Dietary Ethanol-Induced Modifications in Hyperlipoproteinemia and Atherosclerosis in Nonhuman Primates (*Macaca nemestrina*)

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Male *Macaca nemestrina* were studied in an experiment with a 2 × 2 factorial design. Diets contained low vs high cholesterol levels (0.3 vs 1.0 mg/Kcal) and no ethanol or ethanol, as 36% of the calories substituted isocalorically for carbohydrate. After receiving their diets for 18 months, the monkeys had blood samples drawn for lipoprotein analyses, and then were killed for evaluation of the extent of atherosclerosis. Ethanol-fed groups had significantly increased concentrations of serum cholesterol, triglycerides, low density lipoprotein, and high density lipoprotein. The molecular weight of the low density lipoprotein particles was lower in ethanol-fed animals and the cholesterol esters of low density lipoprotein and high density lipoprotein contained relatively more cholesteryl linoleate and less cholesteryl oleate. Dietary cholesterol had the effect of increasing the concentration of low density lipoprotein (primarily via increasing the low density lipoprotein molecular weight) and decreasing the concentration of high density lipoprotein. Significant interactions were found between the effects of ethanol and cholesterol on high density lipoprotein and low density lipoprotein. Ethanol significantly decreased the cholesterol-induced atherosclerosis found in the aorta and coronary arteries. Highly significant correlations between coronary artery atherosclerosis and low density lipoprotein molecular weight, inverse high density lipoprotein concentration, and low density lipoprotein cholesterol ester pattern were found. In contrast, low density lipoprotein molar concentration (number of low density lipoprotein particles per liter of plasma) was not significantly correlated with coronary artery atherosclerosis. Different relationships with aortic atherosclerosis were found; low density lipoprotein molecular weight and cholesterol ester pattern were highly correlated, while high density lipoprotein concentration was not. The high correlations found between lipoprotein characteristics and atherosclerosis severity suggest that the effect of ethanol in reducing the development of atherosclerosis may have been mediated through its effects on the plasma lipoproteins.

(Arteriosclerosis 1981; 1:144–155)

Specific relationships between plasma lipoproteins and diet-induced coronary artery atherosclerosis have been documented in one nonhuman primate species.\(^1\text{-}^3\) Epidemiologic studies of human beings have suggested that ethanol may predispose an individual to have decreased risk of atherosclerosis.\(^4\text{-}^5\) This may be due, in part, to the increased high density lipoprotein (HDL) concentrations in persons ingesting ethanol;\(^6\) HDL is now recognized as a protective factor against coronary artery atherosclerosis.\(^7\) Since HDL levels were inversely correlated to the extent of coronary artery atherosclerosis in our earlier nonhuman primate study,\(^1\text{-}^3\) it seemed appropriate to study the effects of dietary ethanol on the relationship between HDL and coronary artery atherosclerosis in nonhuman primates. Since low density lipoprotein (LDL) molecular weight (mw) was even more highly correlated with the degree of coronary artery atherosclerosis...
sclerosis1,2 we have also investigated the effects of ethanol on the relationship of this measurement to atherosclerosis. We used Macaca nemestrina in this study for three reasons: 1) these monkeys are susceptible to diet-induced atherosclerosis;2 2) the plasma LDL and HDL changes in response to diet are marked;10,11 and 3) a hypercholesterolemic diet has been noted to induce "cirrhosis-like" changes in hepatic morphology in this species,12 suggesting that this animal may be a good model for ethanol-induced liver changes. Because we carried out a detailed study of the lipoproteins of each animal and quantitated the extent of atherosclerosis in each animal, we have been able to use individual animal variability to identify relationships between lipoproteins and atherosclerosis.

**Methods**

**Animals and Diets**

Adult M. nemestrina males were purchased from a commercial supplier (Primate Imports, Port Washington, New York) and were handled and maintained as described elsewhere.13 The animals were fed liquid diets for 18 months. The diets differed in the amount of cholesterol (0.3 or 1.0 mg/kcal, designated "Lo" and "Hi," respectively) and whether they contained ethanol (designated "E"). Each diet contained 38% of the calories as fat (½ corn oil, ½ lard) and 36% of the calories substituted iso-calorically for carbohydrate. Each diet contained 38% of the calories as fat (½ corn oil, ½ lard) and had a caloric density of 1 kcal/ml. Diet consumption and body weights were routinely measured and are reported elsewhere.13 A group of 24 animals was purchased to start the study, and four animals were eliminated during the first month of the study. Two failed to recover from ketamine immobilization, one died from asphyxia related to aspiration, and one died from bronchopneumonia. Subsequently, one animal was removed at 3 months when found to be diabetic. All data in this report are based on the 19 animals that completed the study, including five in the Lo group, six in the Hi group, five in the Lo,E group, and three in the Hi,E group.

