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ACETALDEHYDE-COLLAGEN ADDUCTS IN CCl_4-INDUCED LIVER INJURY IN RATS

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SUMMARY: Circulating AC levels as well as antibodies against AC-protein adducts are increased in non-alcoholic liver injury. To identify the adducts, we used rats with CCl_4-induced cirrhosis. Liver subcellular fractions were analyzed by immunochemical staining of protein slot blots and of electrophoretically separated proteins, transferred to nitrocellulose, using AC-protein adduct-specific antibodies. One reactive protein of about 200 kD was detected in the liver soluble fraction and in the cytosol of isolated hepatocytes and, to a lesser extent in the liver microsomes of CCl_4-treated rats; in control animals, this reactivity was much weaker. The immunopositive AC adduct co-migrated with the β1,2 dimer of rat collagen type I; it was sensitive to digestion by a highly purified collagenase and also reacted with anti-rat collagen type I-specific IgG. In addition, comparison of peptides of the CNBr-digested, immunoprecipitated AC adduct with those of rat collagen type I revealed a high degree of similarity. Thus, AC adduct formation occurs in liver injury of non-alcoholic origin, and a target protein appears to be related to collagen type I, most likely the procollagen precursor.

Antibodies against AC protein adducts are found in sera of alcoholics as well as in patients with non-alcoholic liver diseases, and in both cases, the antibody titer increases with the severity of liver damage (1). In rats chronically fed ethanol-containing diets, AC adducts are formed with the ethanol-metabolizing enzyme, cytochrome P450IIE1 (2) and with an unidentified cytosolic protein with an approximate molecular weight of 37 kD (3). In rats with CCl_4-induced cirrhosis, mitochondrial oxidation of AC is decreased, whereas its production from threonine is increased (4). Target proteins for AC binding in non-alcoholic liver disease are presently unknown. Therefore, this study was undertaken to investigate the possible formation of AC-protein adducts in the liver of CCl_4-treated rats and to identify the target protein(s).

MATERIALS AND METHODS

Animals

Twelve Sprague Dawley rats [CRL-CR(SD)-BR] (Charles River Breeding Laboratories, Wilmington, MA), fed Chow diet ad libitum, were injected intraperitoneally twice weekly with CCl_4 (0.2ml in 0.5ml of peanut oil) or the oil vehicle alone for 6 weeks. After an overnight fast, the rats were sacrificed under pentobarbital (40 mg/kg body weight) anesthesia, at least 3 days after the last CCl_4 dose.

Abbreviations used: AC = acetaldehyde, HCN-AC = acetaldehyde-Keyhole Limpet hemocyanin, RP450-AC = rat P450IIE1-acetaldehyde, IgG = immunoglobulin G, kD = kilo Dalton, CNBr = cyanogen bromide, SDS-PAGE = sodium dodecyl sulfate polyacrylamide electrophoresis, ELISA = enzyme-linked immunosorbant assay, Blotto = phosphate-buffered saline containing 5% dry milk.

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Preparation of liver subfractions

Livers were homogenized in 3 volumes of ice-cold buffer (10 mM Tris-HCl, pH 7.4, in 0.25 M sucrose containing 1 mM EDTA) by 4 up and down strokes in glass homogenizers using a teflon pestle. After centrifugation at 1,100 x g, the sediment was washed in the same buffer and recentrifuged. Pooled supernatants were centrifuged at 8,750 x g for 10 min (4°C) to obtain the mitochondrial pellet. The post-mitochondrial supernatant was centrifuged at 100,000 x g for 70 min to separate the microsomes and the soluble fraction. To prevent *ex-vivo* digestion by proteases, 4 inhibitors were added to all fractions (3 mM p-chloromercuribenzoic acid, 5 mM EDTA, 5 mM n-ethylmaleimide, and 0.2 mM phenylmethylsulfonyl fluoride) and aliquots were stored at -80°C until use. Protein content was determined by the method of Lowry (5).

