Acetaldehyde adducts and autoantibodies against VLDL and LDL in alcoholics

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Abstract Alcohol consumption markedly increases the hepatic output of very low density lipoprotein (VLDL), whereas it decreases the resulting low density lipoprotein (LDL) levels and apolipoprotein B. As ethylation of apoB-lysine renders LDL immunogenic and accelerates their clearance, and as alcoholics develop antibodies against acetaldehyde-protein adducts, we searched for antibodies against lipoproteins. We measured serum IgG, IgA, and IgM titers against VLDL, LDL and high density lipoprotein (HDL) in 10 non-alcoholics and 35 recently drinking alcoholics by ELISA assay. Alcoholics had higher IgG titers than non-alcoholics against VLDL and LDL; these were higher with VLDL than LDL or HDL. Using VLDL and LDL (but not HDL) from alcoholics gave the greatest response. There was no difference in IgA and IgM reactivity. To search for acetaldehyde adducts, we measured the reactivity of VLDL, LDL, HDL, and residual serum proteins against a rabbit anti P4502E1-acetaldehyde adduct IgG, which recognizes the adducts but not the unmodified proteins (except for P4502E1). ApoB-containing lipoproteins from alcoholics (and to a lesser extent non-lipoprotein proteins) reacted with anti-adduct IgG more strongly than those of non-alcoholics. The difference was striking for VLDL, less for LDL, and not detectable for HDL. This suggests that acetaldehyde reacts with apoB prior to its secretion from the liver and that the altered VLDL are partially removed prior to their conversion to LDL. ■ In conclusion, alcoholics develop acetaldehyde adducts in apoB-containing lipoproteins, particularly VLDL. The immune response to these neoantigens could result in accelerated clearance of VLDL and LDL and decreased conversion of VLDL to LDL.—**Wehr, H., M. Rodo, C. S. Lieber, and E. Baraona.** Acetaldehyde adducts and autoantibodies against VLDL and LDL in alcoholics. *J. Lipid Res.* 1993. **34:** 1237–1244.

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Alcohol consumption markedly increases the hepatic output of very low density lipoproteins (VLDL) (1) and accelerates the turnover of VLDL-triacylglycerols (2). One would expect that low density lipoproteins (LDL), being the product of VLDL catabolism, should also be elevated. However, it has been repeatedly reported that LDL cholesterol and apolipoprotein B (apoB) levels remain low in chronic alcohol users after a drinking period (3–5). This suggests that alcoholics may have accelerated clearance of VLDL-apoB, LDL-apoB, or both.

It has been shown both in humans (6) and in rats (7) that in vitro production of LDL adducts with high concentrations of acetaldehyde, under reductive conditions, increases the catabolism of this lipoprotein. It is possible that acetaldehyde may form adducts in vivo with apoB either in the liver or during the circulation of VLDL and LDL. However, the presence of lipoprotein-acetaldehyde adducts has not yet been documented in alcoholics.

In addition, ethylation by acetaldehyde, as well as other in vitro modifications of apoB-lysine, has been shown to render this protein immunogenic (8). Autoantibodies against LDL, circulating immune complexes, and evidence for cell-mediated immune response have been reported in non-alcoholic diseases (9–11). Alcoholics are known to develop antibodies against protein-acetaldehyde adducts prepared in vitro (12–14) and acetaldehyde adducts have been documented in the liver (15) and red blood cells (16). The presence of lipoprotein-acetaldehyde adducts in alcoholics might lead to development of high antibody levels, formation of immune complexes, and acceleration of the clearance of these lipoproteins. However, the presence of antibodies against plasma protein adducts occurring in vivo has not been documented in alcoholics.

Therefore, this study was undertaken to investigate whether alcoholics develop autoantibodies against serum lipoproteins and, in the affirmative, whether lipoprotein-acetaldehyde adducts could be involved as neoantigens and probable immunogens.

