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IN VITRO EFFECTS OF VITAMIN C, THIOCTIC ACID AND DIHYDROLIPOIC ACID ON THE CYTOTOXICITY OF POST-ETHANOL SERUM

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Abstract—The serum of subjects consuming ethanol contains a non-dialysable cytotoxic activity, which is thought to reside in unstable acetaldehyde–protein adducts: the cytotoxic effects have been attributed to the transfer of acetaldehyde molecules from such adducts to target cells. When post-alcohol sera are incubated for 3 hr with ascorbic acid, thioctic acid or dihydrolipoic acid at a concentration of 10–500 μg/mL, their cytotoxicity against A9 cells is reduced. Post-alcohol sera incubated with these concentrations of thioctic acid or dihydrolipoic acid also had reduced cytotoxic activity against phytohaemagglutinin-transformed normal human lymphocytes. Studies with artificially produced [^{14}C]acetaldehyde–^{125}I-albumin complexes showed that treatment with thioctic acid or dihydrolipoic acid resulted in a reduced transfer of [^{14}C]acetaldehyde to K562 cells. If these *in vitro* data also apply *in vivo* and if circulating acetaldehyde–protein adducts play a role in alcohol-mediated tissue damage, vitamin C and, to a greater extent, thioctic acid may have a beneficial effect in patients with acute and chronic alcohol toxicity.

The ingestion of 60–95 g ethanol over 20–35 min is followed by the appearance of a non-dialysable cytotoxic activity in the serum. The cytotoxicity of dialysed post-alcohol serum has been demonstrated *in vitro*, not only against the mouse cell line A9 but also against six human cell lines [1] and against mitogen-stimulated normal human lymphocytes [2]. It is greatest 8–10 hr after the start of alcohol consumption, when blood alcohol levels are below 15 mg/dL, and is still detectable at 24 hr. The serum of individuals chronically consuming an excess of alcohol (140–750 g/day) also displays a non-dialysable cytotoxic activity [3]. The cytotoxic molecules appear largely to consist of acetaldehyde-modified albumin; the cytotoxicity may result from the release of acetaldehyde from unstable adducts formed during the first stage of reaction between the acetaldehyde and albumin and the subsequent binding of the free acetaldehyde to target cells [1]. The possibility that circulating acetaldehyde-modified cytotoxic protein may play a role in the pathogenesis of ethanol-induced damage *in vivo* [4] gains some support from the observation that in a group of 24 heavy drinkers there were statistically significant correlations between serum aspartate transaminase activity or creatine kinase activity on the one hand and an index of serum cytotoxicity on the other [3].

Several studies have shown that the cytotoxic activity in the sera of individuals taking alcohol is markedly reduced following incubation of the sera with the reducing agent sodium borohydride [1–3], presumably because of the stabilization of unstable Schiff bases which are formed by a reaction between the carbonyl group of acetaldehyde and the ε-amino group of lysine and which contain C=N bonds [5, 6]; the stabilization occurs by the addition of hydrogen across the double bond (C=N). This raises the possibility that inebriated patients may benefit from treatment with reducing agents. Another possibility is that the cytotoxicity of post-ethanol serum may be modified by agents capable of binding firmly and irreversibly to acetaldehyde released from unstable acetaldehyde–protein complexes. We have, therefore, studied the *in vitro* effects of vitamin C, dihydrolipoic acid and thioctic acid on the cytotoxicity of post-ethanol serum towards mouse A9 cells and mitogen-stimulated normal human lymphocytes. Thioctic acid is a naturally occurring substance which acts as a co-enzyme in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. It has a disulphide bond and can be converted metabolically to the reduced form, dihydrolipoic acid, which contains two free sulphydryl groups. It has been claimed that both compounds, and particularly the dihydro-form, protect cells from reactive toxic degradation products of some exogenous compounds, and on this basis thioctic acid (Thioctacid) has been advocated for the treatment of fatty liver and cirrhosis, especially due to alcohol ingestion, and of both diabetic and alcoholic polyneuropathy. A prospective randomized control study has shown that Thioctacid has a beneficial effect in the management of diabetic neuropathy [7].

