The epidemiological evidence that moderate, daily consumption of ethanol decreases the risk of the thromboembolic complications of coronary artery disease\(^{1,2}\) has led to interest in the ways in which ethanol may exert this beneficial effect. Suggestions include personality or lifestyle differences between moderate drinkers and nondrinkers, an increase in the high density lipoprotein to low density lipoprotein ratio in those consuming ethanol, increased plasma prostacyclin levels in association with the intake of ethanol, and direct or indirect inhibition by ethanol of platelet functions.\(^{3-5}\) Ethanol inhibits platelet responses to some agonists in vitro\(^{6-10}\) and ex vivo in blood from humans\(^{11}\) and experimental animals.\(^{12}\) Recently, ethanol has been shown to inhibit experimentally induced arterial thrombus formation in rabbits.\(^{13,14}\) Ethanol also inhibits shear-induced thrombosis in injured, stenosed coronary arteries of dogs.\(^{15}\)

In the prediction of the risk of mortality due to coronary artery disease, the possibility of an interaction between alcohol and dietary fats has been considered.\(^{16}\) Some investigators have explored the effects of ethanol on the functions of platelets enriched in saturated fats and have reported that ethanol has a greater inhibitory effect than on the functions of control platelets.\(^{8,17-19}\) However, the effects of ethanol on platelet function in hypercholesterolemia have not been previously investigated, although hypercholesterolemia is well established as a major risk factor in atherosclerosis and its clinical complications.\(^{20}\)

Several groups,\(^{21-24}\) including ourselves,\(^{25}\) have demonstrated an enhancement of platelet responses to some agonists in diet-induced hypercholesterolemia in rabbits, in which the plasma level of very low density lipoprotein is elevated.\(^{26}\) Aggregation and secretion in response to low concentrations of collagen\(^{21-25}\) or thrombin\(^{21,22,25}\) (dependent or independent of thromboxane \(A_2\) [TxA\(_2\)]) are potentiated by diet-induced hypercholesterolemia. In contrast, we found that the genetically determined hypercholesterolemia in Watanabe heritable hyperlipidemic (WHHL) rabbits, in...
which plasma levels of low density lipoprotein are elevated, produces quite different effects. Platelets from WHHL rabbits show reduced responses to agonists such as collagen that act through the generation of TxA2, and responses to thrombin do not differ from the responses of control platelets. Because these platelet functions were investigated with washed platelets in an artificial medium, plasma lipoproteins were not present during the aggregation experiments and thus could have been directly responsible for the differences. This hyposensitivity of washed platelets from WHHL rabbits to collagen but not to thrombin has recently been confirmed by Biancardi and associates. We showed previously that the free cholesterol to phospholipid molar ratio of the platelets was increased in diet-induced hypercholesterolemia but not in genetically determined hypercholesterolemia; an increased cholesterol content of platelets is associated with platelet hypersensitivity.

The purpose of the present study was to investigate and compare the in vitro effects of ethanol on the responses of washed platelets from rabbits with diet-induced hypercholesterolemia and from WHHL rabbits. Aggregation and release of amine-storage granule contents were measured after stimulation with a range of concentrations of ADP, collagen, the TxA2 mimic U46619, and low concentrations of thrombin. In some experiments, aspirin-pretreated platelets were used to explore the influence of hypercholesterolemia on the inhibitory effects of ethanol that are independent of TxA2 formation. The use of washed platelets instead of platelet-rich plasma made it possible to study platelet aggregation without the confounding effects of opaque hypercholesterolemic plasma.