Isolation of hepatocytes

Hepatocytes from 2 control and from 2 CCl_4-treated rats were isolated according to the method of Seglen (6).

Preparation of AC-protein adducts and anti-AC adduct antibodies

HCN-AC adducts were prepared *in vitro* (7), and RP450-AC adducts were isolated from ethanol-fed rats as previously described (2). Antibodies against HCN-AC and RP450-AC adducts were produced in New Zealand white rabbits (2). Total IgG was isolated by the method of McKinney and Parkinson (8). Rabbit anti-HCN-AC and rabbit anti-RP450-AC were further purified by affinity chromatography, using hemoglobin-AC-conjugated and HCN-AC-conjugated sepharose columns, respectively. Because of the lower reactivity of anti-HCN-AC IgG with the antigen detected, anti-RP450-AC IgG was used preferentially in this study.

Immuno-slot blotting

Undenatured liver soluble, mitochondrial, and microsomal proteins were first tested by immuno-slot blotting using Schleicher & Schuell nitrocellulose (pore size 0.45μm) and a Bio.Rad Minifold I apparatus (Bio.Rad, Rockville Center, N.Y.). Equal amounts of protein (0.5 to 2 μg/well), diluted in Tris-NaCl buffer (20 mM Tris-HCl, pH 7.4, in 0.9% NaCl), were applied to the nitrocellulose. After drying at room temperature for 1 hr and at 60°C for 15 min, the nitrocellulose was incubated sequentially in (a) Blotto (1 hr) to block uncoated sites, (b) appropriate dilutions of AC adduct-specific rabbit antibodies in Blotto (1 hr), (c) alkaline-phosphatase-conjugated goat anti-rabbit IgG, diluted in Blotto (2 hr), and (d) alkaline phosphatase substrate (5 mg Nitroblue tetrazolium and 2.5 mg 5-bromo-4-chloro-3-indolyl phosphate in 50ml of 10% diethanolamine buffer, pH 9.8, containing .5 mM $MgCl_2$) (15 to 20 min). In between steps, the nitrocellulose was washed 3 times with 0.05% Tween-20 in saline. Immunoreaction intensity was determined by scanning densitometry.

The immuno-slot blotting method was also used to determine crossreactivity of the anti-RP450-AC IgG with different AC adducts and their carrier proteins (hemocyanin, bovine serum albumin, collagen type I, and cytochrome P450IIE1). For this purpose, 30 ng of AC adduct proteins were used per slot and processed as described above, using anti-RP450IIE1-AC IgG as the primary antibody.

Collagen measurements

To determine the type of collagen and to measure the amount present in the liver soluble fraction of CCl_4-treated and control rats, ELISA microtest plates were coated with 10 μg of soluble liver protein (diluted in carbonate/bicarbonate buffer, pH 9.6) per well and left overnight at 4°C. Purified collagen types I, III, and IV (Collaborative Research Inc., Bedford, MA) were used as standards. After 3 washes with 0.05% Tween-20 in saline, 200 μl of Blotto were added to each well to block uncoated sites. After incubation at room temperature for 1 hr, the samples were incubated sequentially with (a) appropriate dilutions in Blotto of rabbit antibodies against collagen type I, III, or IV (Institut Pasteur, Lyon, France) at 37°C for 1 hr, (b) alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted in Blotto) for 1 hr, (c) alkaline phosphatase substrate (1 mg p-nitrophenyl-phosphate/ml 10 % diethanolamine buffer, pH 9.8, containing .5 mM $MgCl_2$). After color development at room temperature in the dark, optical densities were read at 405 nm with a Bio.Rad ELISA reader (Bio.Rad, Rockville Center, N.Y.). Total liver collagen content was determined by the method of Rojkind and Gonzalez (9).

