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DETECTION OF ANTIBODIES TO ACETALDEHYDE–ALBUMIN CONJUGATES IN ALCOHOLIC LIVER DISEASE

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Abstract — The principal metabolite of ethanol, acetaldehyde, conjugates with various proteins that form antibody-inducing neo-antigens. We have analysed sera from patients with biopsy-proven alcoholic liver disease (hepatitis = 10, cirrhosis = 11, steatosis = 3) and controls = 19 (normal teetotallers and 6 non-alcoholic liver disease). Sera were examined with an enzyme-linked immunoabsorbent assay (ELISA) for antibodies binding preferentially to an acetaldehyde–albumin conjugate. Reactive sera from alcohol misusers were then purified using an amino-hexyl Sepharose affinity column. Antibodies binding to the acetaldehyde–albumin epitopes were significantly raised ($P < 0.005$) in all groups of alcohol misusers, and were present in greatest titre in those with alcoholic hepatitis. These antibodies were successfully purified using the gel affinity column. We conclude that alcohol misusers have significant titres of antibodies reacting to the acetaldadehyde–albumin complex The role of these antibodies remains unclear, but may be related to the initiation of an inflammatory response and tissue damage following ethanol consumption.

INTRODUCTION

Acetaldehyde, the principal metabolite of ethanol, is capable of forming conjugates with various proteins *in vitro,* and in liver slices. Studies by Stevens *et al.* (1981) and Medina *et al.* (1985) have shown that acetaldehyde binding to proteins leads to the formation of both stable and unstable adducts. The latter, postulated to be Schiff bases can be stabilized by various reducing agents such as sodium cyanoborohydride. Acetaldehyde–protein conjugates have been synthesized *in vitro* using limpet haemocyanin, human albumin (Hoerner *et al.*, 1986) and human haemoglobin (Israel *et al.*, 1987; Niemela *et al.*, 1987), in the presence of supra-physiological concentrations of acetaldehyde. These conjugates have been used to detect the presence of antibodies in alcoholics (Israel *et al.*, 1987). When injected into mice, acetaldehyde–protein conjugates prepared *in vitro* have induced the formation of antibodies; these antibodies bind to the acetaldehyde–protein conjugate with much greater affinity than to the unmodified protein (Israel *et al.*, 1986). Similarly, mice (Niemela *et al.*, 1987) and rats (Worrall *et al.*, 1989) which are fed large quantities of ethanol also develop antibodies that bind to acetaldehyde–protein conjugates with greater affinity than to the unmodified protein (Hoerner *et al.*, 1986). Antibodies reacting to a liver membrane lipoprotein (Neuberger *et al.*, 1984; Perperas *et al.*, 1981) and to Mallory's Hyalin (Kanagasundaram *et al.*, 1977) have previously been demonstrated and postulated as a mechanism whereby chronic alcohol consumption, by inducing antigenic changes, could trigger a cell damaging immune reaction. In heavy alcohol drinkers, plasma acetaldehyde concentrations may rise to greater than 100 μM in acute intoxication. (Nutinen *et al.*, 1983; Korsten *et al.*, 1975; Pikkarainen *et al.*, 1980). These observations support the hypothesis that acetaldehyde, formed *in vivo* by the metabolism of ethanol, can form protein conjugates which may behave as neo-antigens stimulating the production of antibodies that bind preferentially to these acetaldehyde-modified epitopes.

We chose to study albumin, a protein synthesized within hepatocytes and present in high concentration in serum. We report the synthesis and evaluation of an acetaldehyde–human albumin conjugate which has not been done previously. We provide further evidence using an enzyme-linked immunosorbent assay (ELISA) that the sera of patients with different forms of alcoholic liver disease contain antibodies which bind preferentially to this acetaldehyde–albumin conjugate. We also report the immuno-affinity purification of such immunoglobulins.

Patients

Heavy alcohol drinkers were recruited between November 1987 and October 1988, from the in-patients of a district general hospital — the principal reason for admission in all cases was physical damage due to alcohol. All had clinical and biochemical evidence of liver damage and in all cases alcoholic liver disease was confirmed on liver biopsy or autopsy, and no alternative cause of liver damage was found. In all cases, the habitual alcohol consumption exceeded 50 g per day for at least 2 years. Non-drinking controls were recruited over the same period. All drank less than 10 g alcohol weekly and none had clinical or biochemical evidence of liver impairment. As disease controls, patients with auto-immune chronic active hepatitis not treated with steroids who did not habitually consume alcohol were used. Serum samples were collected and then stored at −20°C.

