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PROPERTIES OF THE INCREASED GLUTATHIONE S-TRANSFERASE A FORM IN RAT PRENEOPLASTIC HEPATIC LESIONS INDUCED BY CHEMICAL CARCINOGENS

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Summary: Glutathione S-transferase A form (GST-A) is increased markedly in rat preneoplastic hepatic lesions such as hyperplastic nodules induced by diethylnitrosamine followed by administration of N-2-fluorenylacetamide. GST-A was also significantly increased in livers of rats after short-term administration of some drugs. The increased activity and protein content of GST-A were demonstrated by CM-Sephadex C-50 column chromatography as well as by two-dimensional polyacrylamide gel electrophoresis following immuno-affinity column chromatography using antibody against GST-A. Immunologically, GST-A crossreacted strongly with GST-C, weakly with GST-C_2, but not with ligandin, GST-B, or GST-AA. It was confirmed by subunit recombination that GST-C is a heterodimer composed of the subuits of homodimers, GST-A and GST-C_2.

Glutathione S-transferases (GSTs) (EC 2.5.1.18) represent a family of enzymes that perform several roles in hepatic detoxication (1). We have reported that Lig, GST-B and GST-AA are closely related to each other in both immunological and catalytic properties and that GST-B is a hybrid between Lig and GST-AA (2). The induction of Lig or GST-B by agents such as PB and 3-MC is well-documented (3, 4). However, inducibility and immunological and subunit properties of other forms have not been clarified.

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Abbreviations: GSH, glutathione; GST, glutathione S-transferase; Lig, ligandin; SDS, sodium dodecyl sulfate; IgG, immunoglobulin; CDNB, 1-chloro-2,4-dinitrobenezene; DCNB, 1,2-dichloro-4-nitrobenzene; DEN, diethylnitrosamine; FAA, N-2-fluorenylacetamide; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; 3-MC, 3-methylcholanthrene; BHA, butylated hydroxyanisole; PB, phenobarbital; PCB, polychlorinated biphenyl; HN, hyperplastic nodule; GGT, γ-glutamyl transpeptidase.

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Recently, Mannervik and Jensson (5) reported that GST-C is a binary combination of GST-A and GST-C_2.

In this paper we report immunological and subunit properties of the increased GST-A in preneoplastic hepatic lesions induced by chemical carcinogens.

MATERIALS AND METHODS

Assays of GST activities. Activities of GST were determined with two substrates, CDNB and DCNB, as described previously (2).
Purification of Six Forms of GSTs. Sprague-Dawley (SD) male rats weighing 160-250 g were used in this study. Six forms of hepatic GST with the activity towards CDNB were separated by CM-Sephadex C-50 column chromatography (see Fig. 3). GST-A, -AA, -B, -C and Lig were purified as described previously (2). GST-A was purified from livers bearing preneoplastic hepatic lesions. GST-C_2 was purified as follows: A 20 % homogenate of normal liver in 0.25 M sucrose was centrifuged at 105,000 x g for 45 min and the supernatant obtained (100 mg/8.0 ml) was dialyzed against 10 mM Na phosphate buffer (pH 6.7) (Buffer A), applied to a CM-Sephadex C-50 column (2.7x6.5 cm) equilibrated with Buffer A and eluted with the same buffer. Fractions containing the activity towards CDNB were collected, dialyzed against 10 mM potassium phosphate buffer (pH 6.8), applied to a hydroxyapatite column, and eluted with a 10-350 mM linear gradient of potassium phosphate (pH 6.7). Active fractions were collected and applied to a GSH-affinity column, which was prepared according to the method of Koskelo et al.(6). GST was eluted with 20 mM GSH and contained only single protein, which had the same molecular weight as purified GST-A and GST-C on SDS-polyacrylamide gel electrophoresis done by the method of Laemmli (7). The eluate including the activity peak was further passed through a Sepharose 4B column coupled with anti-GST-B IgG to remove a very small amount of contaminated Lig.
Induction of Preneoplastic Hepatic Lesions. The lesions known as enzyme-altered foci detectable by GGT activity staining (8) or HNs were induced according to the system of Solt and Farber (9), except that FAA was given two weeks longer (8).
Two-dimensional polyacrylamide gel electrophoresis. This was performed according to Takami and Busch (10).
Subunit Recombination. The dissociation and recombination of subunits of GST-C and the formation of hybrids between GST-A and GST-C_2 were done as described previously (2).

