NONIDENTITY OF LIVER ALCOHOL DEHYDROGENASE AND THE PRINCIPAL PROTEIN TARGET OF HEPATIC AZOCARCINOGEN

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Received December 7,1973

Summary: During hepatocarcinogenesis in the rat by the aminoazo dyes, a principal carcinogen-protein conjugate (azoprotein) is formed in liver cytosol from a normal target protein, whose identity and function are unknown. Based on similarities of amino acid compositions, molecular weights, and subunit sizes of azoprotein and liver alcohol dehydrogenases, others have proposed that liver alcohol dehydrogenase is the principal normal target protein of azocarcinogens during liver carcinogenesis in the rat.

In the present study, specific antiserum precipitated the principal liver azoprotein and target protein, but failed to precipitate rat liver alcohol dehydrogenase. The ability of the antiserum to distinguish and to separate the azoprotein and target protein from alcohol dehydrogenase shows that this enzyme is not the principal target protein of the azocarcinogens.

The interaction of unknown cellular macromolecules, such as DNA, RNA, or protein, with chemical carcinogens is generally presumed to be essential in chemical carcinogenesis. The feeding of hepatocarcinogenic aminoazo dyes to rats leads to the presence of carcinogen-protein conjugates, the predominant one termed h_2-5S azoprotein, in liver cytosol (1,2). The principal conjugate derives from a normal protein (target protein) whose identity and function are unknown. Liver alcohol dehydrogenase (ADH) has recently been proposed to be the target protein (3,4). The proposal was based in part on the fact that the principal liver azoprotein (h_2-5S) isolated in this laboratory (5,6), the azoprotein purified by Tokuma and Terayama (4), and the ADHs of rat, horse, and human livers, all have similar amino acid compositions, molecular weights, and subunit sizes, as shown in Table 1. The present study therefore undertook to determine by immunological techniques whether or not the principal liver azoprotein of rats fed azocarcinogen does in fact derive from liver ADH.

Table 1. Properties of specific liver azoprotein conjugates of hepatic azocarcinogens compared to the properties of liver alcohol dehydrogenases.

	LIVER AZOPROTEIN CONJUGATES			LIVER ALCOHOL DEHYDROGENASES			
	Ketterer et al.	Tokuma-Terayama	Sorof et al.	Rat	Horse		Human
					E-ADH[a]	S-ADH[a]	
Amino Acid Composition (mole %)							
Lys	9.4[15]	8.4[4]	7.2[6]	8.5[13]	8.0[16]	8.2[17]	7.9[18]
His	1.5	3.1	2.4	2.4	1.9	2.0	1.6
Arg	6.3	2.6	3.0	2.7	3.2	3.1	2.4
Asp	10.3	6.9	7.0	7.2	6.7	7.0	7.7
Thr	3.1	5.8	5.9	6.1	6.4	6.1	6.3
Ser	3.8	6.0	6.4	6.9	7.0	6.9	5.5
Glu	11.1	6.7	6.1	6.9	7.8	8.5	7.1
Pro	4.6	6.4	6.6	5.9	5.3	5.9	5.5
Gly	5.6	10.7	14.2	9.3	10.2	10.8	10.6
Ala	7.6	9.8	9.6	9.0	7.5	8.1	9.0
Cys/2	0.5	2.9	2.9	4.3	3.7	-	3.7
Val	5.1	9.8	9.6	9.3	10.4	11.0	10.8
Met	4.1	1.1	1.1	1.3	2.4	2.5	2.1
Leu	13.8	7.9	7.4	8.0	6.7	7.2	7.7
Ile	5.4	6.2	6.6	6.4	6.4	6.4	5.5
Tyr	3.0	0.9	0.47	1.0	1.1	1.0	1.3
Phe	5.0	4.9	3.0	4.5	4.8	4.8	4.5
Try	-	-	0.52	0.5	0.5	0.5	0.8
Molecular Weight (x 10^4)							
	4.5[15] 5.0±0.6[23]	6.8[4]	6-8[2]	6.5±0.5[19] 8.0[13]	8.0[20]	8.0[21]	8.0[22] 8.7[24]
Subunit Weight (x 10^4)							
	2.3[25]	3.8-4.0[4]	4.4[5,6]	3.7-4.1[19] 4.0[13]	4.0[20]	4.0[21]	4.0[22]

[a] E-ADH and S-ADH indicate the ethanol- and steroid-active isozymes of liver ADH, respectively.

MATERIALS AND METHODS

The principal azoprotein (h_2-5S) was isolated 88-91% pure from the liver cytosols of rats fed the liver carcinogen, 3'-methyl-4-dimethylaminoazobenzene for 15 to 18 days (5,6).

Specific antiserum, exclusively directed against the azoprotein and the

target protein, resulted directly from the use of the early antiserum from rabbits without any absorption. The procedure of immunization was like that previously reported (7), except that i.m. injections were at 0, 2 and 4 weeks. Antisera were collected at the 5th week and twice-weekly thereafter, as long as a single band was present (7). Radial double immunodiffusion analyses were carried out at room temperature in gels of 1% agarose in saline-phosphate buffer (7,8).