MATERIALS AND METHODS

Synthesis of the acetaldehyde conjugate

Using the method of Israel et al. (1986), 25 mg of human albumin (Sigma) were dissolved in 1 ml of phosphate buffered saline (PBS 1.5 mM KH_2PO_4, 6.0 mM $NaHPO_4$, 0.11 mM NaCl, 3 mM KCl, at pH 7.4).

One millilitre of PBS containing varying concentrations of acetaldehyde to a maximum of 420 mM was added, and the sample incubated at 20°C for 30 min or 72 hr at 37°C, before addition of sodium 10 mM cyanoborohydride and further incubation for 1 hr (to stabilize the Schiff bases). The sample was then dialysed for 24 hr against 3 l of 1 mM NaH_2PO_4 at pH 7.0 to remove excess acetaldehyde and cyanoborohydride. The concentration of the modified protein was determined by the Folin–Lowry method (Lowry et al., 1951).

Determination of the extent of acetaldehyde modification

When proteins are conjugated with acetaldehyde, the acetaldehyde reacts with the lysine residues present in the protein, forming *N*-ethyl-lysines. 2,4,6, trinitrobenzenesulphonate (TNBS) reacts with primary amino groups of lysine residues in polypeptide chains, undergoing a colour change that can be measured spectroscopically at 367 nm (Plapp et al., 1971). Consequently, the extent of reaction of TNBS to the acetaldehyde reacted protein is decreased as the degree of *N*-ethyl-lysine formation increases.

This procedure was carried out on aliquots of modified and unmodified proteins of known protein concentration, which were incubated for either 45 min at 20°C or 72 hr at 37°C. The results related to the absorbance value measured for proteins that had gone through the modification protocol in the absence of acetaldehyde.

Detection of antibodies in patients' sera by an ELISA technique

The acetaldehyde–albumin conjugate or unmodified albumin, 50 μg/ml carbonate buffer (NaCl 1.8 g, $NaHCO_3$ 5.86 g, Na_2CO_3 2.12 g in 100 ml, at pH 9.6) was placed in the wells of an ELISA plate. Casein (0.5%) was used to block non-specific binding sites. The patients' sera were diluted 1/10 and 1/100 in PBS, and tested in triplicate. The second antibody was peroxidase linked goat anti-human IgG (Sigma) diluted 1/500 in PBS. The substrate was 30 ml hydrogen peroxide (30%), (0.25 ml azino-bis-3-ethyl benz-thiazoline-6-sulfonic acid) (ABTS) (40 mM) in 25 ml citrate buffer (citric acid 2.5 g, trisodium citrate 2.4 g in 200 ml, at pH 4.0). Plates were read using a spectrophotometer at 420 nm after 30 min incubation.

Characterization of reactive sera by immunoblotting

Samples of acetaldehyde albumin conjugate and fractions from the affinity columns elution profile were loaded onto 9% SDS polyacrylamide gels. After electrophoresis the gels were blotted onto nitrocellulose paper 8, and incubated with peroxidase linked goat anti-human IgG at 20°C for 90 min. The substrate was 25 μl hydrogen peroxide 30% 18 mg 4-chloro-1-Napthol in 6 ml methanol, 50 ml TBS.

Preparation of an N*-ethyl-lysyl albumin affinity column*

Human albumin was modified as described previously and attached to amino-hexyl Sepharose via its free carboxyl groups. The matrix was then placed in four 10 ml plastic syringes and used to isolate *N*-ethyl-lysyl albumin binding proteins from the sera of alcoholics and teetotal controls (Gonyea, 1977; Pharmacia, 1988; Levy and Eveleigh, 1978).

Procedure for affinity purification

The matrix was washed extensively with PBS, pH 7.5, to remove unbound proteins. The serum samples were diluted 1 : 1 in PBS and loaded stepwise onto the column at 4°C. The flow-through fraction was collected and an aliquot taken for ELISA. The remainder was then reloaded for a second cycle. The column was then washed with a further 10 ml PBS and then with PBS containing 1 M NaCl to elute non-specifically bound proteins. The wash was continued until the OD 280 of the effluent was less than 0.05. Tightly bound proteins were eluted with glycine HCl buffer at pH 2.5, continued until the OD 280 of the effluent was less than 0.05.