**Routine Observations**

Blood was collected at regular bimonthly intervals, after the animals were fasted for 18 hours and then immobilized with ketamine-HCl. Serum triglyceride and cholesterol concentrations14 were determined, and agarose electrophoresis of serum lipoproteins15 was performed. Routine clinical evaluations have been described elsewhere13 and included serum urea nitrogen, differential blood counts, total serum protein, bilirubin, serum alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transpeptidase. For histologic evaluation of the liver, biopsies were taken from livers of all animals before selection of diet groups and at 6 and 12 months into the study; later, when the animals had been killed after 18 months of the diets, liver slices were taken.

**Plasma Lipoprotein Analyses**

For lipoprotein studies, fasting blood samples were collected and handled separately from each of the animals at the end of the 18-month experimental period. In the three animals of the Hi,E group, a blood sample was also collected 2 weeks prior to termination. All pairs of values obtained subsequently were similar; thus, their means were calculated for further evaluation. These data served as a control for the small number in this group and as a check on the representativeness of the terminal blood samples. At termination, animals were immobilized with ketamine-HCl and anesthetized with sodium pentobarbital, a ventral abdominal incision was made, and blood was drawn from the caudal vena cava into tubes that contained 5,5-dithiobis-nitrobenzoic acid (DTNB), 0.4 mg/ml; ethylenediamine tetraacetate (EDTA), 1 mg/ml; and NaN₃, 0.5 mg/ml final concentration.

Lipoproteins were promptly isolated from the plasma by ultracentrifugation at a density of 1.225 g/ml and then separated by column chromatography on Bio-Gel A-15 m (Bio-Rad Labs, Richmond, California), as described by Rudel et al.16 A tracer amount of [¹³C]LDL was added to each lipoprotein sample prior to column separation to permit LDL mw determination.17 Density ranges for the material from each of the elution regions were established by subsequent sequential centrifugation at densities of 1.006, 1.019, or 1.063,18 the classical cut-off densities for very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and LDL respectively. Material of Region II (figure 1) was found in each of the density ranges; thus, subfractionation by density was carried out before chemical analyses. Plasma lipoprotein distribution was determined as described previously.2 The material within the various designated elution regions (figure 1) was combined, and the amount of cholesterol,19 protein,20 and phospholipid21 in each region was measured. Each fraction was then extracted in chloroform:methanol, 2:1; lipids were separated by thin layer chromatography; and free cholesterol, ester cholesterol, and triacylglycerol glycerol22 were then assayed. Cholesterol ester fatty acids (CEFA) were methylated with borontrifluoride (Supelco, Inc., Bellefonte,
and the methyl esters were separated and quantitated by gas-liquid chromatography, as described previously. For evaluation of apolipoprotein patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), aliquots of each lipoprotein fraction were dialyzed against 0.01% EDTA in distilled water, lyophilized, and extracted overnight at 4°C in ethanol:diethyl-ether, 3:1. Both solvents were distilled within 1 week of use. Four parts of ether were then added to make the final ratio, ethanol:ether, 3:5, and the solution was centrifuged at 4°C to pellet the protein. This protein pellet was washed 3 to 5 times with cold ether, after which samples were dried under N2. To resolubilize the protein, a solution of 1% SDS, 0.25% mercaptoethanol, 0.5 M urea in 0.025 M barbital buffer, pH 8.6, was added to make the final apoprotein concentration, 1 μg/μl. This solution was mixed overnight at 4°C to completely dissolve the sample.

Evaluation of Atherosclerosis

At the time of necropsy, the animals were exsanguinated, then perfused with normal saline. The heart and coronary arteries were fixed under pressure (100 to 120 mm Hg) with 10% neutral buffered formalin. The aorta, carotid, and iliac arteries were opened and fixed flat on cardboard. These arteries were stained with Sudan IV in isopropanol. Two observers then estimated the percentages of the intimal surface covered by flat sudanophilic areas (fatty streak); flat, opaque, nonsudanophilic areas (diffuse intimal thickening); raised sudanophilic areas (fatty plaque); and raised nonsudanophilic areas (fibrous plaque). The resulting values were averaged.

Sixteen blocks of the major coronary arteries were processed for histological evaluation. These were one block from the left main coronary artery (LMC), and five serial blocks each from the left anterior descending (LAD), left circumflex (LCX), and right coronary arteries (RCA). Two paraffin sections were prepared from each block; one was stained with hematoxylin and eosin and the other by the Verhoeff van Gieson method for elastic tissue. These were also graded by two observers for the percentage of the area bounded by the internal elastic lamina (IEL) that was occupied by atherosclerotic lesions, the percentage of these lesions composed of foam cells, the percentage of the media that was damaged (foam cells, missing muscle, fibrosis, or mineralization), and the percentage of the internal elastic lamina that was damaged. Each of these percentages was derived by visual estimation. Agreement between observers was consistently within 10%.

