PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 325–329 (1981)

Protection of Pyridoxal 5′-Phosphate against Toxicity of Acetaldehyde to Hepatocytes (41280)

SHINICHI KAKUMA,[1] CARROLL M. LEEVY, OSCAR FRANK, AND HERMAN BAKER[2]

Departments of Medicine and Preventive Medicine & Community Health, New Jersey Medical School, Newark, New Jersey 07013

Abstract. Methods were evaluated for annulling the cytotoxicity of acetaldehyde (AcH) for isolated autologous liver cells, obtained by percutaneous liver biopsy, from cases of alcoholic hepatitis. Hepatocytes so obtained were more susceptible to the cytotoxicity of AcH than hepatocytes from normal liver, viral hepatitis, alcoholic fatty liver, and stable alcoholic cirrhosis. Benzylamine (an aldehyde buffer) and pyridoxal 5′-phosphate (PLP) counteracted the cytotoxicity of AcH *in vitro;* pyridoxol did not. AcH cytotoxicity was seen in liver cells from vitamin B_6-deficient alcoholics with alcoholic hepatitis but was reversed when the B_6 deficiency was corrected by intramuscular administration of flushing doses of pyridoxol (150 mg daily). We suggest that *in vitro,* benzylamine neutralizes AcH toxicity through a Schiff-base condensation with AcH, whereas pyridoxal 5′-phosphate protects against AcH toxicity *in vitro* and *in vivo* by probably forming a Schiff base with cellular amino acids, blocking further condensation of these amino groups with AcH.

Hepatocyte malfunction in the alcoholic seems due to toxicity of acetaldehyde (AcH) derived from ethanol (EtOH) oxidation (1, 2). This effect is especially damaging to the liver of chronic alcoholics who nearly all show higher levels of AcH in the blood than nonalcoholics ingesting EtOH (1). Because AcH readily forms Schiff bases, protein synthesis and cell integrity may also be injured by AcH condensation with amino acids (2), and possibly, nucleic acids. We here exploited the ability of AcH to form Schiff bases and show that an aldehyde buffer, e.g., benzylamine (3), is capable of neutralizing AcH cytotoxicity *in vitro;* pyridoxal 5′-phosphate (PLP) can also abrogate AcH cytotoxicity *in vitro* and *in vivo.*

Materials and Methods. *In vitro.* Liver specimens from 58 subjects were obtained by biopsy with the Vim-Silverman needle. Histologic evidence of alcoholic fatty liver was noted in 12; alcoholic hepatitis in 15; stable cirrhosis in 10; acute viral hepatitis in 9; and no liver disease in 12. Each biopsy specimen was minced and incubated in flasks containing dissociation medium, e.g., Roswell Memorial Park Institute (RMPI) medium No. 1640 with 0.1% collagenase plus 0.005% DNAase (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 25 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer, pH 7.4, 10% heat-inactivated fetal calf serum, 50 m*M* glutamine, 4000 U/100 ml penicillin, and 4 mg/100 ml streptomycin. Incubation was at 37° for 15 min in a Dubnoff shaker at 10 strokes/min. This procedure was repeated twice to obtain a suspension of liver cells. Cells (1×10^6), purified by centrifugation over a Ficoll–Hypague gradient, were labeled with 250 μCi $^{51}CrNa_2CrO_4$, specific activity 300 μCi/μg Cr (Amersham, Arlington Heights, Ill.) for 45 min at 37° in a 5% CO_2 atmosphere and then washed three times with RPMI medium. Eighty to ninety percent of the cells were hepatocytes as identified by morphologic appearance; over 90% were viable by trypan blue exclusion.

Cytotoxicity studies. (a) Twenty-five micrograms of AcH (0.2 ml) or 0.2 ml of RMPI medium were added to 1×10^4 ^{51}Cr-labeled

[1] Present address: Third Department of Medicine, Nagoya University School of Medicine, 2-3-16 Higashisakua, Higashi-ku, Nagoya 461, Japan.

[2] Address reprint requests to: 88 Ross Street, East Orange, N.J. 07018

0037-9727/81/110325-05$01.00/0

autologous liver cells (0.2 ml) in 12 × 75-mm culture tubes and incubated 2 hr at 37° in a 5% CO_2 atmosphere. The mixture was centrifuged at 1000 rpm for 10 min and 0.2 ml of the supernatant was pipetted into tubes for gamma counting. Cytotoxicity was expressed as the ratio of experimental release of ^{51}Cr minus spontaneous release to maximum release minus spontaneous release × 100. *Spontaneous release*, ^{51}Cr from autologous liver incubated in media alone; *maximum release*, ^{51}Cr from autologous liver after repetitive freezing–thawings; and *experimental release*, equals the ^{51}Cr from target cells incubated with AcH. Results are expressed as the mean and standard error of triplicate cultures; Student's *t* test was used to estimate the significance of differences (*P*) between the mean AcH-induced cytotoxicity in normal liver as compared to hepatocytes from alcoholics with histological evidence of various liver diseases.

