapted from Duff and McMillan [9]. The length of the abdominal aorta was measured using calipers, from the sixth intercostal to 3 mm from the iliac bifurcation; the intima plus media was then stripped off and weighed prior to biochemical analysis. A sample of operated-femoral artery (including adventitia) was also taken for lipid analysis. Sections of the abdominal aorta (at the iliac bifurcation) and operated-femoral artery were also placed in formol saline prior to staining using Verhoff–Van Giessen stain. Arterial wall intimal thickening was quantified after histology of the transverse sections by projecting the image from a microscope onto graph paper; the outline of the tissue between the lumen and internal elastic lamina was traced, cut out and weighed.

Biochemical analyses. Lipids were measured using a Technicon AutoAnalyser II with enzymatic methods for cholesterol [10] and triglycerides [11], the latter adapted for fluorimetry. Extraction of tissue lipids and measurement of phospholipids was performed by methods described previously [12]. Lipoprotein classes were separated by ultracentrifugation [13]; HDL cholesterol was also measured in the supernatant after selective precipitation of the other lipoproteins using polyethylene glycol 6000 [14] (average mol. wt 6000–7500), a method found to be superior in preliminary experiments on hyperlipidaemic rabbit samples to methods used previously [2]. HDL-total lipids were also measured by agarose-gel electrophoresis [15]. Serum enzymes were measured using test combinations obtained from BCL (Lewes, East Sussex, U.K.). Free and esterified fractions of arterial cholesterol were separated by TLC performed on layers of silica gel $HF_{254+366}$ (Merck) in the solvent system light petroleum (b.p. 40–60°)–diethyl ether (70:30, by vol.). After elution with chloroform, cholesterol was measured by fluorimetry [16] using standard curves of free and esterified cholesterol. After extraction of lipids, the dried arterial residue was weighed and hydrolyzed with 6 N HCl at 120° for 4 hr. Hydroxyproline, taken as an index of collagen content, was measured using chloramine T [17].

The concentration of total bile acids in gall bladder bile was measured by using 3α-hydroxysteroid dehydrogenase [18] after deconjugation using choloylglycine hydrolase and extraction of free bile acids with diethyl ether. Separation of individual bile acids using gas chromatography [2] identified deoxycholic acid as the major bile acid in rabbits on this diet, in agreement with the results of other workers [19].

RESULTS

Optimization of model

In a preliminary experiment, arterial injury was compared in groups of rabbits killed 14 or 21 days after endothelial denudation. In this initial study, rabbits were given a cholesterol-supplemented (10 g/kg diet) stock diet for 28 days. Although serum-cholesterol was similar in the groups (Table 1), the longer post-de-endothelialization period increased the incidence of thoracic sudanophilic lesions and the exacerbated sterol deposition in the abdominal aorta was associated with a 40% increase in the weight of the intima. The concentration of both free

Table 1. Variation in arterial damage depending on time following de-endothelialization

	Post-operative period	
	14 days (n = 6)	21 days (n = 6)
Serum cholesterol (mg/dl)	1430 ± 135*	1408 ± 90
Thoracic sudanophilic lesions	2.4 ± 0.4	3.9 ± 0.6**
Abdominal intimal-medial thickening, by weight (mg)	120 ± 14	174 ± 12***
Abdominal cholesterol (μg/mg wet weight)		
Free	1.62 ± 0.16	2.79 ± 0.40***
Esterified	4.06 ± 0.91	6.08 ± 0.95

* Mean ± S.E.M.
** Indicates a significant difference between groups ($P < 0.05$).
*** Indicates a significant difference between groups ($P < 0.01$).

Table 2. Comparison of diets

	Diet		
	Agar (n = 7)	Agar (n = 10)	Stock (n = 9)
	Cholesterol supplement (g/100 g dry diet)		
	0.25	0.50	0.50
Serum lipid (mg/dl):			
Total cholesterol	1037 ± 101*	1997 ± 157**	1014 ± 225†
Total triglyceride	142 ± 38	123 ± 21	66 ± 11††
VLDL cholesterol (d < 1.006)	477	1138	588
LDL cholesterol (d 1.006–1.063)	467	799	395
HDL cholesterol (d 1.063–1.21)	93	60	30
Tissue cholesterol:			
Liver (mg)	843 ± 156	884 ± 83	1788 ± 243§
Kidney (mg)	50 ± 6.9	54 ± 4.7	72 ± 12.6§
Abdominal aorta (μg/mg wet weight)	6.23 ± 0.70	10.95 ± 1.35**	7.25 ± 0.96†

* Mean ± S.E.M.
** $P < 0.001$ vs agar + 0.25% cholesterol.
† $P < 0.01$ vs agar + 0.50% cholesterol.
†† $P < 0.05$ vs agar + 0.25%, agar + 0.50% cholesterol.
§ $P < 0.01$ vs agar + 0.25%, agar + 0.50% cholesterol.

and esterified cholesterol per unit wet weight of femoral artery was similar in the groups (results not shown) suggesting that a maximal response at this site was obtained relatively earlier after de-endothelialization, which perhaps reflects the greater severity of the insult.