The common carotid arteries were graded in the same manner as the aortas. For statistical purposes, the percent plaque scores from the right and left arteries were averaged for each animal. The carotid bifurcations were graded on a 0 to 5 scale as follows: 0 = no lesion; 1 = fatty streaks present; 2 = extensive fatty streaks; 3 = plaque present; 4 = extensive plaque present; 5 = complicated plaque.

Statistical Analyses

For statistical purposes, we derived a coronary index (CI), which is the mean percentage of the area bounded by the IEL that was occupied by an atherosclerotic lesion ("% stenosis"). We also derived an aortic index (AI), which is the total percentage of the intimal surface covered by raised lesions. Regression and correlation analyses and analysis of variance were carried out essentially as described by Snedecor and Cochran.
Results

Serum Lipid Values

Serum cholesterol and triglyceride concentrations were measured bimonthly throughout the study. For the Lo, Hi, Lo,E, and Hi,E groups, the mean (± SEM) values for cholesterol concentrations were 308 ± 9; 594 ± 13; 365 ± 22; and 949 ± 88; and for triglyceride concentrations, 14 ± 2; 14 ± 2; 96 ± 7; and 200 ± 30 respectively. Dietary cholesterol stimulated a significant increase in serum cholesterol concentration, and an interaction between dietary cholesterol and ethanol occurred, resulting in the highest values in the Hi,E group. Ethanol stimulated a significant increase in serum triglyceride concentration, and again an interaction occurred resulting in the highest values in the Hi,E group.

Lipoprotein Distribution

Agarose gel chromatography elution profiles are shown for representative animals from each diet group in figure 1. In general, four size populations were found, which were pooled separately for further analyses. More material appeared in Regions I, II, and III than in Region IV for both the high cholesterol diet groups and the Lo group. Relatively more Region IV material is apparent for the Lo,E group, and the position of this peak indicates these lipoproteins are larger than Region IV lipoproteins of the other diets. The position of the peak of Region III indicated that these lipoproteins were significantly larger in the Hi group than was the case for the other diets.

The material in Region II was incompletely separated from that of adjacent regions. Thus, further separation of this material was carried out using density centrifugation (table 1). The Region II lipoproteins were predominantly intermediate density lipoproteins (1.006 < d < 1.019), although significant amounts of VLDL (d < 1.006) were also present. Relatively more VLDL was present in the Region II lipoproteins of ethanol-fed groups than in those of groups receiving no ethanol (table 1). All of the Region I material for all groups was VLDL (i.e., floated at d < 1.006 g/ml). More than 80% of the Region III material floated in the LDL density range (1.019 < d < 1.063) for all diet groups except the group that had high cholesterol and no ethanol. In this group, the fraction of the lower density material (1.006 < d < 1.019) increased proportionally to the increase in size, in mw.

To quantitate the effects of diet on lipoprotein distribution, chemical compositions for each of the separate fractions were determined, and the concentrations of individual lipoproteins for each animal were then calculated. Recovery of cholesterol during lipoprotein isolation was monitored in each case, and values were corrected to 100% recovery of cholesterol; cholesterol recovery from the agarose column was consistently over 90%. Mean values for each diet group are shown in figure 2. The concentrations of lipoproteins in all regions were higher in the animals given ethanol than for the respective nonethanol groups. The Region III lipoproteins were present in higher concentrations and the Region IV lipo-
proteins were present in lower concentrations in the Hi groups compared to the respective Lo groups. Two-way analysis of variance was used to show that these effects of ethanol and dietary cholesterol on lipoprotein concentrations were statistically significant (p < 0.01).

Region I and II Lipoproteins

The percentage compositions of the Region I and II lipoproteins were determined to monitor dietary influences on these lipoproteins (figure 3). Significant differences between diet groups were found. The highest percentage of triglycerides was found in the ethanol-treated groups, with the highest percentage of cholesterol ester being found in the Hi,E groups. The percentage of triglycerides was highest in the Region I (VLDL), and progressively decreased in Region II, and LDL lipoproteins for all diet groups. The percentage of cholesterol ester was inversely related to the percentage of triglycerides for all fractions. This pattern occurred in both size populations that were VDL (Region I and II) and in the intermediate-sized low density lipoproteins (ILDL, Region II).