MATERIAL AND METHODS

Materials

Radial immunodiffusion plates and human reference sera for the measurements of human IgG, IgA, and IgM

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were purchased from Kallestad Labs., Inc. (Austin, TX). The IgG fractions of alkaline phosphatase-conjugated goat anti-rabbit IgG, human IgG, IgA (α-chain specific) and IgM (μ-chain specific), as well as peroxidase-conjugated goat anti-human IgG, were obtained from Sigma Chemical Co. (St. Louis, MO).

Subjects

After institutional approval for human studies, blood in excess to that used for the routine clinical assessment was obtained at the time of admission from 35 alcoholics (33 males and 2 females) who had been drinking within the preceding 3 days. In fact, 25 of them had blood ethanol levels of 30.6 $\pm$ 4.1 (SE) mmol/l, assessed by breathalyzer, and only 10 had no detectable alcohol. They had moderate elevations of aspartate (129 $\pm$ 26 U/l or 2.15 $\pm$ 0.43 μkat/l) and alanine (92 $\pm$ 18 U/l or 1.53 $\pm$ 0.30 μkat/l) aminotransferases and of gamma glutamyl-transpeptidase (288 $\pm$ 81 U/l or 4.80 $\pm$ 1.35 μkat/l) activities in the plasma. Four of these alcoholics underwent liver biopsy: two had steatosis (one of them with perivenular fibrosis) and two had morphologic features of alcoholic hepatitis (one with septal fibrosis and the other with cirrhosis). Ten non-alcoholic, healthy subjects provided 10–20 ml of blood, after informed consent, for development of the methods and to serve as controls. All of them consumed alcohol, at least occasionally, in moderate amounts (less than 40 g per week).

Analytical procedures

Plasma was obtained from blood collected in tubes containing EDTA (1 mg/ml). Chylomicrons were removed by centrifugation of the plasma at 5000 g for 30 min (10). VLDL + IDL, LDL, HDL, and non-lipoprotein proteins were sequentially separated at buoyant densities of 1.019, 1.063, and 1.21 g/ml by classical preparative ultracentrifugation (17). Henceforth, VLDL + IDL will be referred as the VLDL fraction. The fractions were dialyzed overnight against phosphate-buffered saline, pH 7.4 (PBS). The purity of the fractions was determined by agarose gel electrophoresis (18). Protein was measured by the method of Lowry et al. (19), using the modification by Kashyap, Hind, and Robinson (20) for the lipoprotein fractions.

Plasma concentrations of IgG, IgA, and IgM were determined by radial immunodiffusion, using the endpoint method (21). IgG, IgA, and IgM reactivities were determined by enzyme-linked immunosorbent assay (ELISA). The antigens consisted of 100 μl of the isolated lipoprotein and non-lipoprotein fractions diluted in coating carbonate-bicarbonate buffer, pH 9.6, to provide 1 μg protein per well. The coating buffer consisted of 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.1 g thimerosal per liter of H_2O. In the non-lipoprotein fractions, endogenous IgG was removed by affinity chromatography with Protein A-