Quantitative immunoprecipitations were carried out using the specific antiserum against the azoprotein in reaction with liver cytosol which was prepared from rats fed the azocarcinogen for 15 days (9,10). Different amounts of the antiserum against the azoprotein were reacted with the liver cytosol. The precipitates were assayed for protein and bound azo dyes. The supernatant fluids were analyzed for steroid ADH activity. Details appear below and in the legend of the appropriate figure.

ADH activity was assayed (11) using both the steroid substrate, etiocholan-17β-ol-3-one (17β-hydroxy-5-androstan-3-one; 5-dihydrotestosterone) (Sigma Chemical Co., St. Louis, Mo.), and the ethanol-related substrate cyclohexanone (Eastman Kodak Co., Rochester, N.Y.). Horse liver crystalline ADH was obtained from Boehringer Mannheim Corp., New York, N.Y. Rabbit antisera against the purified ethanol-active isozyme (E·E dimer) of horse liver crystalline ADH were kind gifts (see Acknowledgments).

RESULTS

Purified $\underline{h_2}$-5S azoprotein exhibited a low level of ADH activity. With the steroid substrate, the azoprotein had an activity of 0.1 U/mg, compared to an estimated 4.4 U/mg of the steroid-reactive isozyme in horse liver crystalline ADH*, compatible with the presence of 9-12% protein impurity in the azoprotein (5,6).

* Computed on the basis that ca 1% of horse liver crystalline ADH is steroid-reactive subunit (11).

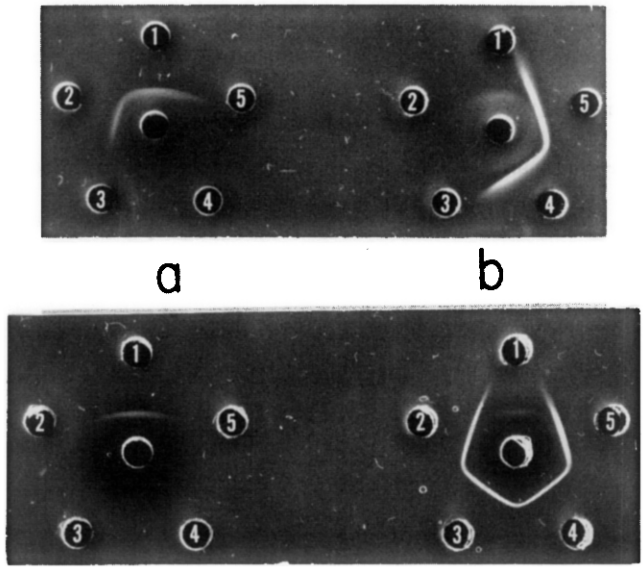

Fig. 1. Gel immunodiffusion of the principal liver azoprotein of rats fed azocarcinogen, of the precursor target protein, and of horse liver ADH. Conditions are given in the text and below.

Panel (a): Center well has antiserum against the principal liver azoprotein (h_2-5S) (73 mg/ml). Outer wells: (1) h_2-5S azoprotein (0.5 mg/ml); (2) normal rat liver cytosol (10 mg/ml); (3) horse liver ADH (0.5 mg/ml); (4) blank; and (5) horse liver ADH (0.5 mg/ml).

Panel (b): Center well has antiserum against horse liver ADH (74 mg/ml). Outer wells: (1), (2) and (5) are like those of panel (a); (2) and (3) are identical; (4) and (5) are identical.

Panel (c): Center well as in panel (a). Outer wells: (1) h_2-5S azoprotein (0.5 mg/ml); (2) horse liver ADH, denaturated at pH 5.0 for 20 min. (0.5 mg/ml); (3) horse liver ADH, mother liquor (0.5 mg/ml); (4) horse liver ADH, crystals and mother liquor (0.5 mg/ml); (5) horse liver ADH, crystals (0.5 mg/ml).

Panel (d): Center well as in panel (b). Outer wells: like those of panel (c).

The specific antiserum against the principal liver azoprotein produced a single, continuous precipitin band in double immunodiffusion reactions with the purified h_2-5S azoprotein (Fig. 1a, well 1), the cytosols of normal livers (well 2), and the cytosols of livers of rats fed the azocarcinogen for 15 to 18 days (not shown) (7). This normal liver protein, thus precipitated, has the same molecular charge (12), molecular and subunit sizes (12), and immunoreactivity (7,12), as does the principal liver azoprotein of rats fed

azocarcinogen (7), and is apparently the target protein of the azocarcinogens (7,8,12). In contrast, the antiserum failed to detectably react with horse liver ADH (wells 3 and 5), despite the known immunological crossreaction between rat and horse liver ADHs (13).