Region III Lipoproteins

The Region III lipoproteins were isolated as a size population, were found to float in the 1.006 to 1.063 density range, and comprised a major subpopulation of LDL. Other lipoproteins in the LDL density range were larger and eluted from the column in Region II (see table 1), but the column excluded these larger sized subfractions of LDL from Region III. We will use the term "LDL" to mean only those lipoproteins of Region III. Table 2 summarizes the data on the effects of dietary cholesterol and ethanol on Region III lipoproteins. Concentrations of these lipoproteins, whether expressed in terms of cholesterol, total mass, or molarity, were increased by dietary cholesterol and ethanol. Two-way analysis of variance showed that the concentration increases due to both ethanol and cholesterol were statistically significant (p < 0.002) and that a significant interaction (p < 0.02) between these dietary factors was also present. The significant interaction term is due to LDL concentration being increased out of proportion to the effects of

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Cholesterol (mg/dl)</th>
<th>Total mass (mg/dl)</th>
<th>Particle (μM)</th>
<th>Mol wt (×10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lo,E</td>
<td>226 ± 27</td>
<td>606 ± 85</td>
<td>1.80 ± 0.24</td>
<td>3.37 ± 0.11</td>
</tr>
<tr>
<td>Hi,E</td>
<td>568 ± 10</td>
<td>1644 ± 62</td>
<td>4.44 ± 0.22</td>
<td>3.72 ± 0.13</td>
</tr>
<tr>
<td>Lo</td>
<td>220 ± 63</td>
<td>504 ± 137</td>
<td>1.26 ± 0.30</td>
<td>3.88 ± 0.30</td>
</tr>
<tr>
<td>Hi</td>
<td>448 ± 67</td>
<td>1034 ± 147</td>
<td>1.95 ± 0.18</td>
<td>5.18 ± 0.30</td>
</tr>
</tbody>
</table>

All values are mean ± SEM for samples from all of the animals in each group. Abbreviations are as in table 1.

Figure 3. Comparison of effects of diets on the percentage compositions of Region I and II lipoproteins. TG = triacylglycerol; CE = cholesterol esters; PL = phospholipids; FC = free cholesterol; Pro = protein. Bars represent mean values on at least two pooled samples per diet group.
ethanol or cholesterol alone in the Hi,E group. In addition to the effects of LDL concentration, dietary cholesterol increase resulted in a significantly ($p < 0.001$) larger LDL mw. In contrast, dietary ethanol resulted in a significantly ($p < 0.005$) lower LDL mw. A significant ($p < 0.05$) interaction term was present, indicating that the LDL mw of the Hi,E group was lower than would be expected based on the effect of ethanol in the lower cholesterol group.

We examined the chemical composition changes that accompanied the mw increase in LDL, determined the percentage compositions for the LDL preparations from each animal, and then calculated the number of molecules of individual constituents based on the mw of the LDL particles. Figure 4 shows the mean values for each of the diet groups. Differences in composition due to diet existed and are clearly correlated to those of LDL mw. The cholesterol esters and free cholesterol of the largest LDL were increased to the greatest extent, while phospholipid and protein were increased to a lesser degree. Increased dietary cholesterol resulted in a per particle increase in each of these constituents, while ethanol consumption resulted in lower values.

The increase in cholesterol esters was then analyzed on an individual ester basis (table 3). Ethanol-fed animals had LDL with a higher proportion of cholesteryl linoleate and lower proportion of cholesteryl oleate than was found in animals not fed ethanol. The data for individual animals were plotted as shown in figure 5. Clearly, the predominant factor in the proportionality between LDL cholesterol ester content and mw was the increase in monounsaturated choles-

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Comparison of the diet effects on the within-particle composition of Region III LDL. Abbreviations are given in legend for figure 3.

### Table 3. Influence of Diet on Cholesteryl Ester Fatty Acids of Plasma Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL)

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Fatty acids (molar percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14:0</td>
</tr>
<tr>
<td>Lo,E</td>
<td>LDL</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
</tr>
<tr>
<td>Hi,E</td>
<td>LDL</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
</tr>
<tr>
<td>Lo</td>
<td>LDL</td>
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<td></td>
<td>HDL</td>
</tr>
<tr>
<td>Hi</td>
<td>LDL</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
</tr>
</tbody>
</table>

All values represent means for determinations on LDL samples from all of the animals of each group. Insufficient amounts of HDL were available to complete these evaluations in all animals; consequently only the HDL samples from 5, 3, 3, and 3 animals for Lo,E, Hi,E, Lo, and Hi groups, respectively, were evaluated. Abbreviations as in table 1.
animals; other differences were small and not statistically significant. We also measured the cholesterol ester content of the HDL. The data in table 3 show that there was an effect on cholesterol ester fatty acids similar to that on those of LDL (i.e., more cholesteryl oleate than cholesteryl linoleate was present in the HDL of animals that had a large LDL mw, namely, the Hi group). The Lo,E animals had the lowest LDL mw and more cholesteryl linoleate than cholesteryl oleate. A significant correlation between LDL mw and HDL cholesterol ester fatty acid pattern (CEFA, expressed as \( \Delta 2 + \Delta 1 + \Delta 0 \)) was found \( (r = 0.8, p < 0.005) \). The level of HDL was also found to be inversely related to the LDL mw \( (r = -0.7, p < 0.01) \). These data suggest that the metabolism of HDL is linked to that of LDL; dietary cholesterol and ethanol apparently exert opposite effects on the HDL levels and on the HDL cholesterol ester composition.