In view of the increased intimal damage found after 3 weeks by comparison with the time chosen by Katocs *et al.* [4] the longer period was used for subsequent experiments. However, it was considered worthwhile to attempt to combine the physical damage with a hyperlipidaemic state of greater physiological relevance. The agar-based, sucrose–butter diet is palatable [5, 6] and rabbits normally adapted rapidly, eating 200–250 g/day within 3–4 days with a gain in body weight similar to that observed using a stock diet. We compared the response of de-endothelialized rabbits to the semi-synthetic diet, supplemented at two levels of cholesterol, with the response to the cholesterol-supplemented stock diet (Table 2). At an equivalent level of dietary cholesterol (0.5%), the agar-based diet induced significantly greater hypercholesterolaemia, and also hypertriglyceridaemia, by comparison with the stock diet. At the equivalent cholesterol supplement, the agar-based diet produced greater sterol deposition in the aorta (mainly cholesteryl esters) whereas the stock-based diet relatively enhanced sterol deposition in the kidneys and liver. At equivalent serum cholesterol concentrations (produced by 0.25% cho-

Table 3. Comparison of standard model with diet alone

	Experimental group		
	Stock diet (n = 10)	Agar diet + 0.15% cholesterol (n = 4)	Agar diet + 0.15% cholesterol + de-endothelialisation (n = 9)
Serum cholesterol (mg/dl)	66 ± 14*†§	827 ± 121	854 ± 64
Thoracic sudanophilic lesions	0†	0.1 ± 0.1†	2.4 ± 0.2
Aortic intimal-medial thickening (mg/cm):			
Abdominal	5.9 ± 0.3†§	13.6 ± 1·9	17.5 ± 1.4
Thoracic	15.7 ± 1.0†	22.2 ± 4.6	26.6 ± 1.5
Abdominal cholesterol (μg/cm):			
Free	3.8 ± 0.7†	6.2 ± 0.9†	15.9 ± 3.0
Esterified	0.20 ± 0.06†	1.2 ± 0.8†	24.6 ± 6.0
Femoral cholesterol (μg/mg weight):			
Free	0.35 ± 0.01†	0.17 ± 0.07†	1.08 ± 0.25
Esterified	0.12 ± 0.03†§§	0.66 ± 0.36†	6.61 ± 1.04
Abdominal collagen (μg/cm)	258 ± 20†§	632 ± 70	618 ± 50

* Mean ± S.E.M.
† $P < 0.001$ vs cholesterol-supplemented diet + de-endothelialization.
§ $P < 0.01$ vs cholesterol-supplemented diet alone.
§§ $P < 0.05$ vs cholesterol-supplemented diet alone.

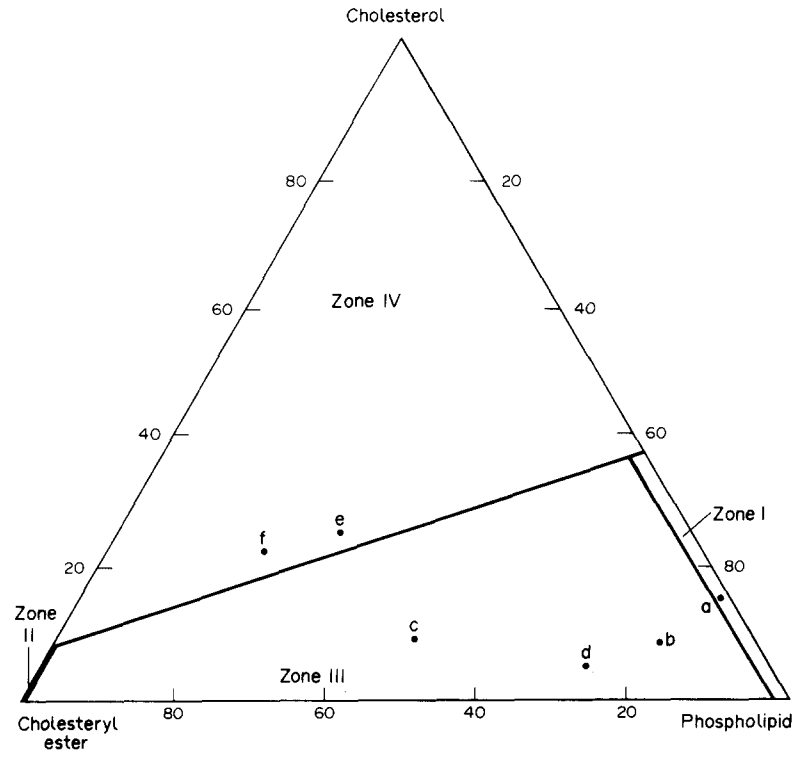

Fig. 2. Phase diagram of arterial intimal-medial lipids. Stock diet, a = abdominal, b = femoral; cholesterol-supplemented diet, c = abdominal, d = fermoral; cholesterol-supplemented diet + de-endothelialisation, e = abdominal, f = femoral.