Sepharose prior to ELISA. The plates were left overnight at 4°C and then the wells were rinsed four times with PBS–Tween 20 buffer, pH 7.4, consisting of 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g $NaHPO_4 \cdot 12H_2O$, 0.2 g KCl, 0.8 mM EDTA, 0.5 ml Tween 20, and 0.1 g thimerosal per liter. Unoccupied sites were coated with 100 μl of 1% bovine serum albumin in PBS for 3 h at room temperature. The solution was then aspirated and the wells were rinsed three times with the washing buffer. The primary antibody consisted of 1:100 to 1:800 (v/v) dilutions of plasma in PBS–1% bovine serum albumin for IgG, and 1:2 to 1:10 dilutions for IgA or IgM determinations. The diluted plasma was added to the plates and incubated at room temperature for 2 h. This was followed by four washings of the wells with 0.05% Tween 20 in PBS. The secondary antibodies were goat anti-human IgG, IgA, or IgM diluted 1:1000, 1:700, and 1:1000 (v/v), respectively, with 1% BSA in PBS. For the lipoprotein fractions, the secondary antibody was conjugated with alkaline phosphatase. For the non-lipoprotein plasma proteins, the secondary antibody was conjugated with peroxidase to avoid the effects of endogenous alkaline phosphatase activity in these fractions. One hundred μl of these enzyme-conjugated antibodies was added to the plates, incubated at room temperature for 2 h, and washed as described above. Alkaline phosphatase activity was measured at 405 nm after 30 min incubation with 100 μl of 0.1 mg p-nitrophenyl phosphate in diethylamine buffer, pH 9.8. Peroxidase activity was measured at 450 nm after 60 min incubation with 0.1 mg 5-aminosalicylic acid and 0.01% H_2O_2 in 20 mM sodium phosphate buffer, pH 6.8. The reactions were stopped with 3 M NaOH and the absorbances were measured in an ELISA reader (Bio-Rad Labs., Richmond, CA; model 2550).

The IgG reactivity of the plasma from alcoholics and non-alcoholics was also tested against VLDL and LDL delipidized with butanol–diisopropylether 40:60 (v/v) according to Cham and Knowles (22) and compared to that against non-delipidized lipoproteins to assess the lipidic or proteic nature of the epitope involved. Furthermore, this reactivity was also tested against several in vitro modifications of the ϵ-amino group of LDL-lysine. Ethylation with acetaldehyde was performed according to Steinbrecher et al. (8), methylation and carbamylation with formaldehyde and potassium cyanate, respectively, as described by Weisgraber, Innerarity, and Mahley (23), and acetylation with acetic anhydride as described by Basu et al. (24). Total, free, and modified amino groups were assessed with trinitrobenzene–sulfonic acid, as reported by Habeeb (25), and the reactivity per equal number of modified groups was compared.

The presence of acetaldehyde-protein adducts in plasma proteins was first assessed by immuno-slot-blots and then quantified by ELISA assay. As antigens, we used 0.25–4 μg protein of delipidated VLDL, LDL, or HDL and the non-lipoprotein plasma fractions. An affinity-

purified rabbit IgG previously described (26) was used as primary antibody, and a goat anti-rabbit IgG (conjugated with alkaline phosphatase) was used as a secondary antibody. The primary anti-adduct IgG was developed against a P4502E1-acetaldehyde adduct isolated from alcohol-fed rats (27). This antibody was shown to recognize acetaldehyde adducts of hemocyanin, hemoglobin, albumin, collagen, and lipoproteins prepared in vitro, but not the corresponding unmodified proteins (except P4502E1). In the present study, we also compared the reactivity of this antibody against in vitro ethylated, methylated, acetylated, and carbamylated human serum albumin, using the same procedures described above for lipoproteins (8, 23–25).

Statistics

The results were expressed as their means ± the standard error of the means (SE). The differences in ELISA assays were tested by analysis of the covariance. All other differences were analyzed by Student's t test.

RESULTS

Alcoholics had significant increases in the plasma concentrations of VLDL and HDL (**Table 1**). However, despite a 28% increase in VLDL-protein and a 21% increase in HDL-protein, LDL-protein was 14% lower in alcoholics than in non-alcoholics.

The plasma concentration of the major immunoglobulins was also significantly increased in alcoholics: IgG, IgA, and IgM were 27, 62, and 56% higher in the alcoholics than in the non-alcoholics (**Table 2**). Even when corrected for the difference in IgG concentrations, the plasma IgG reactivity against autologous VLDL and LDL was significantly higher in alcoholics than in non-alcoholics (**Fig. 1**). By contrast, plasma IgA and IgM reactivities were much smaller and there were no significant differences between alcoholics and non-alcoholics, using up to 1:2 dilutions of the plasma.