RESULTS

Molecular Forms of GST induced in Preneoplastic Hepatic Lesions.

Activities of GST towards both CDNB and DCNB in rat liver cytosol increased with increased number and area of preneoplastic GGT-positive foci or of HNs induced by the administration of DEN followed by FAA (data not shown). It was also noted following CM-Sephadex C-50 column chromatography that activities of

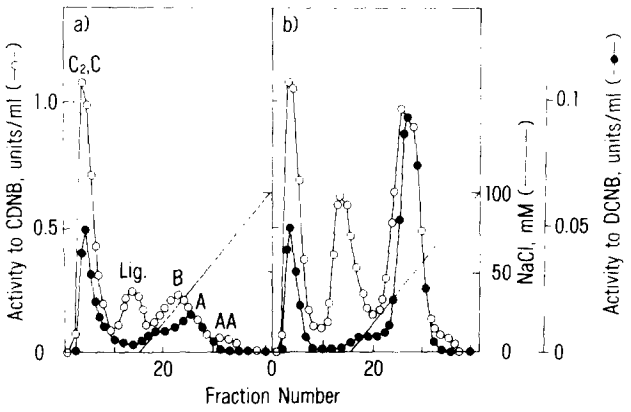

Fig.1. CM-Sephadex C-50 column chromatography of GSTs of normal rat liver (a) and the liver bearing GGT-positive foci (b). 105,000 x g supernatants (40mg/2.5 ml) dialyzed against 10 mM Na phosphate buffer (pH 7.4) were applied to columns (2.7 x 6.5 cm) pre-equilibrated with the buffer and were eluted with 40 ml of the buffer and then with 300 ml of the buffer with a linear gradient of NaCl. Each peak was named according to Hayes et al. (11) except for GST-C_2. C_2, C, A, Lig, B and AA correspond to C_2, CA, A_2, L_2, BL and B_2, respectively, according to the new nomenclature of GST forms proposed by Mannervik and Jensson (5).

GST-A towards both CDNB and DCNB were markedly increased in livers bearing the foci or HNs, and that Lig activity, detectable only with CDNB, was also significantly increased in these livers (Fig. 1). A similar pattern was also observed in livers bearing foci induced by 3'-Me-DAB (0.06%) instead of FAA. At pH 7.4, both GST-C and GST-C_2 were eluted at the breakthrough fraction. Furthermore, it was demonstrated by two-dimensional polyacrylamide gel electrophoresis that GST-A is one of the most highly increased among the cytosol proteins even in an isolated HN (Fig. 2b). The increased protein was identified as GST-A by electrophoresis following immuno-affinity column chromatography using anti-GST-A IgG (Fig. 2c). The preneoplastic foci and HNs were more densely stained immunohistochemically using the anti-GST-A IgG (data not shown). We also examined induction patterns of GSTs in whole livers of rats, which were given either of several agents, including carcinogens and hepatocarcinogenic promoters, for 2 weeks prior to the appearance of special cell