lesterol in agar diet and 0.5% cholesterol in stock diet), the stock diet supported markedly lower HDL cholesterol levels and the very-low-density lipoprotein (VLDL) isolated from these rabbits was poorer in protein (4% vs 7%), phospholipid (7% vs 10%) and triglyceride (8% vs 35%) suggesting the extensive accumulation of chylomicron remnants (βVLDL). Serum cholesterol levels were still high at a supplement of 0.25% so the response to 0.15% dietary cholesterol was also examined in detail. Provision of the atherogenic diet without other damage led to intimal-medial thickening and moderate deposition of both free and esterified cholesterol at both the abdominal and femoral sites (Table 3). The results for abdominal sterol levels are now expressed per unit length to take wall-thickening into account; similar qualitative differences were obtained when results were expressed per unit wet wt. Combination of balloon catheter-induced damage plus diet did not further affect serum lipids but markedly increased the extent and severity of thoracic sudanophilic lesions and sterol deposition at the abdominal and femoral sites. Apparently, under these experimental conditions the diet was the major stimulus to accelerate collagen synthesis. In other experiments, de-endothelialization increased smooth muscle cell proliferation as measured by DNA synthesis from radiolabelled thymidine *ex vivo*. For example, thymidine uptake (dpm/100 mg tissue per 10 min) was 87,000 ± 30,000 in the operated femoral (91% of radiolabelling could be isolated as DNA) by comparison with 27,000 ± 7000 for the unoperated femoral ($n = 5$, $P < 0.01$).

When the lipid composition of the arterial samples was expressed on a phase diagram [20] (Fig. 2), the samples from the normolipidaemic, uninjured rabbits could be assigned either to zone I (the lamellar liquid-crystalline phase), which is also characteristic of normal human intima, or to the border with zone III (containing a second, oily-liquid phase cholesteryl ester). The samples from the unoperated, hyperlipidaemic rabbits could also be assigned to zone III in contrast with the samples from the arteriosclerotic rabbits which appear in zone IV. This latter zone is characterized by three phases (lamellar liquid-crystalline phase saturated with free and esterified sterol; oily cholesteryl ester phase saturated with free sterol; cholesterol crystals) and is typical of advanced plaque in man [20].

BRL 26314

The novel compound BRL 26314 was examined in two separate experiments at a dose of 100 mg/kg body wt per day (Table 4, Figs 3 and 4): the racemic DL-mixture in the first experiment and the L-isomer in the second (similar activity for the two preparations was obtained in rats). BRL 26314 did not significantly affect body weight nor was there any indication of gross toxicity as measured by relative organ weights or serum enzymes or serum urea N (measured only in the second experiment: control = 22.6 ± 1.6 mg/dl, BRL 26314 = 20.6 ± 1.4 mg/dl). As described in Materials and Methods, after adaptation to the diet rabbits were randomized into their experimental groups so that the mean values for serum total cholesterol, triglyceride and

Table 4. Effect of BRL 26314 (100 mg/kg per day) on rabbit atherosclerosis

	Experiment 1		Experiment 2	
	Control (n = 9)	BRL 26314 (DL) (n = 9)	Control (n = 9)	BRL 26314 (L) (n = 9)
Initial body weight (kg)	2.60 ± 0.09*	2.48 ± 0.07	2.77 ± 0.05	2.84 ± 0.08
Final body weight (kg)	2.77 ± 0.09	2.44 ± 0.11	2.80 ± 0.11	2.83 ± 0.12
Food intake (g/day)	—**	—**	132 ± 7	116 ± 9
Liver weight (g/100 g)	3.9 ± 0.2	3.7 ± 0.1	3.2 ± 0.2	3.0 ± 0.3
Kidney weight (g/100 g)	0.54 ± 0.03	0.56 ± 0.04	0.48 ± 0.03	0.54 ± 0.06
Serum enzyme (U/l):				
Alkaline phosphatase	32 ± 2.8	30 ± 3.0	—**	—**
Glutamate-pyruvate transaminase	9.5 ± 1.8	10.6 ± 1.5	6.4 ± 2.1	4.8 ± 0.9
Glutamate-oxaloacetate transaminase	17 ± 1.9	20 ± 1.8	19 ± 3.8	18 ± 1.9
Lipoprotein cholesterol (mg/dl by ultracentrifugation):				
VLDL + chylomicrons (d < 1.006)	406	418	700	590
LDL (d 1.006–1.063)	460	495	400	390
HDL (d 1.063–1.21)	50	107	50	80
HDL protein (mg/ml isolated lipoprotein)	1.48	3.00	1.12	1.72
Thoracic sudanophilic lesions	3.0 ± 0.5	1.3 ± 0.3†	3.1 ± 0.4	1.6 ± 0.4†
Abdominal intimal-medial thickening:				
By weight (mg/cm)	18 ± 2.3	9.6 ± 0.6††	18 ± 1.1	16 ± 0.7
By histology (intimal)	43 ± 11	13 ± 6.7†	74 ± 8.1	27 ± 4.8††
Femoral intimal thickening by histology	27 ± 7.2	17 ± 4.2	41 ± 4.3	23 ± 3.2†
Abdominal lipid (μg/cm):				
Free cholesterol	39 ± 5.1	22 ± 2.5††	29 ± 3.0	21 ± 2.1†
Esterified cholesterol	33 ± 5.5	17 ± 2.7††	68 ± 11	53 ± 7.4
Phospholipid	—**	—**	30 ± 2.5	27 ± 2.6
Abdominal collagen (μg/cm)	—**	—**	511 ± 32	426 ± 24§
Femoral lipid (μg/mg):				
Free cholesterol	3.9 ± 0.32	3.5 ± 0.30	2.8 ± 0.44	1.6 ± 0.20§
Esterified cholesterol	5.9 ± 0.98	5.0 ± 1.30	7.7 ± 1.34	5.3 ± 0.90§
Phospholipid	—**	—**	1.9 ± 0.17	1.4 ± 0.25
Femoral collagen (μg/mg)	—**	—**	57 ± 5.3	68 ± 6.5