Immunoprecipitation of AC-protein adducts and collagenase digestion

Rat liver AC adducts were precipitated by incubating aliquots of the soluble fraction with anti-RP450IIE1-AC IgG (1 μg protein per 10 μg IgG) at 37°C for 1 hr, followed by a 30 min incubation for 30 min with 50 μl protein A-sepharose. The immune complexes were then sedimented by centrifugation in an Eppendorf centrifuge at 15,000 x g for 2 min . Both the supernatant and the immune complex pellet were analyzed by immunostaining of transfer blots of the electrophoretically

separated proteins. The immune complexes were also analyzed after incubation at room temperature for 24 hr with 100 U/ml of highly purified bacterial collagenase (Advance Biofactures Corp., Lynbrook, N.Y.) in 0.12 M HEPES buffer, pH 7.4, containing 5.7 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride. Control samples were incubated under the same conditions, but in the absence of collagenase.

Isolation of collagen type I

Rat tail tendons were removed and washed with PBS followed by distilled water. The tendons were then placed in 0.5 M acetic acid at a ratio of 1:10 (w/v) and stirred at 4°C overnight (10). After centrifugation at 27,000 x g, the supernatant was diluted 1:1 with 0.5 M acetic acid. The purity of the collagen was tested by SDS-PAGE.

SDS-PAGE and protein blotting

SDS-PAGE of liver soluble proteins was performed as described previously (2), using 7.5% acrylamide. for immunochemical staining, the gels were loaded with 25 μg of liver soluble protein per well, and with 20 μg per well for Coomassie-blue staining. Rat collagen type I (Sigma, St Louis, MS) was used at a concentration of 4 μg/well. Protein transfers to nitrocellulose were performed according to Nielson et al (11). Immunochemical staining of protein blots was performed as described under "Immuno-slot blotting".

CNBr treatment of immunoprecipitated AC adducts and rat collagen type I

CNBr fractionation of the immune complexes precipitated from the liver soluble fraction of CCl_4-treated rats (see above) and of rat collagen type I was carried out as described by Epstein et al (12). Five mg protein were dissolved in 4 ml of 70% formic acid. To remove oxygen, the solution was flushed with nitrogen gas and CNBr (approximately 10 times the weight of collagen) added. After solubilization of the CNBr crystals, the mixture was incubated at 30°C for 4 hours and then diluted with 5 volumes of distilled water. To remove acid and CNBr, the digested material was lyophilized, redissolved in 0.5 M acetic acid and stored at -20°C until used for electrophoretic analysis. CNBr-digested fractions of both collagen type I and the AC-protein adduct immune complexes were separated by SDS-PAGE (see above) and the gels stained with Coomassie blue.

RESULTS

Affinity-purified antibodies, produced against RP450-AC adducts, isolated from the liver of alcohol-fed rats, crossreacted with AC adducts of other proteins (such as collagen type I, albumin and hemocyanin) generated *in vitro*, but did not react with the carrier proteins. Aldehyde linkages, normally present in mature collagen did not crossreact with the antibodies. Anti-RP450IIE1-AC IgG reacted strongly with protein slot blots of undenatured liver soluble proteins obtained from CCl_4-treated rats and weakly with the same proteins from control animals. The density of the reactive bands was 2.5 to 4.2-fold higher in experimental as compared to control samples (table 1). Although

Table 1. Density units of the immuno slot blots of undenatured cytosolic protein reacted with anti P450IIE1-acetaldehyde IgG and immunostained with alkaline phosphatase conjugated goat anti rabbit IgG

Pair No.	Control rats	CCl_4-treated rats
1	277	991
2	318	1350
3	285	1203
4	391	1099
5	322	805
6	456	1186
Mean ± SE	341 ± 31	1106 ± 84 *

* $p < 0.01$, by Student's *t* test applied to paired comparisons.