In the alcoholics, the increase in IgG reactivity affected VLDL as well as LDL. By contrast, the IgG reactivity against HDL appeared to be increased in alcoholics only

TABLE 1. Effects of alcohol consumption on plasma lipoproteins

Subjects	VLDL	LDL	HDL
	mg protein/dl plasma		
Alcoholics	12.4 ± 1.2	40.8 ± 2.7	179.8 ± 19.4
Non-alcoholics	9.7 ± 0.1	47.3 ± 1.4	148.2 ± 14.8
P^a	<0.05	<0.05	<0.01

Means ± standard error of protein concentration in lipoprotein fractions floated at densities 1.019, 1.063, and 1.210 g/ml in 20 actively drinking alcoholics and in 10 non-alcoholics.

[a]Significance by Student's t test.

TABLE 2. Effects of alcohol consumption on plasma immunoglobulins

Subjects	IgG	IgA	IgM
	mg/dl plasma		
Alcoholics	1309 ± 88	334 ± 31	213 ± 22
Non-alcoholics	1034 ± 16	207 ± 9	137 ± 4
P^a	<0.01	<0.01	<0.01

Means ± standard error of major immunoglobulin concentrations, measured by radial immunodiffusion, in plasma of 13 alcoholics and 8 non-alcoholics.

[a]Significance by Student's t test.

when expressed per equal volume of plasma, but not per mmol of immunoglobulin. The extent of the reactivity also depended on the source of plasma and the source of lipoproteins. Even in the plasma of control subjects, there was some IgG reactivity against lipoproteins obtained from non-alcoholic subjects. However, the plasma of alcoholics had much higher IgG reactivity against VLDL and LDL from control subjects than the plasma of non-alcoholics. Moreover, both in alcoholics and in non-alcoholics, the reactivity was higher when VLDL and LDL (but not HDL) were obtained from alcoholic than from non-alcoholic subjects.

The plasma from both alcoholics and non-alcoholics produced very high background readings when tested against non-lipoprotein plasma proteins (d > 1.210 g/ml), but these fractions contained endogenous IgG and alkaline phosphatase activities that were recognized by the secondary antibody. After binding the endogenous IgG to Protein A-Sepharose and using a secondary antibody conjugated with peroxidase instead of alkaline phosphatase, the background readings were markedly reduced, although not fully eliminated. Under these conditions, the IgG reactivity against non-lipoprotein proteins was higher in the plasma from alcoholics than from non-alcoholics (**Fig. 2**). In both groups, the reactivity against non-lipoprotein proteins was weaker than that against VLDL or LDL. In addition, the plasma IgG reactivity against non-lipoprotein protein was weaker than that against human serum albumin ethylated with acetaldehyde in vitro and immunostained with peroxidase-conjugated antibodies.

In the non-alcoholics, the reactivity of plasma IgG against delipidized VLDL was similar to that against non-delipidized VLDL (**Fig. 3**). In the alcoholics, who have increased VLDL-lipids, the reactivity against VLDL actually increased after delipidization. Moreover, the human plasma had increased IgG reactivity against delipidized LDL that had been modified in vitro with acetaldehyde under conditions that produce ethylation of ϵ-amino groups of lysine in apoB. Surprisingly, however, the IgG reactivity against other in vitro modifications of LDL-lysine (such as methylation, acetylation, or car-