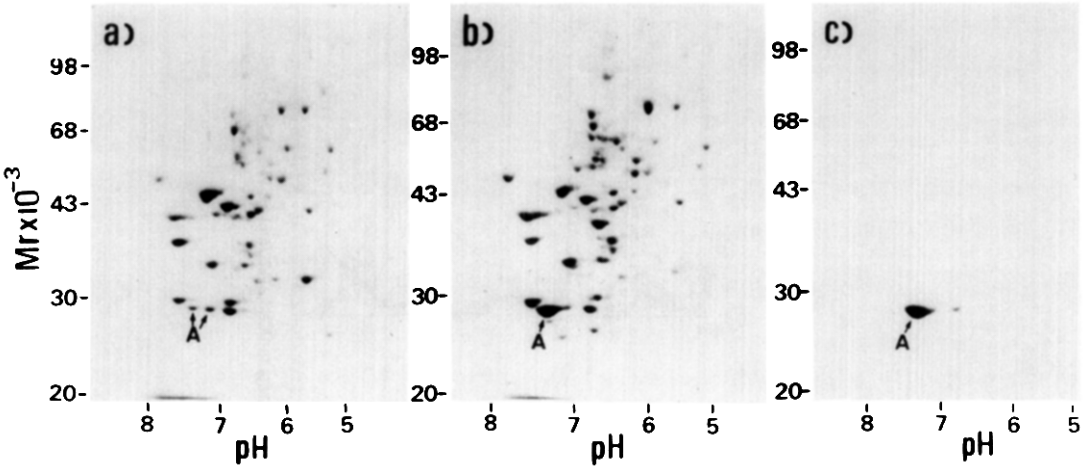

Fig. 2. Two-dimensional electrophoresis of cytosol proteins in normal rat liver and in an isolated HN. 16000 xg supernatants (100 µg protein) from normal liver (5 mg) (Fig. 2a) and from a single HN (2 mg) (Fig. 2b) were used. The 16,000 x g supernatant of isolated HN was applied to an anti-GST-A IgG-Sepharose column. The adsorbed protein(s) was eluted with 1 N acetic acid and then subjected to the electrophoresis (Fig. 2c).

populations. As shown in Fig. 3, neither FAA nor DEN alone induced any form of GST. 3'-Me-DAB and the antioxidant BHA induced both Lig and GST-A, while the promoter PB as well as PCB (the pattern is not shown) markedly induced Lig and slightly induced GST-A. 3-MC induced only Lig. However, the levels of GST-A induced by these agents were much lower than those induced in livers bearing the foci or HNs, or in isolated HNs, and GSTs induced by these agents decreased rapidly to normal levels after the agent was removed from the diet. In contrast, GST-A induced in the foci or HNs remained at high levels so long as the foci or HNs remained, even when the carcinogen or promoter was removed from the diet.

Relationships among GST-A and Its Related Forms.

The relation between GST-A and its immunologically related forms GST-C and GST-C_2 remained unclear, although the relationships among GST-AA, GST-B and Lig had been clarified (2, 12). Mannervik and Jennson (5) first reported the presence of GST-C_2

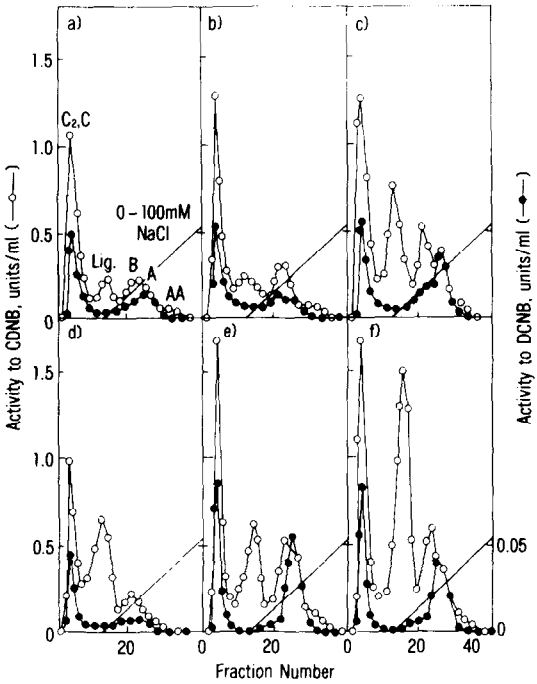

Fig.3. CM-Sephadex column chromatography of hepatic GSTs from rats treated with various chemical agents. FAA (b), 3'-Me-DAB (c), BHA (e) and PB (f) were given to rats weighing 160-170 g with the basal diet at 0.02%, 0.06%, 0.75%, and 0.05%, respectively, for 2 weeks. 3-MC (d) was injected to rats 3 times every other day at a dose of 25 mg/kg. The chromatography was performed as in Fig. 1. Fig. 3a, untreated rat liver.

in rat liver cytosol by chromatofocusing. We also purified this form by two successive CM-Sephadex column chromatography at pH 6.7 followed by pH 7.4, as shown in Fig. 4. Specific activities of the purified GST-A, GST-AA, GST-B, GST-C, GST-C_2 and Lig towards CDNB were 47.3, 18.6, 20.8, 33.5, 8.6 and 23.1, respectively. However, specific activities of GST-A, GST-C and GST-C_2 towards DCNB were 3.94, 2.61 and 0.40, respectively, while those of GST-A, GST-B and Lig were negligible.