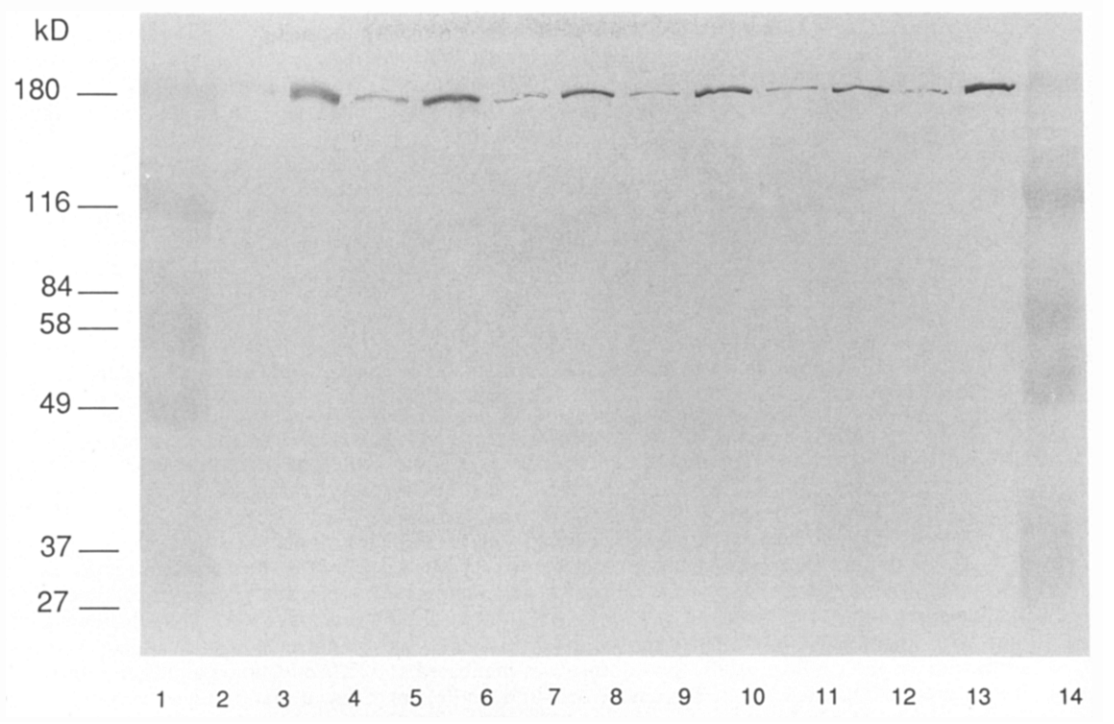

Figure 1. Protein blot of electrophoretically separated soluble liver proteins (25 μg/lane) from CCl_4-treated rats, reacted with anti-RP450-AC IgG. Lanes 1 and 14: prestained molecular weight standards; lanes with even numbers: control samples; lanes with uneven numbers: samples of CCl_4-treated rats.

anti-HCN-AC IgG detected the same antigen, its immunoreactivity was weak and, therefore, anti-RP450-AC was used almost exclusively in the subsequent studies.

Immunochemical staining of transfer blots of electrophoretically separated soluble liver proteins revealed a single band in samples of control and CCl_4-treated rats (figure 1), showing differences in staining intensity similar to those described for immuno-slot blots. A weakly reactive protein band similar to that found in the soluble liver fraction was found in liver microsomes of some

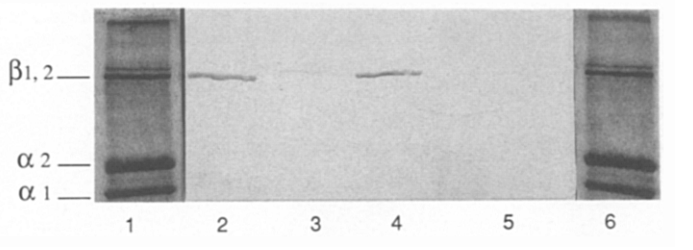

Figure 2. Comparison of rat collagen type I (2 μg/lane) and AC adducts from the liver soluble fraction of CCl_4-treated rats (25 μg/lane), electrophoresed in parallel. Lanes 1 and 6: Coomassie blue-stained rat collagen type I; lanes 2 - 5: protein transfer blot of the AC adducts of the rat liver soluble fraction, immunochemically stained with anti-RP450IIE1-AC IgG. Lane 2: liver soluble proteins, lane 3: the same as lane 2 after immunoprecipitation of the AC adduct with anti-AC adduct IgG, lane 4: sedimented AC adduct immune complexes; lane 5: same as lane 4 after treatment with purified collagenase.