(b) The effects of 0.4, 4.0, and 40 μg (final concentration) benzylamine added to the incubate containing ^{51}Cr-labeled hepatocyte culture from alcoholic hepatitis patients. The cells were incubated with or without 25 μg AcH (vide supra) to determine whether benzylamine prevents AcH-induced cytotoxicity. A similar experiment with 0.25, 2.5, and 25 μg of pyridoxal 5′-phosphate (PLP) instead of benzylamine was also done with hepatocytes from vitamin B_6-deficient patients.

In vivo. Hepatocytes were obtained from the livers of five patients with alcoholic hepatitis who were vitamin B_6 deficient. Three patients were then treated with intramuscularly administered flushing doses of pyridoxol (150 mg daily) for 14 days; one was treated with intramuscularly administered folic acid (1 mg daily) for 14 days; and one patient was not given any vitamin treatment. Hepatocytes from these five patients were incubated with 25 μg AcH (vide supra) to determine the percentage of AcH-induced cytotoxicity before and after vitamin treatment; plasma vitamin B_6 levels of all these patients were determined with *Tetrahymena thermophila;* normal plasma vitamin B_6 is 30–80 ng/ml (4).

Results. Table I shows a dose-related cytotoxic effect of AcH on hepatocytes from patients with alcoholic hepatitis as compared to normal hepatocytes. No significant toxicity was noted on normal or diseased cells without AcH or levels of AcH to 10 μg per culture tube. Compared to normal liver cells, a high significant percentage of AcH cytotoxicity (<.005) was noted in cells from alcoholic hepatitis at a level of 25 μg of AcH per culture tube. Based on this result, we chose 25 μg of AcH per culture tube as the test concentration for studying AcH-induced hepatocyte toxicity.

When comparing hepatocytes from patients with alcoholic hepatitis, we also noted that they were more susceptible to AcH toxicity than subjects with stable cirrhosis, fatty liver, and acute viral hepatitis; all diseased cells were significantly more vulnerable to AcH than cells from normal livers (Table II). Such *in vitro* toxicity was significantly decreased by addition of increasing amounts of benzylamine (Table III) or PLP (Table IV) to the incubated hepatocytes from vitamin B_6-deficient patients with alcoholic hepatitis. Unlike PLP, pyridoxol did not annul the cytotoxic effects of AcH (Table IV). Hepatocytes from patients with alcoholic hepatitis who were

TABLE I. THE CYTOTOXIC EFFECTS OF DIFFERENT AMOUNTS OF AcH ON ^{51}Cr-LABELED AUTOLOGOUS LIVER CELLS

	Concentration of AcH (μg/culture tube)				
Sources of liver cells	0	1	10	25	100
Normal liver (3)	1.0 ± 0.2	1.2 ± 0.3	1.7 ± 0.4	2.6 ± 0.8	19.3 ± 1.9
P	n.s.[a]	n.s.	n.s.	<0.005	<0.05
Alcoholic hepatitis (3)	1.3 ± 0.4	2.0 ± 0.8	8.3 ± 2.8	16.9 ± 3.9	28.3 ± 4.7

Note. The results shown are percentage cytotoxicity (mean ± SE).
[a] Not significant. Number in parenthesis indicates sample number.

TABLE II. CYTOTOXICITY OF ACETALDEHYDE ON ^{51}Cr-LABELED AUTOLOGOUS LIVER CELLS

Sources of liver cells	N^a	Percentage cytotoxicity (mean ± SEM)
Normal liver (N)	12	2.9 ± 1.0
Fatty liver (FL)	12	5.8 ± 1.1
Alcoholic hepatitis (AH)	15	17.3 ± 4.9
Stable cirrhosis (SC)	10	6.5 ± 1.2
Acute viral hepatitis (VH)	9	5.7 ± 1.1

	N	VH	SC	FL
AH	<0.001	<0.01	<0.01	<0.01
FL	<0.025	n.s.	n.s.	
SC	<0.025	n.s.		
VH	<0.025			

Note. The lower half of the table shows the significance levels (P) of diseased and normal liver cells.
[a] N = number of patients.

vitamin B_6 deficient as determined by assay with *T. thermophila* (4) and who then were treated for 14 days with 150 mg pyridoxol, resisted the toxic effects of AcH (Table V) after, but not before, treatment; folic acid treatment, instead of pyridoxol, did not prevent AcH-induced cytotoxicity.