Fig. 1. Effects of the source of plasma and lipoproteins on IgG reactivity. Means ± standards errors (SE) of absorbance at 405 nm wavelength of ELISA assays immunostained with alkaline phosphatase-conjugated anti-human IgG antibody in 16 alcoholics and 8 non-alcoholics. Lipoproteins (1 μg protein per well) from alcoholics and non-alcoholics were used as antigen. Various dilutions of the plasma (1 vol into 100–800 vol of saline–phosphate buffer containing 1% bovine serum albumin) from the same or similar patients were used as primary antibody. The reactivity significantly ($P < 0.01$) increased when either the plasma or the VLDL and LDL (but not HDL) were obtained from alcoholics.
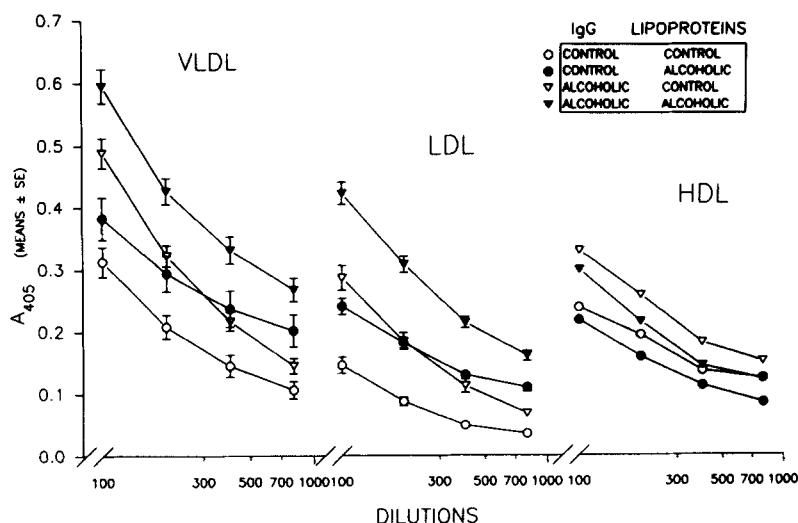
bamylation) was also increased (**Fig. 4**). The plasma from alcoholics tended to have higher reactivity against modified LDL than that from non-alcoholics, but these differences did not reach a level of statistical significance, except in the case of carbamylation ($P < 0.05$).

To assess the in vivo presence of acetaldehyde adducts in lipoprotein and non-lipoprotein plasma proteins, we used a rabbit antibody raised to an acetaldehyde-protein adduct occurring in vivo in alcohol-fed rats (namely, the rabbit anti P4502E1-acetaldehyde adduct IgG). This anti-adduct IgG reacted more strongly with the lipoprotein fractions from alcoholics than from non-alcoholics (**Fig. 5**). The difference was particularly striking for VLDL ($P < 0.01$), less for LDL ($P < 0.05$), and nonsignificant for either HDL or non-lipoprotein proteins. When the rabbit anti-adduct antibody was tested against human serum albumin modified in vitro, the reactivity was much stronger with ethylated than with either methylated, acetylated, or carbamylated albumin.

DISCUSSION

Our results indicate that alcoholics develop increased amounts of autoantibodies, of the IgG class, against apoB-containing lipoproteins. Moreover, they also reveal that acetaldehyde adducts occur in apoB-containing lipoproteins (especially VLDL) and that these modified lipoproteins constitute a major source of neoantigens in the plasma of alcoholics.