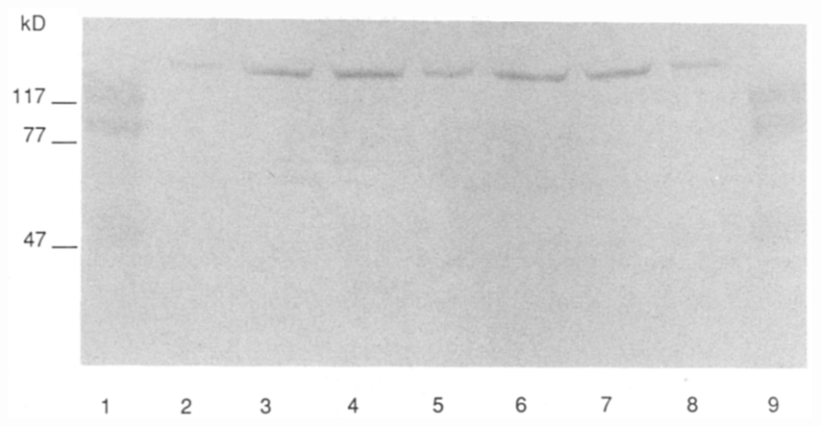

Figure 3. Protein blot, immunochemically stained with anti-rat collagen type I IgG. Lanes 1 and 9 = prestained molecular weight standards; lanes 2, 5 and 8: rat collagen type I (2 μg/lane); lanes 3, 4, 6 and 7: AC-protein adduct immune complex precipitated from the liver soluble fraction of CCl_4-treated rats (200 μg/lane).

CCl_4-treated rats (not shown), whereas the mitochondrial and nuclear proteins showed no reactivity. The molecular weight of the immunoreactive AC-protein adduct was estimated to be near 200 kD when compared to Coomassie blue-stained molecular weight standards, electrophoresed in parallel with the proteins transblotted to nitrocellulose.

The electrophoretic migration characteristics of the β1,2 band (a dimer of the α1 and α2 chains) of rat collagen type I was found to be similar to that of the AC-protein adduct in the soluble liver fraction of CCl_4-treated rats (figure 2). This unidentified AC adduct was undectectable after sequential incubations of liver soluble proteins with the anti-AC adduct IgG and protein A-sepharose, but could be recovered from the sedimented immune complexes; however, it was not recoverable after incubation of the immune complexes with purified bacterial collagenase (figure 2). Furthermore, transfer blots of the electrophoresed AC-protein immune complexes and rat collagen type I, showed a positive reaction with a monospecific antibody against rat collagen type I not only with collagen but also with the liver AC adduct (figure 3), although 200μg as compared to 20 μg adduct protein had to be used to obtain a visible band. The protein concentration of collagen type I used allowed only for the visualization of the β1,2 band.

Table 2. Collagen concentration in rat liver

	Control rats	CCl_4-treated rats	p*
mg collagen/g liver	1.36 ± 0.20	2.25 ± 0.11	< 0.001
mg collagen/g body weight	0.04 ± 0.002	0.11 ± 0.006	< 0.001
μg collagen type I/mg cytosolic protein	1.22 ± 0.17	1.21 ± 0.15	NS**
μg collagen type III and IV/mg cytosolic protein	0	0	

* Student's *t* test, ** NS = non significant.

ELISA assays for collagens Type I, III, and IV on the soluble liver fraction showed that only collagen type I was present, showing no differences in concentration between the control and experimental samples, despite the fact that the collagen content per g liver or per total liver was significantly higher in CCl_4-treated than in control rats (table 2).