* Mean ± S.E.M.
** Analysis not done.
† $P < 0.01$ vs comparable control.
†† $P < 0.001$ vs comparable control.
§ $P < 0.05$ vs comparable control.

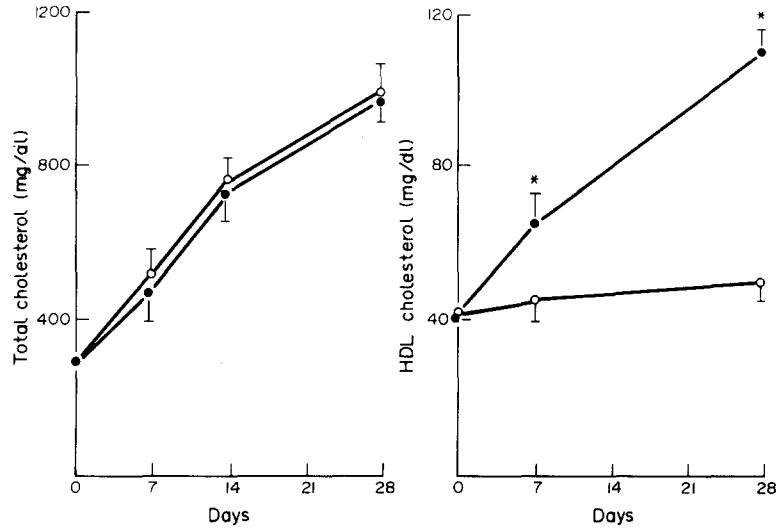

Fig. 3. Serum total and HDL cholesterol after dosing BRL 26314 (DL). Results are means ± S.E.M. for nine rabbits. ○, Control; ●, BRL 26314. * Indicates a significant difference from the control group ($P < 0.001$).