Alcoholics were found to have higher plasma concentrations of the major immunoglobulins (IgG, IgA, and IgM) than non-alcoholics, confirming previous findings (28). However, even when corrected for the increase in immunoglobulins, the plasma of alcoholics had enhanced IgG reactivity against VLDL and LDL, but not against HDL or non-lipoprotein plasma proteins. This suggests that the target antigen is associated with apoB-containing lipoproteins, as this apolipoprotein is common to LDL and VLDL, whereas other apolipoproteins present in VLDL are shared by the nonreactive HDL. Furthermore, the IgG reactivity was not decreased by delipidization, suggesting that the recognized epitope most likely is present in apoB rather than in the lipid moiety.
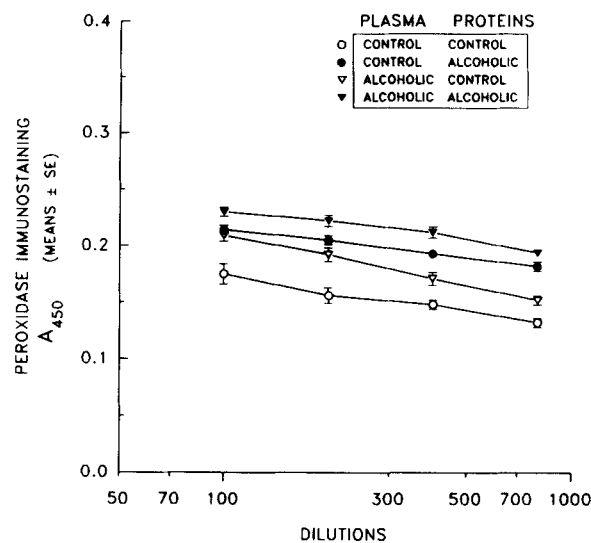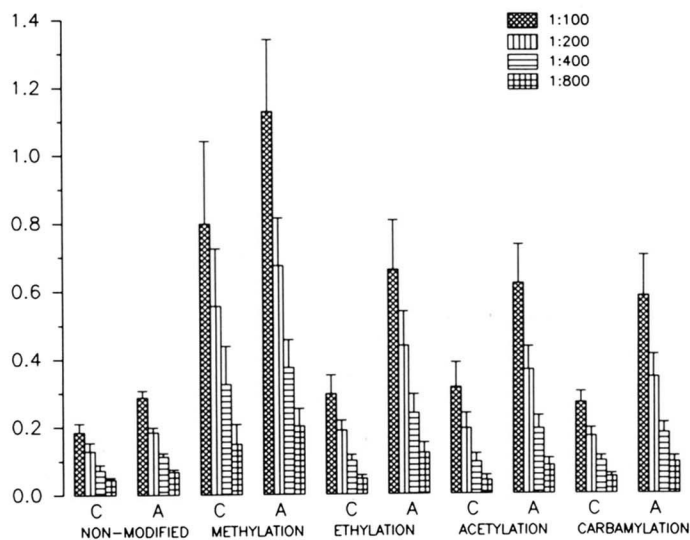

Fig. 2. IgG reactivity of various dilutions of plasma (as in Fig. 1) from seven alcoholics and four non-alcoholics against the lipoprotein-free fraction of the plasma (1 μg protein per well) from the same or similar patients. To decrease the high background readings, the contamination of the non-lipoprotein fraction with IgG and alkaline phosphatase was minimized by absorption of the endogenous IgG to Protein A-Sepharose and by replacement of the phosphatase- for peroxidase-conjugated anti-IgG, as secondary antibody for the immunostaining. The residual reactivity was slightly, but significantly ($P < 0.05$) higher in alcoholics than in non-alcoholics. A_{450} = absorbance at 450 nm wavelength.
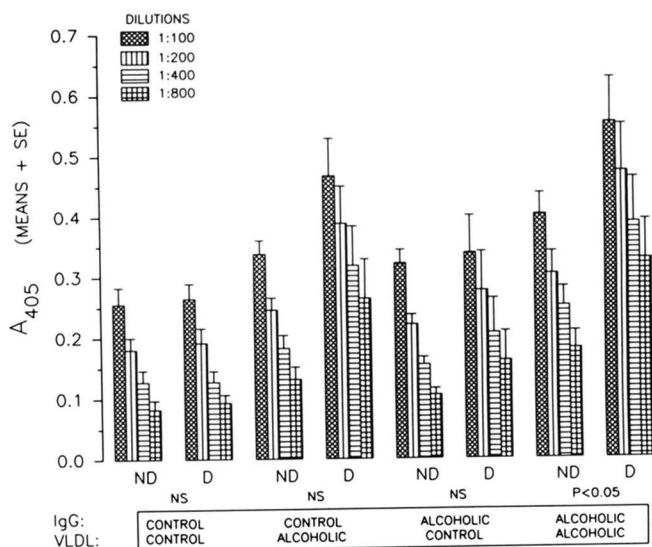
Fig. 3. Comparison of IgG reactivity of various dilutions of plasma (as in Fig. 1) from 14 alcoholics and 8 non-alcoholics against non-delipidized (ND) and delipidized (D) VLDL (1 μg protein per well) from the same or similar patients. VLDL was delipidized with butanol–diisopropylether 40:60 (v/v) and ELISA was carried out as in Fig. 1. NS, nonsignificant difference.