Electrophoretic fingerprinting of CNBr-digested rat tail collagen type I and the immune complexes of the AC adduct revealed a high degree of similarity between the 2 samples, although the immune complex showed additional protein bands due to the presence of small amounts of IgG and protein A and possibly pro-α chains of type I procollagen (figure 4).

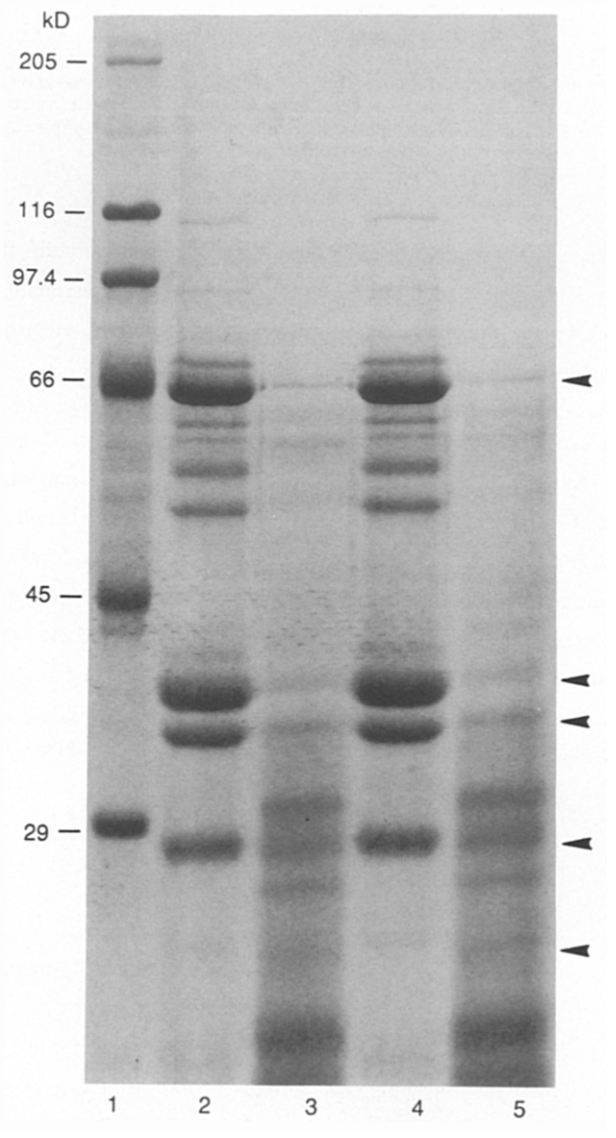

Figure 4. SDS-PAGE of peptides of CNBr-digested rat tail collagen type I and AC-protein adducts immunoprecipitated from the liver soluble fraction of CCl_4-treated rats, immunochemically stained with anti-RP450IIE1-AC IgG. Lane 1: Molecular weight standards; lanes 2 and 4: rat collagen type I peptides; lanes 3 and 5: peptides of AC-protein adduct immune complexes.

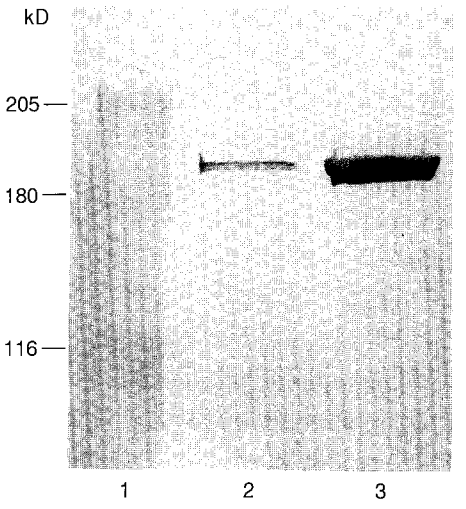

Figure 5. Protein blot of hepatocyte cytosol from a CCl_4-treated and a control rat, immunochemically stained with anti-RP450IIE1-AC IgG. Lane 1: Prestained molecular weight standards; lane 2: cytosol of control hepatocytes; lane 3: cytosol from CCl_4-treated rat.