MATERIALS AND METHODS

Pre-alcohol and post-alcohol sera. Five healthy volunteers who had abstained from consuming any alcohol for at least 3 days drank 700 mL of wine (9.5% ethanol) over 25 min. Venous blood was obtained immediately before alcohol was consumed

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(pre-alcohol serum) and 8 hr later (post-alcohol serum). The sera were separated and dialysed overnight at 4° against 500 mL RPMI medium 1640 (Imperial Laboratories, Andover, U.K.) containing 300 mg L-glutamine/L). The dialysed samples were frozen at −20° until use.

Preparation of solutions of thioctic acid and dihydrolipoic acid. Both compounds were provided by ASTA Pharma, Frankfurt, Germany. Thioctic acid (750 mg; α-lipoic acid, pure substance) was dissolved in 20 mL Tris (tris[hydroxymethyl]-aminomethane; 1.75 g/100 mL) at 45° for 20 min. The solution was cooled to room temperature and its pH adjusted to 7.4 with 1 N NaOH. The total volume was made up to 25 mL with H_2O and the resulting solution filtered through a 0.2 μm millipore membrane filter. This provided a stock solution containing 30 mg/mL. The stock solution was diluted with Tris (pH 7.4) to 1 mg/mL.

A volume of 0.75 mL (i.e. 750 mg) of dihydrolipoic acid (from nitrogen gassed ampoules containing 1 g/mL) was dissolved in 20 mL Tris and a solution containing 1 mg/mL prepared as described for thioctic acid.

Treatment of sera with vitamin C, thioctic acid, dihydrolipoic acid or sodium borohydride. Serum taken before and after alcohol consumption was incubated with an equal volume of Hanks' solution containing various concentrations of ascorbic acid for 3 hr at 37°. The mixtures were then dialysed overnight against RPMI 1640 at 4°.

Two millilitres of pre- or post-alcohol serum was mixed with 2 mL of a solution consisting of varying proportions of Tris (pH 7.4) and the solution containing 1 mg thioctic acid/mL or 1 mg dihydrolipoic acid/mL, such that the final drug concentrations were 0, 10, 20, 100, 200 and 500 μg/mL. The mixtures were incubated at 37° for 3 hr and dialysed overnight against 500 mL RPMI 1640 at 4°.

Aliquots of pre- and post-alcohol sera were also treated with 1.6% (v/v) of 400 mM NaOH or 1.6% (v/v) of 400 mM NaOH containing $NaBH_4$ such that the final concentration of the borohydride is either 0.155 or 1.55 mM (pH 9.5). The mixtures were incubated for 3 hr and then dialysed as described previously [1].

Assay of cytotoxic activity of sera. The cytotoxic activity of treated and untreated pre- and post-alcohol sera was tested against adherent A9 cells as described earlier [4, 8]; 1 mL of each sample was placed undiluted over the adherent cells in all experiments other than those concerned with $NaBH_4$, in which the samples were diluted to 10, 25 or 50% (v/v) in RPMI before testing. The extent of detachment of A9 cells after interaction with the sera for 16 hr was taken as an index of cytotoxicity.

The cytotoxic activity of treated and untreated pre- and post-alcohol sera was also tested against phytohaemagglutinin (PHA*)-stimulated normal human lymphocytes as described by Wickramasinghe and Barden [2]. Lymphocytes were separated from peripheral blood by density gradient centrifugation using Ficoll-Isopaque (Nyegaard & Co, Oslo, Norway). The serum under study was added *ab initio* to PHA-containing microcultures of lymphocytes, and [^{3}H]thymidine incorporation measured 48 hr later. The microcultures were made up of 25% (v/v) of the serum, 75% of lymphocytes suspended in RPMI 1640 containing 0.3 mg L-glutamine/mL, 100 U penicillin/mL and 100 μg streptomycin/mL, and 3 μg PHA/mL (total volume, 200 μL).