### Apolipoproteins

The apolipoprotein patterns were determined using SDS-PAGE analyses for each of the lipoprotein fractions for individual animals from each of the diet groups. The data shown in figure 7 are representative. For individual lipoprotein classes, patterns for samples from animals within each of the diet groups were similar. Perhaps the most noticeable difference was in the apo E content of Region I and Region II lipoproteins of the animals fed the Hi diets, compared to those receiving the Lo diets. Ethanol-fed animals

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**Figure 5.** Relationship between the LDL molecular weight and the within-particle content of individual cholesterol esters. Data for all of the individual animals are shown in regression lines. \( \Delta 0 \) refers to the sum of cholesteryl myristate, palmitate, and stearate; \( \Delta 1 \) refers to the sum of cholesteryl palmitoleate and oleate; \( \Delta 2 \) refers to the sum of cholesteryl linoleate, linolenate, and arachidonate.

**Figure 6.** Comparison of the diet effects on the percentage compositions of Region IV lipoproteins (HDL). Mean values are shown; no significant differences were found among the groups. Abbreviations are given in legend for figure 3.
Figure 7. Comparison of the apolipoprotein patterns for lipoprotein fractions isolated from representative animals in two diet groups. Apolipoproteins were separated by electrophoresis in horizontal 12% polyacrylamide slab gels carried out in the presence of 0.1% SDS. A 5% polyacrylamide spacer gel was present at the top of the gel. Lipoprotein fractions and diet group designations are described in the legends for figures 1 and 2. Apolipoprotein designations are by letter: B = apo B; E = apo E (argin-rich); Al = apo A-I; C = apo C; A-II = apo A-II. The sample well at the origin is designated 0. The position of each of the apolipoproteins was identified by using authentic, purified apolipoproteins.

appeared to have more apoprotein C (apo C) in the Region I and II lipoproteins than those that did not receive ethanol (data not shown). Animals fed the Hi vs Lo diets had relatively less apo C in the Region I and II lipoproteins. Apo E was not seen in any of the HDL fractions. The relatively high apo A-I seen in the Region I lipoprotein sample of the animal from the Lo,E diet group was not seen for any other animal.

Atherosclerosis Evaluations

Data for the averaged measurements of coronary artery and aortic atherosclerosis are shown in tables 4 and 5. Diet had an effect on the extent of atherosclerosis in both arterial systems, but the effect was not the same for both. We found that elevated levels of dietary cholesterol resulted in an increased coronary index (CI); this increase was significant in the Hi groups but not in the Hi,E group. Ethanol significantly reduced the level of cholesterol-induced coronary artery atherosclerosis so that the Hi group had a significantly higher CI (p < 0.005, analysis of variance) than the other three groups (table 4). Ethanol also reduced the percent of foam cells in the coronary lesions of both Hi and Lo groups (p < 0.001). Medial and internal elastic lamina damage was highly correlated with the CI, and the diet effects were the same for these measurements as for CI (table 4). An example of the differences seen in coronary artery atherosclerosis is shown in figure 8.

The effects on aortic atherosclerosis of differing levels of dietary cholesterol and the presence of ethanol are shown in table 5. More than one type of lesion was seen, and the percentage of intimal surface area involved with each type was evaluated. The percentage of intimal surface affected with atherosclerotic plaques (i.e., raised lesions) was termed the "aortic index (Al)" and was used for statistical comparisons. In contrast to the situation for coronary artery atherosclerosis, the data for Al showed that a significant increase in severity occurred due to the increased level of dietary cholesterol (p < 0.001, analysis of variance) even in the presence of dietary ethanol (table 5). A significant decrease in Al due to ethanol (p < 0.025, analysis of variance) was also found. When percentage num-

Table 4. Effect of Ethanol on Diet-Induced Coronary Artery Atherosclerosis among Macaca nemestrina

<table>
<thead>
<tr>
<th>Percentage of intimal surface area</th>
<th>Lo (n = 5)</th>
<th>Hi (n = 6)</th>
<th>Lo,E (n = 5)</th>
<th>Hi,E (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary index*</td>
<td>7 ± 2</td>
<td>45 ± 2</td>
<td>2 ± 1</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Foam cells in plaque (%)</td>
<td>62 ± 8</td>
<td>52 ± 4</td>
<td>30 ± 8</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>Damaged media</td>
<td>2 ± 1</td>
<td>30 ± 8</td>
<td>9 ± 4</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Damaged internal elastic lamina</td>
<td>8 ± 4</td>
<td>37 ± 7</td>
<td>14 ± 5</td>
<td>11 ± 4</td>
</tr>
</tbody>
</table>

*The mean percentage of the area bounded by the internal elastic lamina that was occupied by an atherosclerotic lesion. Determined from 16 sections from each animal. Lo, Hi, Lo,E, Hi,E indicate diet groups (see table 1).