Methods

Animals and Diets

Male New Zealand White (NZW) rabbits (2.6±0.1 kg, mean±SD, n=30) were fed a regular chow diet (150 g/day, Purina Rabbit Chow Checkers Performance Blend, Ralston Purina Canada Inc., Longueuil, Canada) for a 1-week run-in period. Then, starting at week 0, half of the rabbits remained on the regular chow diet (control diet that contained negligible amounts of cholesterol) for a further 8 weeks, while the others were fed a diet enriched with 0.25% (wt/wt) cholesterol (Sigma Chemical Co., St. Louis, Mo.) for a further 8 weeks. The cholesterol content of platelets was labeled with [14C]serotonin (as 5-hydroxy[3'-14C]tryptamine creatinine sulfate, 60 mCi/mmol, Amersham Corp., Oakville, Canada; 0.05 μCi/ml platelet suspension). The extent of uptake of the radioisotope (80%) was similar in all groups of rabbits. For the preparation of aspirin-treated platelets, 500 μM aspirin (Sigma) was included in the first washing fluid. (Aspirin-treated platelets did not aggregate in response to collagen [1 μg/ml].) Platelet suspensions (0.5×10^9/ml in Tyrode's solution containing 0.35% albumin, 5 μM imipramine [Geigy Canada, Dorval, Canada], and apyrase, pH 7.35) were incubated at 37°C for at least 20 minutes before testing. Platelet aggregation was initiated by the following agonists: ADP (Sigma); acid-soluble collagen prepared from bovine tendon collagen (Sigma); U46619 (a generous gift of The Upjohn Co., Kalamazoo, Mich.); and thrombin (bovine thrombin [topical], Parke-Davis, Scarborough, Canada). Ethanol (final concentration, 4 mg/ml) or saline was added 2 minutes before the aggregating agent. (This concentration of ethanol is physiologically tolerable, and previous experiments have shown that when platelet aggregation is inhibited by ethanol, inhibition is consistent and marked at 4 mg/ml.) Aggregation was studied at 37°C in an aggregometer (Payton Associates, Scarborough, Canada). For all of the experiments with U46619 and some of the experiments with thrombin, the platelets were pretreated with aspirin as described above. The extent of aggregation was calculated as previously described, setting 100% aggregation as the maximum possible increase in light transmission with the platelet-suspending medium. Three minutes after the addition of ADP or U46619 or 5 minutes after the addition of collagen or thrombin, supernatant samples were prepared by centrifugation of the platelet suspension for 1 minute at 12,000 g in an Eppendorf centrifuge. These samples were used to measure secretion of [14C]serotonin from prelabeled platelets. In all experiments, platelets from a hypercholesterolemic rabbit and a control rabbit were tested on the same day with the same reagents.

Statistical Analyses

Values are reported as mean±SEM, with the number of experiments indicated. Nonorthogonal two-way analyses of variance were used to determine differences in
responses of the platelets. Differences were deemed to be statistically significant when \( p < 0.05 \).

**Results**

**Diet-Induced Hypercholesterolemia**

As reported previously,\(^\text{25}\) the plasma cholesterol of rabbits fed the cholesterol-enriched diet had reached a steady-state level of 15.2±1.4 mM (\( n = 15 \)) by 8 weeks, whereas the plasma cholesterol of the control rabbits was unchanged (1.5±0.1 mM, \( n = 15 \)). Platelet characteristics have been reported elsewhere\(^\text{25}\); the free cholesterol to phospholipid molar ratio of the platelets increased from 0.60±0.04 to 0.68±0.05 (\( p < 0.05 \), \( n = 9 \), paired \( t \) test) during the 8-week cholesterol-feeding period.

**Responses of platelets to ADP.** With platelets from the cholesterol-fed and the control rabbits, aggregation induced by ADP (1–5 \( \mu \text{M} \)) was essentially identical and was not affected by the presence of 4 mg/ml ethanol (data not shown). ADP did not induce appreciable secretion of \( [\text{\^{14}C}]\)serotonin from the platelets of either cholesterol-fed or control rabbits.