Effects of thioctic acid and dihydrolipoic acid on the transfer to K562 cells of [^{14}C]acetaldehyde from double-labelled acetaldehyde–albumin complexes. Double-labelled cytotoxic acetaldehyde–albumin complexes were prepared *in vitro* as described previously [1] by incubating [1,2-^{14}C]acetaldehyde with ^{125}I-labelled human serum albumin at 37° for 3 hr and dialysing the resulting solution. K562 cells were incubated for 3 hr with the double-labelled complexes and the extent of binding of radioactivity to K562 cells studied, measuring both the bound ^{14}C and ^{125}I in cells reacted with the complexes. The effects of pre-incubating the double-labelled complex for 3 hr with 500 μg/mL thioctic acid or 500 μg/mL dihydrolipoic acid on the binding of radioactivity to K562 cells was also investigated.

RESULTS

The number of residual adherent A9 cells was always lower in wells containing untreated post-alcohol sera than untreated pre-alcohol sera and the difference was statistically significant (Table 1).

Treatment of post-alcohol sera with 10–500 μg/mL ascorbic acid caused a statistically significant increase in the number of residual adherent A9 cells (i.e. a decrease in cytotoxic activity) (Table 1). The average percentage increase with 500 μg/mL ascorbic acid was 31%. Treatment of pre-alcohol sera with this substance caused slight increases in the number of residual adherent cells but the changes were not statistically significant, at any concentration of vitamin C.

The treatment of post-alcohol sera but not pre-alcohol sera with thioctic acid and dihydrolipoic acid (Table 1) at concentrations of 10–500 μg/mL caused a statistically significant increase in the number of residual adherent A9 cells (i.e. a decrease in cytotoxic activity). The average increase in adherent cells with 500 μg/mL thioctic acid and 500 μg/mL dihydrolipoic acid were, respectively, 43% and 53%. The average increase caused by the strong reducing agent $NaBH_4$ plus NaOH was 41% and 71% when the serum concentration in the test wells was 25% and 50% (v/v), respectively (Table 2).

When compared with pre-alcohol serum, post-alcohol serum caused inhibition of [^{3}H]thymidine incorporation by PHA-stimulated lymphocytes (i.e. inhibition of lymphocyte transformation) (Table 3). Treatment of post-alcohol sera with thioctic acid (20–500 μg/mL) or dihydrolipoic acid (10–500 μg/mL) caused a statistically significant and concentration-dependent reduction in this inhibitory activity (Table 3). Microcultures containing untreated and treated pre-alcohol sera incorporated [^{3}H]-thymidine to similar extents.

When [^{14}C]acetaldehyde/^{125}I-albumin complexes were incubated with K562 cells for 3 hr, an average

* Abbreviation: PHA, phytohaemagglutinin.

Table 1. Effects of treating pre- and post-alcohol serum from four or five healthy volunteers with various concentrations of ascorbic acid, thioctic acid or dihydrolipoic acid for 3 hr on their cytotoxicity towards A9 cells