All values, mean ± SEM.
bers are used, as in the measurements of the intimal surface area shown in table 5, it should be remembered that, as the severity increases, interrelationships are implied by the method of estimation (i.e., as any one measurement or set of measurements approaches 100%, the other values have to approach zero).

Atherosclerosis in the common carotid artery, although lower in the total percentage of intimal surface involved, was distributed among the groups about the same as atherosclerosis in the aorta. Carotid bifurcation data were derived by averaging the scores from the left and right sides for each animal. In this site an effect of ethanol was not detected; the Lo and Lo,E scores were nearly identical (2 ± 0.6; 1.8 ± 0.6) and were lower than the Hi and Hi,E groups (3.4 ± 0.2; 3.7 ± 0.3).

Among the 19 animals, the posterior interventricular septum was supplied by the left circumflex (LCX) artery in 12 animals, the right coronary artery (RCA) in five animals, and by branches of about equal size from both the LCX and RCA in two animals. There was no apparent relationship between these patterns of coronary circulation and atherosclerosis. The section of coronary artery with the greatest percent of stenosis was from the left anterior descending (LAD) artery in about 50% of the animals, from the LCX in 40%, and from the RCA in 10% of the animals. When all sections from all animals were considered, there was a trend for stenosis to be more severe in the order, LCX > LAD > RCA > LMC, but single samples from an artery or a site in an artery would not have predicted the order of scores determined from all sites.

Table 5. Effect of Ethanol on Diet-Induced Abdominal Aortic Atherosclerosis among Macaca nemestrina

<table>
<thead>
<tr>
<th></th>
<th>Percentage of intimal surface area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lo (n = 5)</td>
</tr>
<tr>
<td>Fatty streak</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>[1]</td>
<td></td>
</tr>
<tr>
<td>DIT</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>[2]</td>
<td></td>
</tr>
<tr>
<td>Fatty plaque</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>[3]</td>
<td></td>
</tr>
<tr>
<td>Fibrous plaque</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>[4]</td>
<td></td>
</tr>
<tr>
<td>Total plaque*</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>[3+4]</td>
<td></td>
</tr>
<tr>
<td>Total sudano-</td>
<td>35 ± 11</td>
</tr>
<tr>
<td>philia</td>
<td>[1+3]</td>
</tr>
<tr>
<td>Total lesion</td>
<td>71 ± 9</td>
</tr>
<tr>
<td>[1+2+3+4]</td>
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</table>

The numbers in brackets identify the type of measurement. All values are mean ± SEM. DIT = diffuse intimal thickening (non-sudanophilic); other abbreviations are as in table 1. *Aortic index (AI).

Lipid accumulation in the heart valves was associated with cholesterol consumption and did not appear to be greatly affected by alcohol consumption. We found that 2 of 5 Lo, 0 of 5 Lo,E, 5 of 6 Hi, and 2 of 3 Hi,E animals had grossly detectable lipid accumulation in one or more heart valves.

Figure 8. Representative photomicrographs of coronary arteries showing diet effects. A. Animal fed high cholesterol diet alone. The artery has a relatively thick plaque which involves most of the circumference of the vessel. B. Animal fed the high cholesterol diet plus ethanol. The artery has only a small, focal lesion.
Table 6. Simple Correlation Coefficients for Measurements of Plasma Lipoproteins Versus Those of Atherosclerosis

| Predictors | No. | Coronary Index | | Aortic index | |
|------------|-----|----------------|----------------|----------------|
|            |     | Correlation Coefficient | Significance | Correlation Coefficient | Significance |
| Lipoproteins |     |               |               |               |               |
| LDL (µM) | 19  | -0.18 | ns | 0.43 | ns |
| HDL (mg/dl)^-1 | 19 | 0.60 | p < 0.01 | 0.37 | ns |
| LDL mol wt | 19 | 0.71 | p < 0.001 | 0.72 | p < 0.001 |
| LDL CEFA ratio† | 19 | 0.81 | p < 0.0001 | 0.53 | p < 0.02 |
| Lipids |     |               |               |               |               |
| Cholesterol (mg/dl) | 19 | 0.17 | ns | 0.72 | p < 0.001 |
| Triglycerides (mg/dl) | 19 | -0.48 | p < 0.05 | 0.09 | ns |

*Evaluated by the test statistic t = r√[(N−2)/(1−r^2)]. ns = not significant at the p < 0.05 level.
†The ratio was calculated from the molar distribution of cholesterol esters (see table 3) in which the value for polyunsaturated cholesterol esters was divided by the sum of the values for saturated and monounsaturated cholesterol esters. Therefore, this ratio was based on the cholesterol ester fatty acids (CEFA).