Data analysis and statistical methods

The mean of triplicate ELISA absorbance readings was determined and used to establish difference scores (Israel *et al.*, 1987). Difference scores were obtained by subtracting the immunoassay value obtained from a reaction between the sample and unconjugated protein, and the value obtained with the sample and the protein–acetaldehyde conjugate. This allows an inherent control for non-specific binding to the albumin. The means of the different scores were compared using an unpaired *t*-test. Significance was taken at the 5% level.

The triplicate measurements of absorbance for unmodified albumin and acetaldehyde albumin conjugates were transformed into means, and the difference determined. This gives a measure of specific binding to the conjugate.

RESULTS

In vitro *albumin modification*

Incubation at 20°C for 45 min produced significant modification only at 24 mM and 240 mM acetaldehyde, while incubation at 37°C for 72 hr produced modification at the lowest acetaldehyde concentrations as low as 24 μM.

Patients

Among the alcohol misusers, eleven had cirrhosis, ten had alcoholic hepatitis, and three had fatty change (steatosis). The results of the ELISA on the patients sera are shown in Fig. 1 and 2. The difference between the controls and all alcohol misusers is significant ($P < 0.005$), and for alcoholic hepatitis, highly significant ($P < 0.001$).

Affinity purification of N*-ethyl-lysyl albumin binding proteins from sera of alcoholics and controls*

A random sample of sera was selected and *N*-ethyl-lysyl binding proteins were extracted using the affinity matrix. A typical elution profile for an alcohol misuser and control are shown in Fig. 3. A comparison of the binding activities of the original sera and the peak acid wash fractions recovered from the affinity matrix is shown in Fig. 4.

Analysis of acid washed fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Aliquots of peak acid washed fractions from the sera of alcoholics were applied to 7.5% SDS-PAGE gels and run at 4 mA for 12 hr (Towbin *et al.*, 1979). Gels were stained with Coomassie brilliant blue. The most marked proteins had molecular weights of approxi-

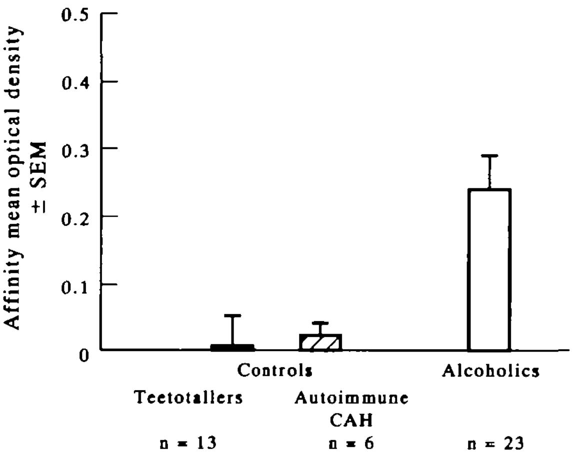

Fig. 1. ELISA OD ratio for original sera and peak fractions after affinity chromatography.

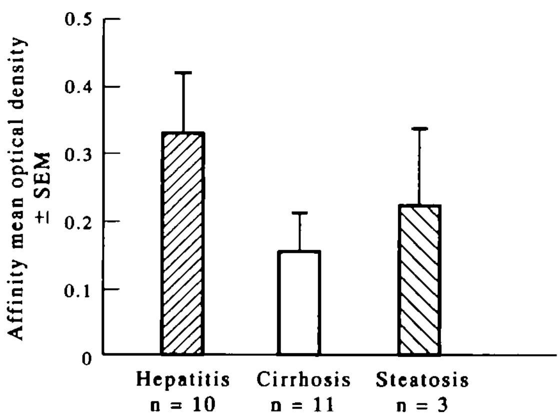

Fig. 2. Serum protein elution profile from *N*-ethyl-lysyl albumin affinity matrix.

mately 72, 50 and 25 kD. Immunoblotting of identical gels using an anti-human IgG revealed that the 50 and 25 kD proteins bands were heavy and light chains.