Treatment and concentration of chemical (μg/mL)	Residual adherent A9 cells per well × 10^{-5}						
	Pre-alcohol serum			Post-alcohol serum			
	N	Mean	SD	N	Mean	SD	P*
Ascorbic acid							
0	4	2.04	0.35	4	1.41†	0.32	—
10	4	2.04	0.40	4	1.58	0.30	<0.01
50	4	2.09	0.39	4	1.74	0.39	<0.005
100	4	2.13	0.45	4	1.84	0.38	<0.005
500	4	2.15	0.48	4	1.85	0.33	<0.005
Thioctic acid							
0	5	2.34	0.91	5	1.39‡	0.60	—
10	5	2.37	0.94	5	1.55	0.56	<0.02
20	5	2.33	0.92	5	1.69	0.61	<0.005
100	5	2.27	0.88	5	1.82	0.64	<0.005
200	5	2.21	0.79	5	1.89	0.65	<0.001
500	5	2.16	0.75	5	1.99	0.64	<0.001
Dihydrolipoic acid							
0	4	2.40	0.30	4	1.69‡	0.20	—
10	4	2.42	0.28	4	2.00	0.20	<0.01
20	4	2.45	0.24	4	2.11	0.23	<0.025
100	4	2.59	0.21	4	2.37	0.27	<0.05
200	4	2.57	0.16	4	2.49	0.13	<0.01
500	4	2.65	0.09	4	2.59	0.10	<0.01

The cytotoxic activity of treated and untreated sera was tested against mouse A9 cells and was considered to be inversely proportional to the number of residual adherent A9 cells per well.

N = number of sera studied.

* For significance of difference from values given by untreated post-alcohol serum, determined using a paired *t*-test.

Significance of difference from value given by untreated pre-alcohol serum: † $P < 0.001$, ‡ $P < 0.005$ (paired *t*-test).

Table 2. Effects of treating pre- and post-alcohol serum from four healthy volunteers with NaOH or $NaBH_4$ for 3 hr

Treatment	No. of experiments	% (v/v) serum in test wells	Residual adherent A9 cells per well × 10^{-5}			
			Pre-alcohol serum		Post-alcohol serum	
			Mean	SD	Mean	SD
None	3	10	2.66	0.30	1.80	0.08
	4	25	2.94	0.16	1.67*	0.06
	2	50	3.65	—	1.58	—
6.4 mM NaOH	3	10	2.60	0.31	1.71	0.05
	4	25	2.73	0.20	1.59	0.08
	2	50	3.19	—	1.38	—
6.4 mM NaOH + $NaBH_4$†	3	10	2.54	0.07	2.29	0.18
	4	25	2.50	0.12	2.35‡	0.10
	2	50	3.09	—	2.70	—

The cytotoxic activity of treated and untreated sera was tested against mouse A9 cells and was considered to be inversely proportional to the number of residual adherent A9 cells per well.

* Significance of difference from value obtained in the presence of 25% (v/v) untreated pre-alcohol serum: $P < 0.001$ (paired *t*-test).

† $NaBH_4$ at a concentration of 1.55 mM was used in experiments in which 10% (v/v) serum was used in the test wells and 0.155 mM $NaBH_4$ in the others.

‡ Significance of difference from value obtained in the presence of 25% (v/v) NaOH-treated post-alcohol serum: $P < 0.001$ (paired *t*-test).

Table 3. Effects of treating pre- and post-alcohol serum from two healthy volunteers with various concentrations of thioctic acid or dihydrolipoic acid for 3 hr, on the PHA-induced transformation of blood lymphocytes from eight healthy blood donors

Treatment (μg/mL)	[^{3}H]Thymidine incorporation (cpm/well)				
	Pre-alcohol serum		Post-alcohol serum		
	Mean	SD	Mean	SD	P*
Thioctic acid					
0	5881	4384	4006†	2822	—
10	5916	4505	4202	3051	>0.05
20	5881	4458	4297	3053	<0.05
100	5811	4310	4404	3012	<0.01
200	5872	4424	4640	3218	<0.01
500	5826	4416	5112	3845	<0.05
Dihydrolipoic acid					
0	3597	2023	2511‡	1332	—
10	3539	2032	2693	1491	<0.05
20	3561	1989	2790	1551	<0.05
100	3516	1890	2813	1451	<0.01
200	3474	1906	2942	1588	<0.01
500	3522	1959	3093	1697	<0.01

The lymphocytes were cultured for 3 days in the presence of 3 μg/mL PHA and 25% (v/v) of the serum under study.