Lipoprotein Relationships to Atherosclerosis

Since lipoprotein and atherosclerosis measurements were available in each of the animals of the study, we were able to relate the extent of atherosclerosis to the lipoprotein measurements. The effects of diet gave a wide range in the CI and AI and in values for lipoproteins, suggesting that correlation and regression analyses would be appropriate to establish relationships. Table 6 shows the correlation coefficients found for the lipoprotein measurements vs the CI. Statistically significant correlations were found for HDL expressed as the inverse of mass concentration, for LDL mw, and for the cholesterol ester fatty acid (CEFA) ratio of LDL. Surprisingly, no significant correlation existed between the CI and the LDL molar concentration (number of LDL particles per milliliter of plasma). When multiple regression analysis was used to further clarify the relationships, it was found that the LDL CEFA ratio explained the maximum amount of variance in the CI (R^2 = 0.62) and that the other measurements did not contribute additional significance to the regression coefficient R^2. This is due to the highly significant relationships between the LDL CEFA ratio and the inverse of the HDL concentration (r = 0.73) and LDL mw (r = 0.86). Significant correlations were not found between CI and Region I and II lipoprotein concentrations. For comparison, the relationship of the CI to plasma cholesterol and triglyceride concentrations was determined. An interesting finding was the significant negative correlation of CI with triglyceride concentration. No significant correlation between CI and plasma cholesterol concentration was found.

The relationships between AI and plasma lipoproteins were not the same as those found for the CI, as shown in table 6. For the lipoprotein measurements, only LDL molecular weight and LDL CEFA ratio were significantly correlated to AI. The correlation coefficients for LDL molar concentrations and the inverse of the HDL concentration did not reach the 5% confidence limit for statistical significance, although they were close (p < 0.07 and p < 0.10 respectively). When multiple regression analysis was performed to evaluate measurements important in explaining the AI variability, both LDL mw and LDL CEFA ratio contributed significantly to the regression equation. Together, the R^2 for these measurements was 0.53, meaning that they explained about 53% of the AI variability. The R^2 increased to 0.64 when the inverse of the HDL concentration was added to the regression equation. When the correlation of AI with plasma cholesterol and triglyceride concentrations was examined for comparison to the lipoprotein measurements, a highly significant correlation between AI and cholesterol concentration was found.

Discussion

These data indicate that ethanol modified the metabolic response of both the plasma lipoproteins and atherosclerosis to dietary cholesterol. The alcohol-induced modification resulted in a LDL of lower molecular weight and in higher HDL levels. These changes appear to be linked through an alteration in cholesterol ester metabolism, resulting in an increased lipoprotein content of cholesteryl oleate proportional to LDL size and to the inverse of HDL concentration. The corollary to the change in plasma lipoproteins was alcohol's effect on atherosclerosis. Ethanol appeared to prevent much of the cholesterol-induced coronary artery atherosclerosis, and ethanol-fed animals had less aortic atherosclerosis than those not receiving ethanol.
The influence of ethanol on atherosclerosis was not the same for these different arteries, nor were the same relationships found between the plasma lipoproteins and the severity of atherosclerosis for these arteries. The increased LDL size was highly correlated with CI in a fashion similar to that found in our previous studies. However, in the present study the cholesterol ester composition of the LDL, expressed as the CEFA ratio (\(\Delta 2+/\Delta 0 + \Delta 1\)), was even more highly correlated, and with multiple regression analysis it was shown to account for all of the variability in the relationship of LDL mw (and of HDL concentration) to the CI of atherosclerosis. In this study, as earlier, we found that the LDL molar concentration (i.e., the number of LDL particles per milliliter of plasma) was not correlated with the CI. In contrast, LDL mw was the lipoprotein measurement most highly correlated to the AI, being higher than for the LDL CEFA ratio. The correlation between the inverse HDL concentration and AI was not statistically significant, in contrast to the situation for CI. Although cause and effect relationships cannot be assigned from these data, the results strongly suggest the possibility that dietary ethanol and cholesterol are influencing the extent of atherosclerosis through their effect on plasma LDL composition and on HDL levels. The very high correlation coefficients between the lipoprotein and atherosclerosis measurements, the discovery of which was made possible by studying both aspects in each experimental animal, emphasize the likelihood of this possibility.