**Responses of platelets to collagen (Figure 1).** As shown previously,\(^\text{25}\) platelets from cholesterol-fed rabbits aggregated more extensively (Figure 1A) and secreted a higher percentage of \( [\text{\^{14}C}]\)serotonin (Figure 1B) in response to collagen than did platelets from control rabbits. With platelets from both groups of rabbits, ethanol inhibited aggregation and secretion (Figures 1A and 1B). To determine whether the extent of inhibition by ethanol was different with platelets from cholesterol-fed rabbits in comparison with platelets from control rabbits, inhibition was expressed as a percentage at equal extents of aggregation for each rabbit. Inhibition by ethanol of collagen-induced aggregation expressed in this way showed no difference between platelets from cholesterol-fed rabbits and control rabbits (Figure 1C).

**Responses of platelets to U46619.** In response to the TxA\(_2\) mimetic U46619, aspirin-pretreated platelets from cholesterol-fed rabbits aggregated more extensively than did aspirin-pretreated platelets from control rabbits.\(^\text{25}\) Ethanol did not significantly affect either the extent of aggregation or the secretion of granule contents induced by U46619 with platelets from either group of rabbits (data not shown).

**Responses of platelets to thrombin (Figures 2 and 3).** As shown previously,\(^\text{25}\) platelets from cholesterol-fed rabbits aggregated more extensively (Figure 2A) and secreted a higher percentage of \( [\text{\^{14}C}]\)serotonin (Figure 2B)
than did platelets from control rabbits in response to low concentrations of thrombin. Ethanol inhibited thrombin-induced aggregation and secretion from the platelets of both groups of rabbits (Figures 2A and 2B). Inhibition expressed at equal extents of aggregation was less with platelets from cholesterol-fed rabbits than with platelets from control rabbits (Figure 2C). Ethanol also inhibited thromboxane-independent thrombin-induced aggregation (Figure 3A) and secretion of [14C]serotonin (Figure 3B) from aspirin-pretreated platelets from both groups of rabbits. Again, inhibition of aggregation was significantly less with platelets from cholesterol-fed rabbits than with platelets from control rabbits (Figure 3C).

Watanabe Heritable Hyperlipidemic Rabbits

As reported elsewhere, the plasma cholesterol of the WHHL rabbits was 13.9±1.7 mM (n=7), whereas the plasma cholesterol of the control rabbits was 2.2±0.3 mM (n=7). Platelet characteristics have been reported previously; the free cholesterol to phospholipid molar ratios were not significantly different between platelets from WHHL and control rabbits.

Responses of platelets to ADP. Aggregation induced by ADP (1–5 μM) of platelets from WHHL rabbits and control rabbits did not differ and was unchanged by 4 mg/ml ethanol (data not shown). Appreciable secretion of [14C]serotonin in response to ADP did not occur with platelets from either group of rabbits.

Responses of platelets to collagen (Figure 4). As shown previously, platelets from WHHL rabbits aggregated less extensively (Figure 4A) and secreted a lower percentage of [14C]serotonin (Figure 4B) than did platelets from control rabbits. With platelets from both groups of rabbits, ethanol inhibited aggregation and secretion (Figures 4A and 4B).

Responses of platelets to U46619. Aggregation and secretion of granule contents stimulated by U46619 were less extensive in aspirin-pretreated platelets from WHHL rabbits than in aspirin-pretreated platelets from control rabbits. Ethanol did not significantly inhibit U46619-induced aggregation or secretion by aspirin-pretreated platelets from either group (data not shown).