* For significance of difference from values obtained in the presence of untreated post-alcohol serum, determined using a paired *t*-test (N = 8 lymphocyte donors); in this analysis, the results of experiments with the sera from the two healthy volunteers were pooled.

Significance of difference from value obtained in the presence of untreated pre-alcohol serum: † $P < 0.01$, ‡ $P < 0.005$ (paired *t*-test).

of 0.150 nmol [^{14}C]acetaldehyde (N = 3) bound per 10^6 K562 cells. Following pre-treatment with 500 μg/mL of thioctic or dihydrolipoic acid for 3 hr, the average quantity of [^{14}C]acetaldehyde bound per 10^6 K562 cells was reduced to 0.082 and 0.089, respectively (N = 3); a reduction was seen in each of the three experiments and varied from 29.7 to 50.8%. The average molar ratio of [^{14}C]acetaldehyde to ^{125}I-albumin in the dialysed double-labelled complexes was 1.7. By contrast, the average molar ratio of material bound to K562 cells was 12.4. This fell to 5.7 and 5.1, after the complexes were treated with thioctic acid and dihydrolipoic acid, respectively.

DISCUSSION

The recommended daily dose of thioctic acid for diabetic and alcoholic polyneuropathy is 300–600 mg/day i.v. over a period of at least 14 days, followed by 200–300 mg/day orally. A much higher dose of 2–4 g/day has recently been administered to patients with AIDS [9]. After a single i.v. dose of 100 mg, the average initial concentration is 44.9 μg/mL of serum and the average half-life in the blood is 0.24 hr (ASTA Pharma Internal Report 1983, unpublished). The initial drug concentration achieved in the serum after single i.v. doses of 300 and 500 mg may, therefore, be expected to be of the order of 135 and 225 μg/mL, respectively. The results of the present study thus indicate that thioctic acid, at concentrations achieved therapeutically in the plasma *in vivo*, and dihydrolipoic acid reduce the *in vitro* cytotoxicity of post-ethanol serum towards both A9 cells and normal human lymphocytes.

High concentrations of vitamin C also caused a reduction of cytotoxicity against A9 cells but the extent of reduction was less than with 500 μg/mL of thioctic acid or dihydrolipoic acid. It is noteworthy that the two lowest concentrations of vitamin C that had some effect are within the range of whole blood vitamin C levels seen in individuals receiving 5 g ascorbic acid orally daily [10]. Previous studies have shown that vitamin C promotes the formation of stable adducts between acetaldehyde and the lysine moieties of bovine serum albumin by a mechanism which does not involve the reduction of Schiff bases [6]. It is likely that the reduction of cytotoxic activity by vitamin C is dependent on the stabilization of unstable adducts by the same mechanism.

The studies with the double-labelled acetaldehyde–albumin complexes and K562 cells suggest that the effects of thioctic and dihydrolipoic acids may be at least partly mediated via alterations to the cytotoxic acetaldehyde–albumin complexes such that the acetaldehyde which is reversibly bound to albumin can no longer be transferred to and exert cytotoxic effects on a target cell. This could well be due to a drug-related reduction and stabilization of unstable Schiff bases or some other unstable adducts formed between acetaldehyde and albumin. Alternatively, the drugs may combine strongly with free acetaldehyde and thus remove the acetaldehyde from the unstable adducts, thereby rendering the post-ethanol serum non-toxic. However, there is as yet no evidence that a chemical reaction between thioctic or dihydrolipoic acid and acetaldehyde does occur.

If the above *in vitro* data also apply *in vivo*, one mechanism by which thioctic acid has a beneficial effect in patients with acute and chronic alcohol toxicity may be by reducing the cytotoxic activity of circulating acetaldehyde-modified plasma proteins and thereby minimizing continuing tissue damage. Furthermore, the data on vitamin C raise the possibility that at least some of the reported protective effects of this vitamin against alcohol-related abnormalities [11, 12] may result from its effects on unstable acetaldehyde–protein adducts.

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