Discussion. Nutrient repletion, especially with folic acid and pyridoxol, enhances liver cell regeneration in the alcoholic (4–7); alcoholism tends to deplete circulating folate and vitamin B_6 (4–8). Indeed, AcH, a product of EtOH oxidation, impairs vitamin B_6 metabolism in human erythrocytes and hepatocytes *in vitro* (6, 7, 9). Our present study shows that AcH significantly injures liver cells from alcoholics (Table II); PLP protected against the cytotoxicity of AcH *in vitro* (Table IV) as well as protecting hepatocytes from pyridoxol-treated alcoholics (Table V). This suggests that hepatotoxicity induced by AcH is elicited by the combination of EtOH ingestion and vitamin B_6 deficiency and is attributed to AcH produced from EtOH; EtOH cannot so act until yielding AcH (9).

TABLE III. ABROGATION OF INHIBITION OF ACETALDEHYDE (AcH)-INDUCED CYTOTOXICITY BY BENZYLAMINE IN HEPATOCYTES FROM SIX PATIENTS WITH ALCOHOLIC HEPATITIS

Additives	Percentage cytotoxicity (mean ± SEM)	P^a
Benzylamine, 40 μg	0.1 ± 0.6	
AcH, 25 μg	14.3 ± 3.5	
AcH, 25 μg; benzylamine, 0.4 μg	8.2 ± 2.2	n.s.[b]
AcH, 25 μg; benzylamine, 4.0 μg	3.3 ± 0.9	<0.05
AcH, 25 μg; benzylamine, 40.0 μg	1.2 ± 0.4	<0.01

[a] As compared to AcH alone.
[b] Not significant.

TABLE IV. PROTECTION AGAINST ACETALDEHYDE (AcH)-INDUCED CYTOTOXICITY IN HEPATOCYTES FROM THREE PATIENTS WITH ALCOHOLIC HEPATITIS WITH VITAMIN B_6 DEFICIENCY BY PYRIDOXAL 5'-PHOSPHATE (PLP) AS COMPARED WITH PYRIDOXOL

Additives	Percentage cytotoxicity (mean ± SEM)	P^a
PLP, 25 μg	1.4 ± 0.4	
Pyridoxol, 25 μg	1.2 ± 0.1	
AcH, 25 μg	12.8 ± 2.2	
AcH, 25 μg; PLP, 0.25 μg	8.8 ± 1.2	n.s.[b]
AcH, 25 μg; PLP, 2.5 μg	4.0 ± 0.7	<0.05
AcH, 25 μg; PLP, 25 μg	0.8 ± 0.2	<0.01
AcH, 25 μg; pyridoxol, 25 μg	12.0 ± 2.2	n.s.

[a] As compared to AcH alone.
[b] Not significant.

As noted, AcH is very reactive, forming Schiff-base condensates with many amines. Thus quantitative data concerning AcH toxicity are dependent upon the condensation product with which AcH forms a Schiff base. The cytotoxicity of AcH shown in this study suggests a Schiff-base condensation of AcH with cell amino acids and, quite likely nucleic acid purines and pyrimidines to form a complex that has a decreased affinity for PLP (2, 9, 10). This condensation inhibits protein synthesis at the level of RNA synthesis thus producing cytotoxicity (10). The cytoprotective effect of PLP (Table IV) is thus explicable. PLP also forms a Schiff base with amino acids (2, 11, 12). When sufficient PLP is present to saturate, i.e., by binding the amino groups through Schiff-base formation, AcH condensations with these groups are blocked

TABLE V. COMPARISON OF ACETALDEHYDE-INDUCED CYTOTOXICITY ON LIVER CELLS FROM FIVE PATIENTS WITH ALCOHOLIC HEPATITIS AND VITAMIN B_6 DEFICIENCY BEFORE AND AFTER INTRAMUSCULAR TREATMENT WITH PYRIDOXOL OR FOLIC ACID FOR 14 DAYS

Case No.	Treatment	Percentage cytotoxicity		Plasma vitamin B_6 (ng/ml)[a]	
		Before	After	Before	After
1	None	14.0	12.4	24	28
2	Pyridoxol 150 mg daily	18.4	6.0	19	125
3	Pyridoxol 150 mg daily	22.7	5.1	14	169
4	Pyridoxol 150 mg daily	17.0	7.2	21	194
5	Folic acid 1 mg daily	17.6	15.9	24	21

[a] Normal range, 30–80 ng/ml (95% confidence limits).

