Correlations between serum proteins modified by acetaldehyde and biochemical variables in heavy drinkers

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SUMMARY A strong and highly significant correlation was observed between serum aspartate transaminase (AST) activity and an index of the cytotoxic activity associated with serum proteins modified by acetaldehyde in a group of 24 heavy drinkers. A weaker but significant correlation ($R = 0.564$, $p = 0.008$) was found between total serum creatine kinase activity and this index of serum cytotoxicity.

As it is likely that the concentration of circulating modified protein was largely determined by the quantity of free acetaldehyde generated in the liver and that the AST activity was mainly derived from damaged hepatocytes, the data indicate a correlation between hepatic acetaldehyde generation and hepatocyte damage. This correlation may indicate either that increased quantities of acetaldehyde are released by damaged hepatocytes or that acetaldehyde is hepatotoxic *in vivo*. As only the creatine kinase isoenzyme present in skeletal muscle (CK-MM) was demonstrable in the serum in all but one of our patients, the data also suggest that circulating modified serum proteins may be toxic towards skeletal muscle cells.

The mechanisms underlying tissue damage induced by alcohol are still uncertain. It is generally accepted that the susceptibility of the liver to damage induced by ethanol is related to the fact that over 90% of ethanol metabolism takes place in this organ.¹ More recently it has been suggested that damage to certain extrahepatic tissues may be a consequence of the ability of tissue macrophages to metabolise ethanol at substantial rates.²⁻⁵ The eventual mediators of hepatic and extrahepatic tissue damage which have been proposed include acetaldehyde,^{6,7} free radicals,⁸⁻¹⁰ antibodies against neoantigens induced by alcoholism,¹¹ and serum proteins modified by acetaldehyde.⁵ The last of these propositions is based on the finding that the consumption of ethanol is followed by the appearance of circulating acetaldehyde protein complexes which are cytotoxic towards various cell types *in vitro*.^{12,13} In this study we investigated the importance of circulating cytotoxic proteins in the pathogenesis of tissue damage resulting from alcohol abuse in a group of heavy drinkers by correlating two indices of the

cytotoxic activity of serum proteins with various biochemical markers of hepatic and extrahepatic tissue damage.

Material and methods

Twenty two male and two female patients were studied on the morning of admission to the Regional Alcoholism and Drug Dependency Unit, St Bernards Hospital, Ealing. All had consumed excessive quantities of ethanol and had continued to drink heavily until admission to the Unit. Table 1 gives demographic details. Clinical history and blood samples were obtained before administration of drugs (chlormethiazole, phenytoin) for suppression of the withdrawal reaction.

Blood specimens were collected using the vacutainer system. An aliquot was mixed with ethylenediamine tetra-acetate and used to determine a full blood count. Clotted samples were centrifuged within 12 hours of phlebotomy. The serum was transferred under aseptic conditions into 10 ml sterile containers and frozen at

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Table 1 Essential clinical and laboratory variables in the 22 male and two female heavy drinkers

Variable	(n =)	Mean	Observed range	Reference values
Age (years)	24	45	23-68	
Duration of excessive drinking (years)	24	13	1-40	
Daily intake of alcohol (g)	24	333	140-750	
AST (IU/l)	22	48.9	10-125	0-25
Alkaline phosphatase (IU/l)	22	167.7	107-399	10-210
GGT (IU/l)	18	157.1	23-856	0-37
Bilirubin (mmol/l)	22	12.3	6-18	0-17
Total CK (IU/l)	21	198	44-1371	<200
Total amylase (IU/l)	21	111.6	20-241	<220
Amylase—P (IU/l)	21	41.8	0-113	M 35-115 F 50-120
Amylase—S (IU/l)	21	68.9	9-207	<125
Haemoglobin concentration (g/dl)	23	15.3	12.0-17.0	M 13.4-17.0 F 11.9-15.3
Mean cell volume (fl)	22	94.3	80-100	82.5-99.0

–20°C until required for biochemical and cytotoxicity studies.

Blood counts were performed using a Coulter Counter, model S plus IV (Coulter Electronics, Luton, Bedfordshire).