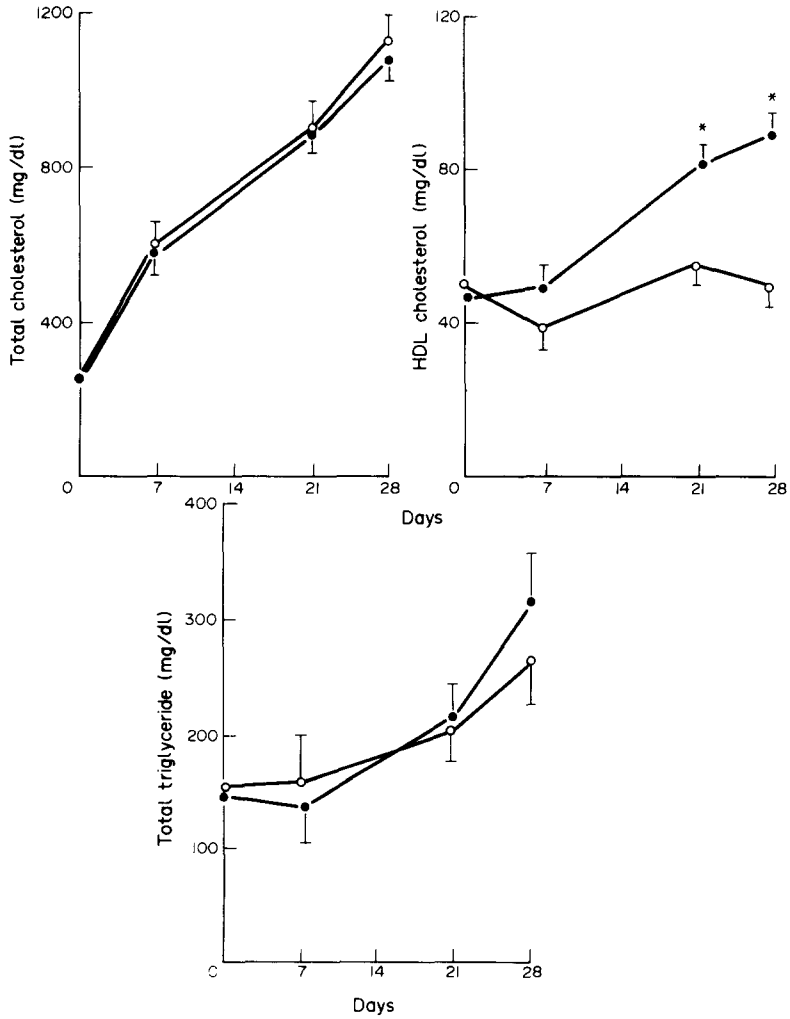

Fig. 4. Serum total and HDL cholesterol and total triglyceride after dosing BRL 26314 (L). Results are means ± S.E.M. for nine rabbits. ○, Control; ●, BRL 26314. * Indicates a significant difference from the control group ($P < 0.01$).

HDL cholesterol were similar at the start. Serum total cholesterol and triglyceride levels increased throughout the experiment (Figs 3 and 4) but were not affected by BRL 26314. However, BRL 26314 did specifically increase the concentration of HDL cholesterol whether measured after VLDL and LDL precipitation (Figs 3 and 4), ultracentrifugation (pooled samples, Table 4) or by electrophoresis (for example after 7 days dosing the fraction with α-mobility as measured by lipid-staining accounted for $50 \pm 8\%$ of the total lipids by comparison with $9 \pm 6\%$ for the control rabbits, $P < 0.001$). BRL 26314 also markedly elevated HDL protein (+60–100%, Table 4) and phospholipid (+40–50%, results not shown). The concentrations of the other lipoprotein classes were not consistently altered.

In both experiments, BRL 26314 significantly reduced the extent and severity of the thoracic sudanophilic lesions and both abdominal and femoral wall-thickening was lessened. In association with these changes, BRL 26314 decreased both free and esterified cholesterol concentrations at both the abdominal and femoral sites although there was some variation between the experiments with respect both to the basal levels and the response to the compound. BRL 26314 also significantly decreased abdominal collagen content. BRL 26314 did not affect the concentration of liver lipids (in the second experiment: total lipid = 59 ± 1.8 mg/g vs 57 ± 2.5 mg/g in control rabbits; cholesterol = 10.0 ± 1.1 mg/g vs 10.8 ± 0.8 mg/g; triglyceride = 7.2 ± 0.5 mg/g vs 6.7 ± 0.6 mg/g).

The concentration of gall bladder bile acid was also unchanged (total bile acid: control, 8.58 ± 1.72 mg/ml; BRL 26314, 7.25 ± 0.91; deoxycholic acid: control, 7.10 ± 1.50 mg/ml; BRL 26314, 6.28 ± 0.82) although bile phospholipid increased from a control level of 2.52 ± 0.50 mg/ml to 5.20 ± 1.05 mg/ml ($P < 0.01$). Histological analysis of the livers disclosed no abnormalities.

Standard compounds

The model has also been used to evaluate representative compounds from classes of commercially available hypolipidaemic agents. Results for two recently introduced drugs are shown in Table 5. Bezafibrate, at 100 mg/kg body wt per day for 28 days, slightly but not significantly raised HDL cholesterol. A modest, albeit statistically significant, reduction in abdominal intimal-medial thickening was observed and this was accompanied by a significant decrease in the content of collagen but not cholesterol.

Clofibrate at 100 mg/kg was without effect on either serum lipoproteins or atherosclerosis (results not shown). At this dose procetofene was toxic (60% deaths) and impaired kidney function.

Probucol at 100 mg/kg (Table 5) decreased HDL cholesterol in agreement with clinical results [21] and this may explain the tendency for increased sterol deposition which was statistically significant in the femoral artery. Other work [22] suggests probucol is relatively ineffective in the rabbit at this dose and that a much higher intake (1% in diet) is required for efficacy.

Nicotinic acid at 100 mg/kg was significantly anti-lipolytic (serum free fatty acid decreased by 45%) but produced no other effect on serum lipids or lipoproteins or on atherosclerosis (results not shown). When testing nicotinic acid and analogues, other workers [23] have used much longer periods of dosing to obtain an effect on experimental atherosclerosis.

DISCUSSION

Many experimental models of atherosclerosis employ dietary cholesterol but a stock diet supplemented with 1–2% cholesterol leads to cholesterol-storage disease in rabbits rather than to specific arterial damage [24]. High levels of dietary cholesterol also induce lipoprotein changes characteristic of type III hyperlipidaemia rather than the clinically common types, and the low levels of circulating HDL together with saturation and suppression of hepatic lipoprotein receptors [25] limits the usefulness of this model for evaluating the clinically relevant consequences of therapeutic intervention. We believe that the present model is an improvement by providing a palatable semi-synthetic diet which maintains basal HDL levels with hyperbetalipoproteinaemia without massive accumulation of βVLDL and where physical damage to the arterial endothelium initiates a rapid response leading to smooth muscle cell proliferation [26]. Under these experimental conditions, arterial sterol deposition results from both active lipoprotein uptake by the smooth muscle cells [4] and from passive diffusion. There is variation in the response to the combination of atherogenic stresses both within and between experiments. Part of the latter variance can be attributed to seasonal patterns in food intake and hormone levels. The resulting metabolic state is a demanding but realistic test of any agent proposed to affect atherosclerosis by means of lipoprotein changes, and the present experiments have used this model to investigate the possibility that raising the circulating level of HDL is associated with reduction of atherosclerosis.