thus obviating AcH cytotoxicity *in vitro* and *in vivo* (Tables IV, V). Since *pyridoxol* and *pyridoxamine* do not contain an aldehyde group, they cannot act like PLP in Schiff-base formation unless converted to PLP *in vivo*. The phosphate group of PLP makes PLP more reactive in aldehydic condensations (12) which is the reason PLP is more reactive than pyridoxol (Table IV). Further proof for cytotoxicity of AcH by Schiff-base formation is suggested by the results with benzylamine (Table III). Benzylamine, an aldehyde buffer (3), acts by reversible condensation with aldehyde groups. When sufficient benzylamine is added *in vitro* to couple with AcH, benzylamine neutralizes the toxic action of AcH by blocking further Schiff-base formation. There could also have been an aldol-type condensation of AcH with PLP, thereby removing AcH from the field of action. A test of this latter possibility would demand a comparison of the detailed kinetics of condensation of AcH and benzylamine with PLP. However, it should be noted that the original proposal for use of benzylamine as an aldehyde buffer rested on the rapidity of condensation of benzylamine with several low-molecular aldehydes, e.g., AcH and formaldehyde (3).

These results point to the likely importance of PLP in protecting liver cells against AcH toxicity. Our study shows that it is advisable to protect alcoholic hepatitis patients with vitamin B_6 to initiate such protection (Table V). This is especially significant when alcoholics are vitamin B_6 deficient. Unlike a previous report (7) suggesting that alcoholic patients with liver disease do not convert pyridoxol to PLP, our results show that these alcoholics do convert pyridoxol to PLP, else pyridoxol therapy would not overcome AcH cytotoxicity (Table V). Furthermore, since conversion of pyridoxol to PLP was not possible in the *in vitro* system used here (Table IV), pyridoxol could not counteract the toxicity of AcH as PLP did, hence conversion to PLP must have occurred *in vivo* to annul AcH toxicity. This is substantiated by assays of circulating vitamin B_6 (Table V). The procedure used (4) is specific for only metabolically active forms of B_6, e.g., PLP and pyridoxamine 5′-phosphate; the increased levels of these vitamers seen in the alcoholics after treatment with pyridoxol (Table V) would not have been detected unless pyridoxol had been converted to active forms.

The reason for a decrease in metabolically active vitamin B_6 in alcoholics may be acceleration of PLP clearance, thereupon depleting circulating PLP (13)—which further jeopardizes hepatocytes in respect to AcH. Others have also shown that protein binding of PLP protects PLP against degradation (9, 14). This is a further indication that protein binding of PLP is an im-

portant mechanism for protecting the cells against AcH destruction by blocking protein-binding sites available to AcH during ethanolism.

1. Lieber, C. S., Baraona, E., Matsuda, Y., Salaspuro, M., Hasamura, Y., and Matsuzaki, S., Advan. Exp. Med. Biol. **126**, 397 (1978).
2. Perin, A., and Sessa, A., *in* "The Role of Acetaldehyde in the Actions of Ethanol" (K. O. Lindros and C. J. P. Eriksson, eds.), Satellite Symposium, 6th International Congress of Pharmacology. Finn. Found. Alcohol Stud. **23**, 105 (1975).
3. Jakoby, W. B., and Narrod, S. A., J. Bacteriol. **77**, 410 (1959).
4. Baker, H., Frank, O., Ning, M., Gellene, R. A., Hutner, S. H., and Leevy, C. M., Amer. J. Clin. Nutr. **18**, 123 (1966).
5. Leevy, C. M., and Baker, H., Med. Clin. N. Amer. **54**, 467 (1970).
6. Veitch, R. L., Lumeng, L., and Li, T. K., J. Clin. Invest. **55**, 1026 (1975).
7. Hines, J. D., and Cowan, D. H., N. Engl. J. Med. **283**, 441 (1970).
8. Baker, H., and Frank, O., World Rev. Nutr. Diet. **9**, 124 (1968).
9. Lumeng, L., J. Clin. Invest. **62**, 286 (1978).
10. Kakuma, S., Levinson, R., Baker, H., and Leevy, C. M., Gastroenterology **71**, 914 (1976).
11. Fasella, P., Annu. Rev. Biochem. **36**, 185 (1967).
12. Braunstein, A. E., Vitam. Horm. (N.Y.) **22**, 451 (1964).
13. Mitchell, E., Wagner, C., Stone, W. J., Wilkinson, G. R., and Schenker, S., Gastroenterology **71**, 1043 (1976).
14. Li, T. K., *in* "Human Vitamin B_6 Requirements," Proceedings of a Workshop, p. 210. National Academy of Sciences, Washington D.C. (1978).

Received March 5, 1981. P.S.E.B.M. 1981, Vol. 168.