Aspartate transaminase (AST, EC 2.6.1.1), alkaline phosphatase (AP, EC 3.1.3.1), gamma-glutamyl transferase (GGT, EC 2.3.2.1) activities and bilirubin concentration were assayed on a Kone Progress Discretionary Analyser, using standard methods. Creatine kinase (CK, EC 2.7.3.2) was measured at 37°C by an optimised method¹⁴ with commercial reagents (CK-NAC opt.; BCL, Lewes, Sussex) using a centrifugal analyser (Encore, Baker Instruments). CK-MB activity was measured by electrophoresis and densitometric scanning.¹⁵ CK-MB concentration was determined using the Hybritech Tandem-E CK-MB assay system (Hybritech UK, Nottingham). This uses monoclonal antibodies to the B and M subunits in a two-site sandwich assay specific for CK-MB protein. The laboratory upper reference limits for these values were CK, 200 U/l; CK-MB activity, 12 U/l and 5% of total CK; CK-MB concentration, 12 g/l. Total amylase (EC 3.2.11) was determined using Boehringer reagents (paranitrophenyl-malto-heptaoside) and amylase isoenzymes by the method of Foo and Rosalki.¹⁶

CYTOTOXICITY STUDIES

An aliquot of each serum sample was dialysed against 500 ml of RPMI 1640 (Gibco Europe Ltd, Paisley, Scotland) containing 100 U penicillin/ml and 100 µg streptomycin/ml at 4°C for 24 hours. The cytotoxicity of both the undialysed and dialysed serum was tested against A9 cells as described below.

In other experiments aliquots of each serum sample were incubated at 37°C for one hour with 1.6% (v/v) of a solution containing 400 mmol/l NaOH or 400 mmol/l NaOH plus 10 mmol/l sodium boro-

hydride (NaBH₄); the pH of both reaction mixtures was 9.5. After the incubation the mixtures were cooled on ice and dialysed against two 1000 ml volumes of RPMI 1640 at 4°C over 24 hours. The dialysed solutions were tested for cytotoxic activity.

The cytotoxicity of the untreated and treated serum samples was assessed on the basis of their capacity to cause detachment of adherent mouse A9 cells, as described previously.³ The A9 cells were suspended to a concentration of 1.5×10^5 /ml in Eagle's Minimum Essential Medium with Earle's salts and L-glutamine (Gibco Europe Ltd), 100 µg streptomycin/ml, 100 U penicillin/ml, and 10% (v/v) fetal calf serum. Aliquots (2 ml) of this cell suspension were placed within each of the wells of a 24-well tissue culture plate (Sterilin Ltd, Teddington, Middlesex) and the plates incubated at 37°C (5% carbon dioxide, 100% humidity) for six hours to permit the A9 cells to adhere. Non-adherent cells were removed and the adherent cells washed once with Hanks's solution at 37°C. Equal volumes (0.5 ml) of each serum sample to be tested and of RPMI 1640 (with penicillin and streptomycin) were placed within a well containing adherent cells and the culture plates incubated for 16 hours at 37°C (5% carbon dioxide, 100% humidity). The wells were then emptied and the residual adherent cells washed once with Hanks's solution at 37°C. The adherent cells were detached by treatment with 1 ml 0.1% (w/v) lyophilised trypsin (Gibco Europe Ltd) in calcium and magnesium-free phosphate buffered saline. When all the cells were detached, the trypsin was neutralised by the addition of 200 µl newborn calf serum to each well. The cells present in 1 ml of the cell suspension were counted after dilution with 9 ml Isoton (Coulter Electronics Ltd), using a Coulter Counter, Model ZF (Coulter Electronics Ltd) and the total number of residual adherent cells in each well calculated.

The extent of cytotoxicity of a serum sample was considered to be inversely proportional to the number

of residual adherent A9 cells (NRAC) after exposure to the sample being tested. Another index of cytotoxicity was derived from the experiments in which serum was treated with NaOH or NaOH plus NaBH₄ and was based on evidence that treatment with NaBH₄ (pH 9.5) results in detoxification of post-alcohol serum because of the reduction and consequent stabilisation of cytotoxic unstable Schiff bases formed between acetaldehyde and proteins.¹³ This index, termed the "percentage increase in residual adherent cells", was determined by subtracting the NRAC in the presence of NaOH from the NRAC in the presence of NaOH plus NaBH₄ and expressing the difference as a percentage of the NRAC in the presence of NaOH. The cytotoxicity of a serum sample was considered to be directly proportional to the percentage increase in residual adherent cells (PIRAC).

When both variables to be correlated were normally distributed, Pearson's correlation coefficient (*r*) was determined. When this was not the case, Spearman's correlation coefficient (*R*) was calculated.

Results

Table 1 summarises the clinical and laboratory data in the patients studied. All had consumed an average of 140 g ethanol or more a day for at least one year. None of the patients showed clinical evidence of chronic liver disease. AST, GGT, alkaline phosphatase, total CK and amylase activities were raised in 63% (14/22), 66% (12/18), 18% (4/22), 13% (3/23) and 17% (4/23) of cases, respectively. The serum bilirubin concentration was raised in only one of the 22 patients investigated. The haemoglobin concentration was within the normal range except in two male patients in

whom it was slightly reduced. None of the patients had reticulocytosis.