Responses of platelets to thrombin (Figures 5 and 6). As shown previously, platelets from WHHL rabbits did not differ significantly from platelets from control rabbits in their responses to low concentrations of thrombin (Figures 5A and 5B). Ethanol inhibited aggregation (Figure 5A) and secretion of [14C]serotonin (Figure 5B) induced by low concentrations of thrombin with both WHHL rabbits and control rabbits. However, when inhibition was expressed at equal extents of aggregation (Figure 5C), the extent of inhibition did not differ between platelets from WHHL rabbits and platelets from control rabbits. Similar results were obtained for thrombin-induced aggregation and secretion by aspirin-pretreated platelets (Figures 6A and 6B). Although ethanol was inhibitory, the extent of inhibition was similar with platelets from the two groups of rabbits (Figure 6C).
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FIGURE 3. Inhibition by ethanol (4 mg/ml) of thrombin-induced responses of aspirin-pretreated platelets from cholesterol-fed and control rabbits. Panel A: Bar graph showing inhibition of the extent of aggregation of aspirin-pretreated platelets 5 minutes after the addition of thrombin to platelets from cholesterol-fed and control rabbits (n=5, p<0.001 for inhibition by ethanol for both groups). Panel B: Bar graph showing inhibition of the extent of secretion of [14C]serotonin 5 minutes after the addition of thrombin to platelets from cholesterol-fed and control rabbits (n=5). Panel C: Line plot showing that inhibition by ethanol at equal extents of thrombin-induced aggregation of aspirin-pretreated platelets was significantly less (n=8, p<0.001) with platelets from cholesterol-fed rabbits (•) than with platelets from control rabbits (○). Chol., cholesterol; U, units.

Discussion

These studies with ethanol were done to investigate the effects of two types of hypercholesterolemia on the magnitude of the inhibitory effect of ethanol on platelet function. When ethanol was inhibitory, its effect was most apparent with low concentrations of agonists that caused less than maximal responses. As noted previously, when the concentration of an agonist was high,
inhibition of platelet responses by ethanol was not demonstrable.

It has been reported that the extent of the inhibitory effect of ethanol on platelet function is related to the lipid composition of the platelet membranes, with ethanol having a greater inhibitory effect on platelets enriched in saturated fats. However, experiments designed to investigate the inhibition by ethanol have not been previously reported for hypercholesterolemia, nor has the degree of inhibition in any type of hypercholesterolemia been assessed at equal extents of aggregation over ranges of concentrations of a variety of agonists. This method of analysis of the results was necessary to compensate for the hypersensitivity to some aggregating agents of platelets from rabbits with diet-induced hypercholesterolemia and the hyposensitivity of platelets from WHHL rabbits. Several unexpected findings arose during these investigations.

As in previous studies, ADP-induced responses of platelets from all the rabbits were unaffected by ethanol. ADP stimulates a primary, reversible aggregation of rabbit platelets that is not associated with appreciable secretion of granule contents, formation of TXA₂, or formation of the second messenger, inositol trisphosphate. Thus, phospholipase A₁ and phospholipase C do not appear to play a major role in ADP-induced primary aggregation, and because it appears to be the activation or activity of these enzymes that is inhibited by ethanol, it is not surprising that ethanol would not affect ADP-induced responses of platelets.

In the present study, inhibition by ethanol of the responses of platelets from both types of hypercholesterolemic rabbits (diet-induced and genetically determined) was observed with collagen, but (as with normal, aspirin-pretreated platelets) responses to U46619 were not significantly altered by the presence of ethanol. These observations with U46619 (under conditions in which feedback amplification by TXA₂ is blocked by aspirin) indicate that ethanol does not inhibit collagen-induced responses by affecting TXA₂-induced responses. Collagen-induced aggregation is mediated largely via the combination of TXA₂ that is formed from arachidonate primarily by phospholipase A₁ and by ADP that is secreted when platelets are stimulated by collagen. Because ADP-induced primary aggregation is not inhibited by ethanol, the evidence indicates that ethanol inhibits collagen-induced platelet responses by inhibiting the activation or activity of phospholipase A₁; this conclusion is in accord with earlier findings. When the hypersensitivity of platelets from cholesterol-fed rabbits was compensated for by examining the percentages of inhibition by ethanol at 25%, 35%, 50%, 65%, and 75% aggregation induced by collagen, it was evident that collagen-induced responses of platelets from rabbits with diet-induced hypercholesterolemia were inhibited by ethanol to the same extent as these responses of platelets from control rabbits. It may be that in diet-
induced hypercholesterolemia, the extent to which ethanol inhibits the mobilization of arachidonate is not affected.