To determine the intra- or extracellular location of the AC adduct, the cytosol of isolated hepatocytes was tested, and immunochemical staining of protein blots with anti-RP450IIE1-AC IgG revealed the presence of the same AC adduct as that found in the liver soluble and microsomal fractions. The cytosolic adduct of CCl_4-treated rats showed an intense reaction as compared to much weaker staining in control samples (figure 5).

DISCUSSION

Our results indicate that severe liver injury, such as that produced by CCl_4 administration to rats, is associated with increased formation of an AC-protein adduct, which appears to be related to procollagen type I. This AC adduct was readily demonstrable in the hepatic soluble fraction and to a lesser extent in the liver microsomes of CCl_4-treated rats. The same adduct was found in the cytosol of isolated hepatocytes, showing an intensely stained band in samples from CCl_4-treated as compared to a much weaker band in control samples. No crossreactivity of the anti-RP450IIE1-AC IgG with aldehyde linkages in mature collagen type I was observed, which precludes the possibility that the antibody merely reacted with collagen and not with an AC-collagen adduct.

A significant amount of AC is generated from physiological substrates, and we have recently shown that endogenous AC markedly increases in patients with non-alcoholic liver diseases (13) as well as in CCl_4- or demethylnitrosamine-induced liver injuries (4). Moreover, increased antibody titers against AC-protein adducts were found in the serum of patients with non-alcoholic liver diseases (1). Results obtained in rats with dimethylnitrosamine-induced cirrhosis (Liu et al., unpublished observation) indicate that AC adduct formation is unrelated to the agent used to produce liver injury.

Since AC has been shown to readily bind to collagen (14) and to stimulate collagen formation in cultured liver cells (15-17), we considered collagen as a possible target protein. The estimated

molecular weight of the adduct (approximately 200 kD) was similar to that of the β1,2 dimer of collagen type I, and the evidence presented here indicates that the liver cytosolic AC adduct contains a collagen component. Not only was the adduct sensitive to digestion by a highly purified bacterial collagenase, but also reacted with anti-collagen type I-specific IgG. The increased amount of adduct protein necessary to obtain a positive reaction with the latter antibody is most likely due to the AC-altered epitopes of the collagen component of the adduct or to its lower affinity of a collagen precursor. The fact that the AC adduct co-migrated with the β1,2 band of collagen I, makes one wonder whether dimerization of primary α chains can be produced by AC during the intracellular transport of collagen, since AC is known to induce crosslinking of proteins (15). On the other hand, the adduct may be an AC ligand of procollagen which has a molecular weight not significantly different from that of the β dimer of α1 and α2 chains.

The presence of the AC-collagen adduct in the cytosol of isolated hepatocytes does not exclude the possibility that the same adduct is formed in other types of liver cells as well as in the extracellular space. Evidence given by other investigators indicates that hepatocytes contribute to the formation of collagen (16). The intracellular location of the adduct suggests that procollagen is involved in AC adduct formation. The concentration of AC derived from endogenous sources as well as exogenous ethanol are likely to be highest in the hepatocyte. The stronger immunoreactivity of the adduct in the liver of cirrhotic rats was not merely the consequence of increased amounts of collagen, since the main difference in adduct concentration was detected in the cytosol where the amount of collagen in treated and control rats was similar. We cannot explain why the presence of the adduct was more readily demonstrable in the cytosol than in the microsomes, which are the site of collagen synthesis and represent the normal transport pathway for this export protein; however, the possibility of preferential retention of the altered protein in the cytosol has to be considered. It remains to be determined whether the AC-collagen adduct can be detected at specific stages of alcoholic liver injury associated with active fibrogenesis and whether AC adduct formation plays a pathogenetic role in the enhanced collagen accumulation in alcoholic as well as non-alcoholic liver diseases.

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