In addition to the epidemiological evidence from heterogeneous populations, it is known that familial hyperalphalipoproteinaemia is associated with prolonged longevity and decreased morbidity from myocardial infarction [27]. Patients with Tangier disease (familial HDL deficiency) show clinically evident vascular disease [28] although atherosclerosis is not strikingly accelerated, perhaps because of the low LDL level. Premature atherosclerosis is also absent in familial hypoalphalipoproteinaemia in fish-eye disease but these findings are similarly difficult to interpret because of concomitant changes in other lipoproteins [29]. Other evidence [30] does associate hypoalphalipoproteinaemia *per se* with coronary artery disease and myocardial infarction, and the cause of the low HDL levels could be important [31].

During maturation in the circulation, HDL accumulates cholesterol either by transfer from triglyceride-rich lipoproteins during their catabolism or from stores in peripheral tissues [32]. HDL may, therefore, function as an anti-atherosclerotic agent by promoting the egress of cholesterol from the

Table 5. Evaluation of standard compounds (100 mg/kg per day) on rabbit atherosclerosis

	Experiment 1		Experiment 2	
	Control (n = 10)	Bezafibrate (n = 11)	Control (n = 8)	Probucol (n = 8)
Serum lipid (mg/dl):				
Total cholesterol	827 ± 64*	936 ± 83	775 ± 238	502 ± 55
HDL cholesterol	32 ± 4.2	42 ± 7.4	49 ± 4.8	23 ± 3.2†
Triglyceride	118 ± 16	158 ± 32	264 ± 108	269 ± 74
Thoracic sudanophilic lesions	—**	—**	2.4 ± 0.2	2.8 ± 0.3
Abdominal intimal-medial thickening (mg/cm)	18.8 ± 0.7	16.2 ± 0.8§	17.5 ± 1.4	16.9 ± 1.5
Abdominal cholesterol (μg/cm):				
Free	32 ± 2.6	37 ± 5.3	16 ± 3.0	23 ± 2.2
Esterified	45 ± 4.2	36 ± 5.8	25 ± 6.0	38 ± 4.6
Femoral cholesterol (μg/mg):				
Free	1.6 ± 0.18	2.0 ± 0.26	1.1 ± 0.25	2.5 ± 0.17†
Esterified	3.8 ± 0.76	3.4 ± 0.74	6.6 ± 1.04	4.7 ± 0.60
Abdominal collagen (μg/cm)	702 ± 20	523 ± 25†	—**	—**

* Mean ± S.E.M.
** Analysis not done.
† $P < 0.001$ vs comparable control.
§ $P < 0.05$ vs comparable control.

arterial wall or perhaps by impairing the uptake of LDL [33].

Other studies using rabbits have identified anti-atherosclerotic hyperalphalipoproteinaemic compounds, e.g. BR-931 [4-chloro-6-(2,3-xylidino) -2-pyrimidinylthio-(*N*-β-hydroxyethyl)-acetamide] [34], but hypobetalipoproteinaemic effects were also obtained and the relative contribution made by changes in HDL concentration cannot be evaluated. When interpreting results from animal studies it must be emphasized that the HDL population is chemically and functionally heterogeneous and that there is insufficient evidence regarding the role of HDL in rabbits, although it is known that BR-931 is hyperalphalipoproteinaemic in man [35] as well as in rabbits [34] and in the present studies the response to certain compounds (probucol, bezafibrate) parallels the response by patients.

BRL 26314, whether DL-, or L-, markedly elevated serum HDL (cholesterol, protein, phospholipid) in rabbits, without significant effect on other lipoproteins, in association with a clear reduction in atherosclerosis. By contrast to results in rats [2], BRL 26314 is not hypotriglyceridaemic in rabbits, nor is there evidence for accumulation of HDLc (or LDL). The response to BRL 26314 in rabbits probably did not involve the reactions catalysed by either lipoprotein lipase or hepatic lipase because changes in these enzyme activities would have affected the other lipoprotein classes. Thus, during HDL maturation the additional cholesterol was probably obtained from peripheral tissues rather than accelerated triglyceride-rich lipoprotein catabolism. The activity of lecithin-cholesterol acyltransferase, measured as the endogenous esterifying capacity of plasma at 37° (free and esterified cholesterol fractions were separated by TLC and measured by fluorimetry), was not affected: absolute catabolic rate (mg/hr per 100 ml plasma) after BRL 26314 (L) was 10.3 ± 2.1 by comparison with the control level of 10.0 ± 1.6 (normo-lipidaemic level was 3.1 ± 0.7), but this enzyme is of doubtful importance in rabbits. An action of BRL 26314 in the liver or small intestine to promote the synthesis of nascent HDL, which can then enhance cholesterol egress from the arterial wall, seems possible in both rabbits and rats but the compound is not a general inducer of protein synthesis, nor does it affect other circulating proteins of hepatic origin, e.g. albumin (R. Fears, unpublished results). It should be noted that BRL 26314 is effective in rabbits with marked hyperlipidaemia and this observation may contrast with the conclusions by other workers from experiments using purified lipoproteins *in vitro* [36] where HDL did not appear to promote cholesterol egress when levels of other lipoproteins were high. This is an area of metabolism where results obtained *in vitro* need to be interpreted cautiously [37].