In non-alcoholic subjects, there was some reactivity between their plasma IgG and autologous lipoproteins, but this reactivity against lipoproteins from non-alcoholics was markedly exaggerated when the plasma of alcoholics was used as a source of antibodies. This indicates enhanced recognition by alcoholics of an antigen, which is present in non-alcoholics. Moreover, both in alcoholics and non-alcoholics, the plasma IgG reactivity increased when VLDL or LDL were isolated from recently drinking alcoholics. This indicates that both the formation of the antigen and the humoral immune response are increased by alcohol consumption.

Acetaldehyde, the first product of ethanol oxidation, forms adducts with proteins and phospholipids, generating neoantigens that have been shown to induce a humoral (8, 29, 30) and tissular (31) immune response in experimental animals. Moreover, increased plasma antibody titers against in vitro-produced acetaldehyde adducts have been extensively documented in alcoholics (12–14). Also in the present study, the plasma of alcoholics had enhanced IgG reactivity against human serum proteins (such as LDL or albumin) when they were ethylated in vitro with acetaldehyde. However, in vivo, the reactivity against the non-lipoprotein fraction of the plasma (which includes albumin) was much weaker than that against the naturally occurring apoB-containing lipoproteins. This suggested that, compared to other plasma proteins, the apoB-containing lipoproteins may constitute a major in vivo source of acetaldehyde-induced neoantigens in alcoholics. In keeping with this interpretation, a rabbit antibody developed against a P4502E1-acetaldehyde adduct isolated from alcohol-fed rats (26, 27) reacted more strongly with apoB-containing lipoproteins from alcoholics than from non-alcoholics (Fig. 3). The difference was particularly striking for VLDL, less for LDL, and nonsignificant for either HDL or non-lipoprotein fractions. A surprising finding was that human IgG showed enhanced recognition not only of acetaldehyde-induced modifications of LDL, but also of other in vitro modifications of the ε-amino group of LDL lysine (such as methylation, acetylation, and carbamylation). This is at variance with observations in the rabbit, a species that develops specific antibodies against each of the above modifications (8). Also, the rabbit anti-adduct IgG, used in the present study, showed specificity for the acetaldehyde-induced modification. It is uncertain whether the chemical modifications tested, except that produced by acetaldehyde, could occur in vivo to a significant extent in alcoholics.

Fig. 4. IgG reactivity of various dilutions of plasma (as in Fig. 1) from 12 alcoholics (A) and 8 non-alcoholics (C) against in vitro modifications of LDL-lysine by the procedures described in Methods. The percentage of modified lysines, as determined by the method of Habeeb (25), was 77% for methylation, 18% for ethylation, 50% for acetylation, and 34% for carbamylation. One μg of LDL protein was used per well and ELISA was carried out as in Fig. 1. In all subjects, the reactivity to modified LDL was higher ($P < 0.01$) than that to nonmodified LDL. These changes tended to be greater in the alcoholics than in the non-alcoholics, but reached statistical significance ($P < 0.05$) only with carbamylated LDL.