An interaction occurred between ethanol and dietary cholesterol in producing hyperlipoproteinemia. The group with the highest serum cholesterol and triglyceride concentrations was the Hi,E group. The effect was more than that of ethanol or cholesterol alone, and resulted in a significant interaction term in the two-way analysis of variance. When the plasma lipoprotein distribution for this group was determined (figure 2), it was seen that the hyperlipoproteinemia resulted from an elevation of all lipoprotein classes except HDL. The lipoprotein fraction that increased the most was the LDL of Region III, the mass concentration of which was 1644 mg/dl, significantly higher than that of any other group. The extent of this increase was apparently due to the interaction of cholesterol and ethanol, as evidenced by the significant interaction term in the analysis of variance, and was higher than would be expected due to ethanol or cholesterol alone. These LDLs were intermediate in size, and had a significantly lower mw than those of the Hi group. As a result, the micromolar (\(\mu \text{M}\)) concentration for the Hi,E group was the highest of any group, with the mean value being more than twice that of any other group. These data appear to define the effects of dietary ethanol and cholesterol on LDL as being distinct. It appears that one effect of ethanol is blocking of the dietary cholesterol-induced LDL mw increase, at the same time that ethanol's primary effect seems to be stimulation of an increase in the number of LDL particles in plasma.

The mechanisms by which these factors exert their influence are as yet unclear. For LDL mw increases, the data suggest that cholesterol ester metabolism may be involved. Dietary cholesterol had the effect of increasing LDL choleseryl oleate content in proportion to LDL size; ethanol seemed to limit the extent to which LDL cholesteryl oleate content increased and to limit LDL size. An increase in the cholesteryl oleate content of LDL will change the physical state of the cholesteryl ester phase, a factor that may have significance with regard to the eventual rates of LDL catabolism, but that presumably is a result of an alteration in synthesis. We have postulated that the origin of much of the LDL cholesteryl oleate is from synthesis in a tissue, with subsequent transport into plasma in chylomicra (intestine) or VLDL (liver), which is then subsequently degraded to LDL. Should this represent the pathway by which LDL cholesterol esters are derived in cholesteryl-fed monkeys, we would expect dietary ethanol to influence cholesterol ester metabolism in the liver or the gut. We measured the liver CEFA patterns in the animals of the present study. Although small differences were found, with the highest percentage of cholesteryl oleate being present in the livers of the Hi group, differences among diet groups reflective of those of plasma LDL were not seen. In the animals of the diet group with large mw LDL, the fasting plasma LDL and HDL showed a higher percentage of cholesterol esters (figure 3) than was the case for the other groups. Presumably, these lipoproteins of fasting plasma are of liver origin. Their increased cholesterol ester content could lead to LDL of increased cholesterol ester content. Unfortunately, limited amounts of these lipoproteins were available and their CEFA patterns were not determined.

Little is known about the cholesteryl esters derived from the gut in monkeys. If an analogy exists with species such as the rat, in which cholesteryl oleate is the preferred product of intestinal acyl CoA cholesterol acyl transferase, an increase in the cholesterol ester content of chylomicra via cholesterol feeding would result in an increase in the cholesteryl oleate content. If chylomicra degradation results in LDL formation, as has been proposed for VLDL, it is possible that the increased cholesteryl oleate of large mw is derived from this source. For ethanol to exert its effect in this scheme, it would need to decrease the content of cholesteryl oleate and other cholesterol esters in chylomicra. No data supporting such a possibility are available.
This study was not designed to assess the risk-to-benefit ratio of alcohol consumption, although the findings are consistent with a protective effect of alcohol, especially on coronary atherosclerosis. It may be that, while ethanol would have the effect of reducing coronary atherosclerosis, the cerebral circulation may not be protected. There was little indication that atherosclerosis of the carotid bifurcation was reduced by ethanol consumption, but the time course of this study was too short for intracranial artery atherosclerosis to develop, or for full evaluation of the effect of alcohol on the liver and other organs. The need for further work in this area is readily apparent. Other important areas needing study include determination of the lowest level of ethanol consumption that has the beneficial effect on coronary atherosclerosis. The work of other investigators has suggested that, in most species of experimental animals, 7% to 10% of calories as ethanol has little effect on coronary atherosclerosis. If this is true for Macaca nemestrina, the lowest dose would be somewhere between this and the 36% of calories used in the present study. Some epidemiological studies suggest that females may be more susceptible than males to the adverse effects of ethanol. There may be important differences between the sexes in the lipid metabolic and arterial responses, as well.

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References

8. Bond MG, Bullock BC, Clarkson TB. Diet induced coronary atherosclerosis of pig-tailed macaques. Am J Pathol 1976;82.68a
13. Leathers CW. Pathologic effects of dietary cholesterol and ethanol in Macaca nemestrina (PhD thesis). Winston-Salem, North Carolina: Bowman Gray School of Medicine, Wake Forest University, 1977
27. Eisenberg S, Bilheimer DW, Levy RI, Lindgren FT. On the metabolic conversion of human plasma very low density lipoprotein to low density lipoprotein. Biochim Biophys Acts 1973;326.361-377

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