The increase in circulating HDL, when associated with depletion of arterial cholesterol, would be expected to enhance cholesterol return to the liver for disposal but in rabbits the accelerated flux is small by comparison with total cholesterol turnover (e.g. dietary input is approximately 300 mg/day). Thus, it has not been possible to determine the fate of that cholesterol which would otherwise have accumulated in the arterial wall (and possibly other extrahepatic tissues). It has been suggested [38] that HDL-free cholesterol is the primary precursor for bile acid synthesis in man. It is not known if substrate requirements are the same in rabbits but microsomal cholesterol 7α-hydroxylase (the rate-limiting enzyme in bile acid synthesis), when measured by methods described previously [39], was not affected by the marked increase in HDL-cholesterol caused by BRL 26314 (W. R. Rush and R. Fears, unpublished results). Similarly, the concentration of gall bladder bile acid was not affected although biliary phospholipid did increase. There was no evidence for sterol accumulation in the liver. Comparison of the present rabbit results with the results obtained previously using rats [2] discloses differences, for example, with respect to the concentration of triglyceride in serum and bile acids in bile. These disparities may reflect either interspecies variation in the metabolism of HDL or the difficulty of measuring relatively small changes superimposed upon large daily sterol fluxes. Although these changes in cholesterol transport can be difficult to quantify, there is little doubt both in rats and rabbits that the rise in HDL is associated with accelerated centripetal transport and with reduction in extrahepatic cholesterol deposition, particularly in the arterial wall.

In addition to the action on arterial lipids in rabbits, BRL 26314 decreased the concentration of collagen and this response may also be HDL-mediated for it has been shown that HDL inhibits both smooth muscle cell proliferation in monkeys [40] and synthesis of both DNA and sulphated glycosaminoglycans in human smooth muscle cells [41], and stimulates porcine arterial endothelial cell prostacyclin biosynthesis [42]. The observation that elastase-type activity is associated with HDL [43] suggests that elevating HDL levels might also promote a proteolytic attack on matrix and cell constituents within the arterial lesion.

In conclusion, a model of atherosclerosis should be able to compare agents potentially capable of acting by a variety of mechanisms. It is possible that the present model favours a compound which, by raising HDL, is able to reverse the lipid accumulation induced by de-endothelialization but underestimates hypolipidaemic compounds because the relatively large contribution by dietary cholesterol tends to overwhelm normal regulatory mechanisms. However, clinical experience with the commercially available hypolipidaemics has also been relatively disappointing although the combination of agents with different mechanisms of action may give synergy. The possibility of therapeutic treatment of atherosclerosis by promoting HDL cholesterol transport is an exciting prospect and we believe that the present studies, using BRL 26314 as a pharmacological tool, provide one of the first pieces of convincing evidence *in vivo* for an association between raised HDL and reduction of arterial disease.

Acknowledgements—We thank Mrs. R. Carter and Mr. G. Bond for technical assistance, Miss S. Smith for the histological analyses and Mr. M. Moran for the care of the animals. We would also like to thank Ms. A. M. Spence for her help in the preparation of this manuscript.