Fig. 5. Acetaldehyde-protein adducts in delipidized lipoproteins and in the non-lipoprotein fraction of the plasma in 20 alcoholics and 9 non-alcoholics. The adducts were measured by ELISA using a polyclonal rabbit anti-adduct IgG, previously described (26), as primary antibody and alkaline-phosphatase-conjugated goat anti-rabbit IgG as secondary antibody. The changes were significant for VLDL ($P < 0.01$) and LDL ($P < 0.05$), but not for HDL or non-lipoprotein proteins (NLP).
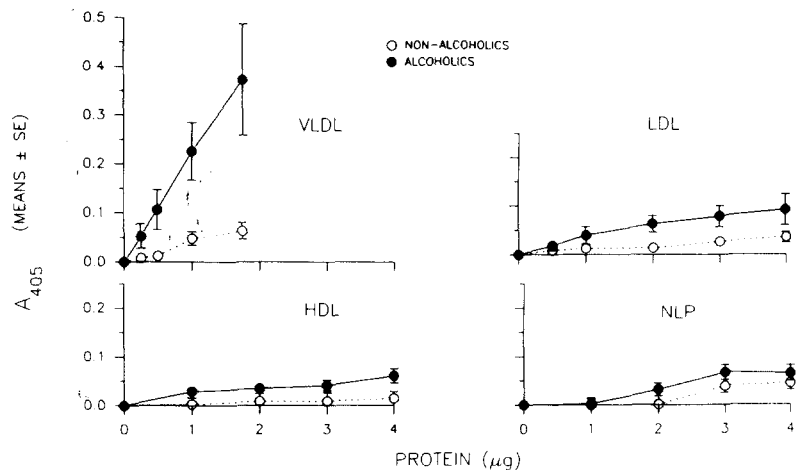
The finding that acetaldehyde adducts occur predominantly in VLDL suggests that the in vivo modification of apoB takes place in the liver rather than in the circulation. This is also consistent with the fact that the concentrations of acetaldehyde during alcohol consumption are highest in the liver (32). Moreover, the lesser increase in acetaldehyde adduct in LDL suggests that only part of the modified VLDL completes its conversion to LDL. In alcoholics, the increase in circulating antibodies against these adducts could accelerate the removal of VLDL from circulation. This is consistent with the reported increases in VLDL-triacylglycerols (2) and VLDL-apoB (33) fractional catabolic rates in alcoholics. The effects of alcohol administration on the catabolism of LDL-apoB are less clear: whereas one study reported an increase in the fractional catabolic rate after alcohol administration to normal volunteers (34), another failed to detect changes in alcoholics after the withdrawal of alcohol (35), and a third one reported a decrease in the fractional catabolic rate of LDL-apoB in squirrel monkeys fed 25% of energy as ethanol (36). At variance with most studies in alcoholics, however, squirrel monkeys developed a substantial increase in the plasma levels of apoB after alcohol. These conflicting results could depend on the degree of alcohol consumption and the extent of acetaldehyde-modification of LDL-apoB. The accelerated clearance of LDL-acetaldehyde adducts reported in non-alcoholic volunteers (6) could be due, at least in part, to the presence of antibodies against modified apoB-containing lipoproteins even in non-alcoholic individuals. It must be pointed out that we reported a similar increased clearance of LDL-acetaldehyde adducts by normal rats (7), but the possibility of antibodies capable of recognizing the modified LDL cannot be excluded as acetaldehyde is also produced endogenously from non-alcoholic sources and acetaldehyde-protein adducts have indeed been detected in rats not fed alcohol (26).

Enhanced removal of acetaldehyde-modified VLDL and LDL immune complexes could contribute to the modest elevation of VLDL and to the decrease in LDL levels found in alcoholics, despite a marked increase in the output of VLDL from the liver, as documented in the hepatic venous blood of animals chronically fed alcohol-containing diets (1). It is difficult to predict to what extent an immune-mediated removal of acetaldehyde-modified VLDL and LDL may affect the development of atherosclerosis. Most of the epidemiological evidence (37) and some results in experimental animals (38) favor a preventive role of moderate, but not of excessive, alcohol consumption on the development of coronary atherosclerosis. There is no evidence that the acetaldehyde-induced modification of LDL could be atherogenic, as has been postulated for the oxidized LDL (39). In any event, the removal of immune complexes of apoB-containing lipoproteins may contribute to maintain lower than expected levels of VLDL and LDL in alcoholics. Moreover, the likelihood that this removal may take place in the reticuloendothelial system of the liver and spleen may explain, at least in part, our previous observations of increased splanchnic extraction of IDL and LDL cholesterol esters (40) and triacylglycerols (1) in alcohol-fed baboons. ▮▮

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