Isoenzyme studies showed only CK-MM activity (the skeletal muscle isoenzyme form) in all cases except one, in which 95% of the total CK activity consisted of CK-MM and 5% of CK-MB (CK-MB $\geq$ 5% of total CK suggests cardiac damage). All the patients with increased amylase activity showed an increase in isoamylase-S but not in isoamylase-P, indicating that the salivary gland rather than pancreas was the source of this increase.

Correlations were sought between NRAC with undialysed serum, NRAC with dialysed serum, or PIRAC on the one hand, and the various variables listed in table 1 (except age and duration of drinking) on the other. The only significant correlations observed are given in table 2, and two of these are illustrated in figs 1 and 2. The strongest correlation seen was between PIRAC and AST (*r* = 0.814; fig 2), the variability in PIRAC accounting for 64% of the variability in AST. Table 2 also gives details of the few significant correlations observed between the various biochemical variables estimated.

Discussion

In heavy drinkers there is a remarkably strong and highly significant correlation between PIRAC and AST. There are less strong but significant correlations between NRAC with undialysed or dialysed serum and AST. As both NRAC and, particularly, PIRAC reflect the cytotoxic activity of serum proteins modified by acetaldehyde, these correlations point to a direct or indirect relation between the concentration of such proteins and damage to one or more cell types

Table 2 Pearson correlation coefficients (*r*) or Spearman correlation coefficients (*R*) for all significant relations observed between various variables in heavy drinkers

<i>Coordinates</i>					
<i>x</i>	<i>y</i>	(<i>n</i> =)	<i>r</i>	<i>R</i>	<i>p</i> Value
Residual A9 cells with undialysed serum	AST	22	-0.435		0.043
		21*	-0.434		0.049
Residual A9 cells with dialysed serum	AST	22	-0.569		0.006
		21*	-0.562		0.008
Percentage increase in residual A9 cells after borohydride treatment	AST	22	0.814		0.0001
		21*	0.801		0.001
	Total CK	21		0.564	0.008
		19*		0.480	0.037
AST	GGT	18†		0.734	0.005
		22	-0.482		0.023
	Bilirubin	21*	-0.468		0.032
		22		0.598	0.003
GGT	AP	21*		0.546	0.010
		15†		0.624	0.013
	AP	18†		0.674	0.002

*Excluding female patients.

†No data available for female patients.

Fig 1 Correlation between serum AST activity in heavy drinkers and number of residual adherent cells after incubation of A9 cells with dialysed serum ($r = -0.569$).

Fig 2 Correlation between serum AST activity in heavy drinkers and percentage increase in residual adherent A9 cells after treatment of serum with NaBH_4 ($r = 0.814$).

containing AST. Cells which release AST when injured are hepatocytes, cardiac and skeletal muscle cells, and erythrocytes. As only one of the cases studied had raised CK-MB activities and only two cases showed increased CK-MM activities, it seems unlikely that damaged cardiac or skeletal muscle cells

were the major source of the increased AST activities in the sera of our patients. It is also unlikely that damage to circulating red cells was an important source of serum AST as no patient showed haematological or biochemical evidence suggestive of a substantial degree of haemolysis. For these reasons, and because there was a highly significant correlation between AST and GGT, we conclude that the high serum AST activities observed in our patients were largely derived from hepatocytes and that our data show some association between the concentrations of circulating cytotoxic protein and hepatocellular injury. As there is probably a close relation between the quantity of free acetaldehyde released into the hepatic sinusoids and the concentrations of circulating cytotoxic protein, our data point to a strong correlation between AST and hepatic acetaldehyde generation. This correlation may merely result from the fact that both AST and acetaldehyde are released at an increased rate from damaged hepatocytes. Alternatively, the observed correlations may represent evidence of the hepatotoxicity of acetaldehyde generated in the liver. Up to now the hypothesis that acetaldehyde is hepatotoxic has been based largely on evidence from *in vitro* studies.¹⁷⁻²²

Previous workers have found increased amounts of conjugated dienes in the plasma and liver in chronic alcoholics and have suggested that free radical activity may have a role in the pathogenesis of liver damage induced by alcohol.^{10,23} The increased free radical activity in the livers of heavy drinkers, however, does not correlate either with serum AST activity or the concentration of glutathione-S transferase, a sensitive marker of liver damage (RS Fink *et al*, unpublished observations). The present finding that concentrations of serum proteins made cytotoxic by acetaldehyde correlate with serum AST is, therefore, especially interesting.

Despite the fact that only three of the 23 cases had raised total CK activities, there was a relatively weak but significant correlation between PIRAC and total CK (largely CK-MM). This raises the possibility that modified serum proteins have a toxic effect on skeletal muscle cells *in vivo*. Thus our data suggest that both acetaldehyde and serum proteins modified by acetaldehyde have a role in causing tissue damage in alcoholics.

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