Although platelets from the WHHL rabbits were much less responsive to collagen than were platelets from control rabbits, the extent of the inhibitory effect of ethanol on collagen-induced aggregation and secretion of granule contents appeared to be similar. Analysis of the inhibition of aggregation by ethanol at equal percentages of aggregation was not possible because of the limited aggregation responses of platelets from WHHL rabbits to the range of collagen concentrations that caused greater responses by control platelets. Again, ethanol did not affect U46619-stimulated responses of aspirin-pretreated platelets from either WHHL or control rabbits.

The inhibitory effect of ethanol on aggregation and secretion in response to low concentrations of thrombin was significantly less with platelets from the cholesterol-fed rabbits than with control platelets. This difference was also observed with aspirin-pretreated platelets, so thrombin-induced responses that are independent of TxA2 are also less inhibited by ethanol when the animals have been fed a cholesterol-enriched diet.

We have compared our results with cholesterol-fed rabbits with those of McGregor and Renaud, who investigated platelets from rats fed a diet high in saturated fat. When we plotted our results in the same way as these investigators, it appeared as if ethanol was more inhibitory when the rabbits had been fed the cholesterol-enriched diet. However, neither McGregor and Renaud, nor Fenn and Littleton compensated for the hypersensitivity of platelets enriched with saturated fats before concluding that ethanol had a greater inhibitory effect on platelets enriched in saturated fats. The analysis that we have performed of our data at equal extents of aggregation shows that ethanol was actually less inhibitory of thrombin-induced responses with platelets from the cholesterol-fed rabbits than with platelets from the control rabbits. Thus, although ethanol inhibits the activation or activity of phospholipase C in thrombin-stimulated platelets, it may do so to a lesser extent in platelets from cholesterol-fed rabbits.

In contrast, the thrombin-induced responses of platelets from WHHL rabbits were inhibited by ethanol to the same extent as the responses of platelets from control rabbits. All these observations indicate that the extent of the inhibitory effect of ethanol on the responses of platelets from hypercholesterolemic rabbits to collagen and thrombin differs in the two forms of hypercholesterolemia that were investigated. This difference is not directly attributable to plasma lipoproteins because the studies were done with isolated platelets in an artificial medium. It should be noted that the free cholesterol to phospholipid molar ratio was greater in platelets from the cholesterol-fed rabbits than from the control rabbits. An increased cholesterol content is associated with a de-
crease in membrane fluidity.\textsuperscript{50} Enrichment of platelets with cholesterol by incubation with cholesterol-enriched liposomes in vitro has been shown to decrease membrane fluidity.\textsuperscript{50,51} and Chin and Goldstein\textsuperscript{2,52} have shown that the addition of cholesterol to phospholipid liposomes or to erythrocyte or synaptosomal membranes reduces the fluidizing effect of ethanol. Thus, the attenuated effect of ethanol on the inhibition of thromboxane-independent thrombin-induced aggregation of platelets from cholesterol-fed rabbits that we have observed is consistent with the results of Chin and Goldstein.

With platelets from the WHHL rabbits, the free cholesterol to phospholipid molar ratio was the same as that of platelets from the control rabbits. This observation is in accord with the finding that thrombin-induced responses were equally inhibited by ethanol with platelets from these two groups of rabbits, regardless of whether the platelets were pretreated with aspirin.

The finding that ethanol has a different effect on platelets from cholesterol-fed rabbits compared with platelets from WHHL rabbits is a further indication that the hypercholesterolemia of WHHL rabbits does not cause the same changes in platelet function as does diet-induced hypercholesterolemia.

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M L Rand, P L Gross, D V Barrow and M A Packham

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