Furthermore, rabbit anti-horse liver ADH strongly precipitated horse liver ADH (Fig. 1b, wells 4 and 5). However, that precipitin band did not fuse with any band that was formed by reaction with either the $\underline{h_2}$-$\underline{5S}$ azoprotein (well 1), or with normal liver cytosol proteins (wells 2 and 3). [The anti-horse liver ADH also precipitated a small impurity in the horse liver ADH (wells 4 and 5), which was also present in the $\underline{h_2}$-$\underline{5S}$ azoprotein (well 1) and in normal rat liver cytosol (wells 2 and 3)].

In addition, horse liver ADH adjusted to pH 5.0 for 20 min with acetic acid, and mother liquor of commercial horse liver ADH were used as sources of marginally denatured enzyme. Both preparations gave the same results in immunodiffusion with both types of antisera as did the more native horse liver ADH (Fig. 1c and 1d). These results and the strong precipitin reaction with target protein in normal liver cytosol both dispute any assumption that the antiserum against azoprotein may actually be directed against denatured ADH.

The ability of the anti-azoprotein serum to distinguish between the principal liver azoprotein and ADH was further substantiated by quantitative immunoprecipitations from rat liver cytosol. Increasing amounts of the specific antiserum precipitated increasing amounts of protein and bound azo dyes from the liver cytosol of rats fed azocarcinogen, but left all the ADH activity in the supernatant fluid (Fig. 2). The principal liver azoprotein and its precursor target protein were thus molecularly separated from, and immunologically unrelated to, rat liver ADH.

DISCUSSION

Specific antiserum not only distinguishes, but also separates, ADH from the principal liver protein target of the hepatic azocarcinogens. The anti-

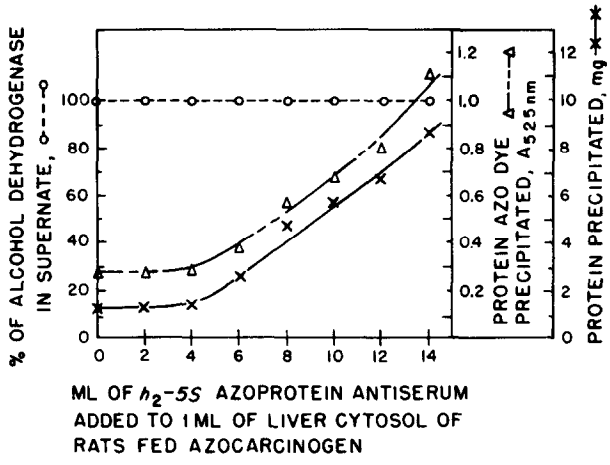

Fig. 2. Lack of precipitation of rat liver ADH during quanitative immunoprecipitation of the principal azoprotein and target protein from the liver cytosol of rats fed azocarcinogen for 15 days. The cytosol was prepared by homogenization of perfused livers in 0.08 M sodium phosphate buffer, pH 7.8,containing 0.075 M NaCl, and stored at -60° until used (9,10). The cytosol was then dialyzed extensively against the phosphate-chloride buffer at 1-3°. Increasing volumes (0 to 14 ml) of clarified specific antiserum (73 mg/ml), and decreasing volumes (14 to 0 ml) of non-immune rabbit serum (76 mg/ml), were added to 1 ml of the liver cytosol (53 mg protein), so that the reaction mixtures contained increasing amounts of antisera and equal total amounts of sera. After immunoprecipitation (23°, 1 hr; 1-3°, 65 hrs) and centrifugation, the supernatant fluids were assayed for ADH activity with steroid substrate, 5-dihydrotestosterone, in the range of activity that linearly increased with time and the amount of the supernatant fluids. The ADH activity of the supernatant fluid from the mixture of 1 ml of liver cytosol and 14 ml of non-immune serum was taken as the total (100%) activity present. The precipitates, twice washed with the phosphate-chloride buffer, were analyzed for protein by the Folin method, and for azo dyes in 88% formic acid at 525 nm (9).

serum precipitates the principal target protein and its azoprotein, but fails to give any detectable precipitin band with horse liver crystalline ADH. This lack of reaction with horse liver ADH is so despite the approximate 80% homology in the amino acid sequence and peptides of the ADHs of horse and rat livers (14), and the known crossreactivity of the two dehydrogenases in gel double immunodiffusion (13). In addition, while the specific antiserum precipitates sizeable amounts of principal liver azoprotein, as well as of the target protein from which it derives (7,8), the antiserum fails to precipitate any protein from rat liver cytosol with ADH activity. ADH thus

appears not to be the principal liver protein target of the hepatic azo-carcinogens. Inherent in this conclusion is the reasonable assumption that all hypothetical conformers of ADH of normal rat liver would react with the specific antiserum to form precipitates, if ADH were the target protein.

Supported in part by grants CA-05945, CA-06927, and RR-05539 from the National Institutes of Health, and an appropriation from the Commonwealth of Pennsylvania. The authors thank Dr. Regina Pietruszko of the Center for Alcohol Studies, Rutgers University, New Brunswick, N.J., for generous gifts of antisera against horse liver alcohol dehydrogenase, and for helpful discussions.

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