DISCUSSION

The extent of *in vitro* acetaldehyde modification of human albumin is dependent on the acetaldehyde concentration — and it is clear that the relatively low concentration of acet-

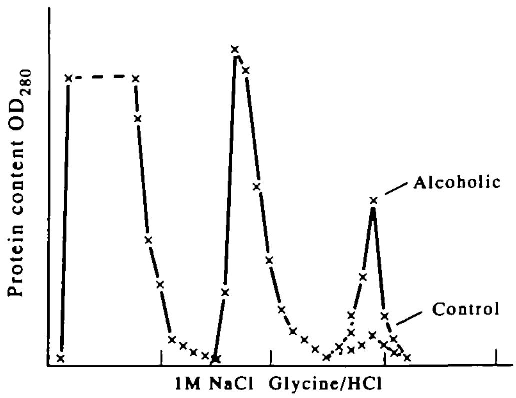

Fig. 3. Difference between affinities for patient groups.

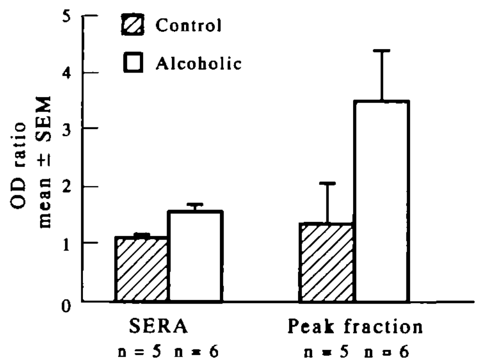

Fig. 4. Difference between affinities for patient subgroups.

aldehyde (120 μM) which can be found in the peripheral blood of heavy drinkers during acute intoxication, will cause modification of albumin.

The sera of heavy drinkers and controls contained antibodies which bound to unmodified albumin to a variable extent. We have no explanation for this but compensated for it by expressing results of the ELISA as the difference between the absorbance with the conjugate to the absorbance with unmodified albumin. In this way each sample tested acted as its own control. Our results show that the sera of heavy alcohol drinkers contain antibodies which bind with greater affinity to the acetaldehyde–albumin conjugate than to unmodified albumin. The mean absorbance ratio for heavy drinkers was significantly greater than that for non-drinking controls. When analysed according to the histological severity of liver damage, the patients with alcoholic hepatitis showed the highest absorbance difference ratio and the greatest significance of difference from the controls.

These findings support those of Hoerner *et al.* (1986), who detected in the sera of heavy drinkers antibodies against an acetaldehyde–protein conjugate, and Niemela *et al.* (1987) who detected antibodies against acetaldehyde–protein conjugates in the sera of alcoholics; the antibody titres were highest among the patients with alcoholic hepatitis. However, our findings differ from a more recent report of Hoerner *et al.* (1988), that antibodies against acetaldehyde–protein conjugates may be formed in a variety of non-alcohol related liver diseases, and that the highest titres were seen in patients who had advanced liver damage. Our non-drinking patients who had autoimmune chronic active hepatitis did not have antibodies against the acetaldehyde–albumin conjugate; however, we only studied three of these patients.

It is interesting that patients with earlier, less severe histological forms of alcohol-induced liver damage tend to have higher levels of antibody against the acetaldehyde–albumin conjugate on the ELISA, compared to patients with cirrhosis. As yet the role of antibodies against acetaldehyde–protein conjugates in the pathogenesis of alcoholic liver disease is unclear (Hoerner *et al.*, 1988). We are now studying whether the presence of these antibodies can be used prognostically in heavy drinkers to predict the subsequent development of more severe liver damage, and whether the level of antibody in each patient changes as the liver damage either resolves with abstinence, or worsens with continued drinking.

One control patient had an unexpectedly high level of antibody against the acetaldehyde–albumin conjugate on the ELISA. This was corroborated by applying her serum on the immunoblot, where it bound preferentially to the conjugate. Where her serum was subjected to protein blotting, the polyclonal serum of a positive control bound to the protein bands, raising the possibility that her serum contained acetaldehyde-modified proteins. Although she may have been drinking much more alcohol than she admitted, she had no clinical or biochemical evidence of heavy alcohol intake. An alternative explanation is that she might produce relatively high acetaldehyde concentrations in response to modest alcohol ingestion, as has been noted among some Japanese subjects (Mizio *et al.*, 1979).

Immunoblotting therefore confirmed that the major proteins present in sera that bound to acetaldehyde-modified albumin were immunoglobulins, but also revealed that another protein species of higher molecular weight was retained by the affinity matrix.

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