REFERENCES

1. H. Ferres, Eur. Pub. Appl. No. 31653 (1981).
2. R. Fears, W. R. Rush, P. Walker and H. Ferres, *Biochem. Pharmac.* BP 875 (in press).
3. G. J. Miller and N. E. Miller, *Lancet* i, 16 (1975).
4. A. S. Katocs, Jr., E. L. Largis, L. W. Will, D. K. McClintock and S. J. Riggi, *Artery* **2**, 38 (1976).
5. R. B. Wilson, P. M. Newberne and M. W. Conner, *Archs Path.* **96**, 355 (1973).
6. R. B. Wilson, C. C. Middleton and G. Y. Sun, *J. Nutr.* **108**, 1858 (1978).
7. H. Imai, N. T. Werthessen, C. B. Taylor and K. T. Lee, *Archs Path.* **100**, 565 (1976).
8. H. R. Baumgartner, *Z. Gesamte exp. Med.* **137**, 227 (1963).
9. G. L. Duff and G. C. McMillan, *J. exp. Med.* **89**, 611 (1949).
10. Method sheet 35/76/5C, The Boehringer Corporation (London) Ltd.
11. Method sheet 578.114.6, The Boehringer Corporation (London) Ltd.
12. R. Fears, D. H. Richards and H. Ferres, *Atherosclerosis* **35**, 439 (1980).
13. R. J. Havel, H. A. Eder and H. J. Bragdon, *J. clin. Invest.* **34**, 1345 (1955).
14. J. Viikari, *Scand. J. clin. Lab. Invest.* **36**, 265 (1976).
15. R. Fears and A. M. Umpleby, *Biochem. J.* **182**, 803 (1979).
16. G. Bondjers and S. Bjorkerud, *Analyt. Biochem.* **42**, 363 (1971).
17. J. E. Woessner, Jr., *Archs Biochem. Biophys.* **93**, 440 (1961).
18. M. J. Sheltawy and M. S. Losowsky, *Clinica chim. Acta* **64**, 127 (1975).
19. E. H. Mosbach, M. A. Rothschild, I. Bekersky, M. Oratz and J. Mongelli, *J. clin. Invest.* **50**, 1720 (1971).
20. D. M. Small and G. G. Shipley, *Science* **185**, 222 (1974).
21. T. A. Miettinen, J. K. Huttunen, C. Ehnholm, T. Kumlin, S. Mattila and V. Naukkarinen, *Atherosclerosis* **36**, 249 (1980).
22. D. Kritchevsky, H. K. Kim and S. A. Tepper, *Proc. Soc. exp. Biol. Med.* **136**, 1216 (1971).
23. M. R. Parwaresch, H. Haacke and Ch. Mäder, *Atherosclerosis* **31**, 395 (1978).
24. J. T. Prior, D. M. Kurtz and D. D. Ziegler, *Archs Path.* **71**, 672 (1961).
25. P. T. Kovanen, M. S. Brown, S. K. Basu, D. W. Bilheimer and J. L. Goldstein, *Proc. natn. Acad. Sci. U.S.A.* **78**, 1396 (1981).
26. R. Ross and L. Harker, *Science* **193**, 1094 (1976).
27. C. J. Glueck, P. Gartside, R. W. Fallat, J. Sielski and P. M. Steiner, *J. Lab. clin. Med.* **88**, 941 (1976).
28. E. J. Schaefer, L. A. Zech, D. E. Schwartz and H. B. Brewer, Jr., *Ann. intern. Med.* **93**, 261 (1980).
29. G. Utermann, *Eur. J. clin. Invest.* **12**, 5 (1982).
30. L. Toth and W. Koenig, *Atherosclerosis* **42**, 121 (1982).
31. R. A. Norum, J. B. Lakier, S. Goldstein, A. Angel, R. D. Goldberg, W. D. Block, D. K. Noffze, P. J. Dolphin, J. Edelglass, D. D. Bogorad and P. Alaupovic, *New Engl. J. Med.* **306**, 1513 (1982).
32. N. B. Myant, *Clin. Sci.* **62**, 261 (1982).
33. N. E. Miller, D. B. Weinstein, T. E. Carew, T. Koschinsky and D. Steinberg, *J. clin. Invest.* **60**, 78 (1977).
34. C. R. Sirtori, P. Gomarasca, G. D'Atri, S. Cerutti, G. Tronconi and C. Scolastico, *Atherosclerosis* **30**, 45 (1978).
35. G. Nunes, *Curr. ther. Res.* **31**, 193 (1982).
36. G. Bondjers, O. Wiklund, P. I. Norfeldt and S. O. Olofsson, in *Metabolic Risk Factors in Ischaemic Cardiovascular Disease* (Eds. L. A. Carlson and B. Pernow), p. 133. Raven Press, New York (1982).
37. R. J. Daniels, L. S. Guertler, T. S. Parker and D. Steinberg, *J. biol. Chem.* **256**, 4978 (1981).
38. L. G. Halloran, C. S. Schwartz, Z. R. Vlahcevic, R. M. Nisman and L. Swell, *Surgery* **84**, 1 (1978).
39. W. R. Rush, R. Fears, J. Green and D. V. Parke, *Comp. Biochem. Physiol.* **69B**, 493 (1981).
40. M. Tammi, T. Rönnemaa, T. Vihersaari, H. Saarni, A. Lehtonen and J. Viikari, *Med. Biol.* **57**, 118 (1979).
41. Y. Yoshida, K. Fisher-Dzoga and R. W. Wissler, *Circulation* **55** (Suppl. III), 100 (1977).
42. L. N. Fleisher, A. R. Tall, L. D. Witte, R. W. Miller and P. J. Cannon, *J. biol. Chem.* **257**, 6653 (1982).
43. M. P. Jacob, G. Bellon, L. Robert, W. Hornebeck, M. Ayrault-Jarrier, J. Burdin and J. Polonovski, *Biochem. biophys. Res. Commun.* **103**, 311 (1981).