With IgGs prepared against the six forms of GST, no precipitin line was observed between anti-C IgG and any member of the GST-B group, AA, B, and Lig, while A, C and C_2 formed fused precipitin lines with the anti-C IgG (Fig. 5a). However, the lines between the anti-C IgG and A or C_2 did not fuse (Fig. 5b).

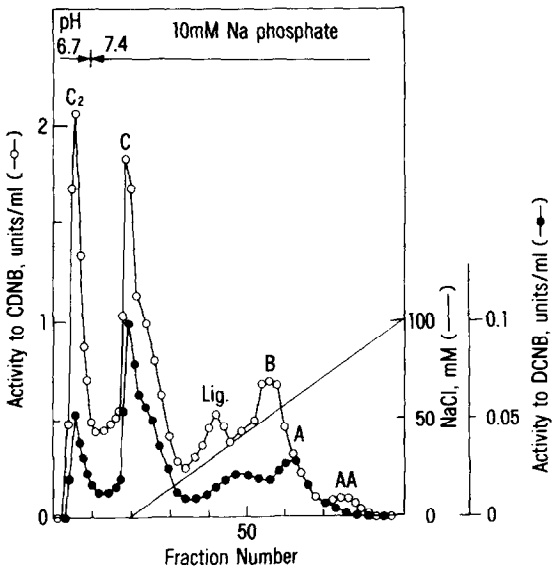

Fig.4. Separation of GST-C_2 by CM-Sephadex C-50 column chromatography at pH 6.7. 105,000 x g supernatant (100 mg/8.0 ml) from a normal male rat was dialyzed against 10 mM Na phosphate buffer (pH 6.7) and applied to a CM-Sephadex C-50 column (2.7x6.5 cm) pre-equilibrated with the same buffer. GSTs were eluted with 40 ml of the buffer, then eluted with the same volume of 10 mM Na phosphate buffer (pH 7.4), and further eluted with 300 ml of the buffer with a linear gradient of NaCl.

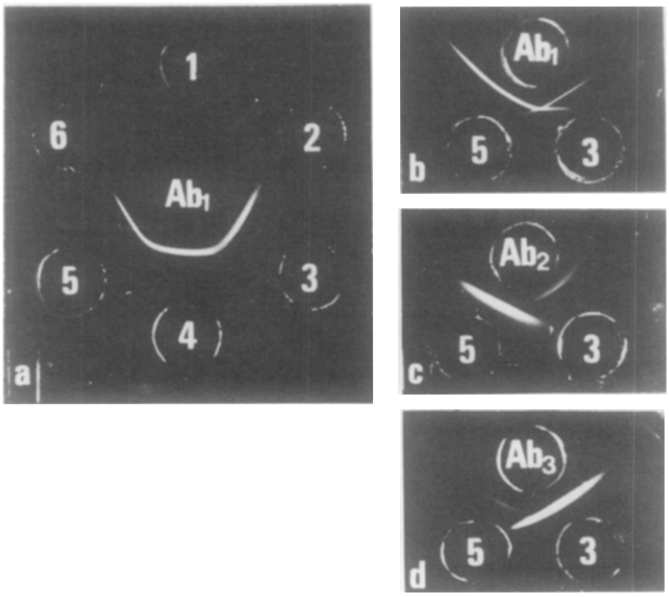

Fig.5. Double immunodiffusion of GSTs in 1% agar gel. The wells contain 2 μg of purified Lig (1), GST-B (2), GST-C_2 (3), GST-C (4), GST-A (5) and GST-AA (6), respectively. The other wells contain 40 μg of anti-C IgG (Ab_1), anti-A IgG (Ab_2) and anti-C_2 IgG (Ab_3), respectively. IgGs were prepared as described previously (2).

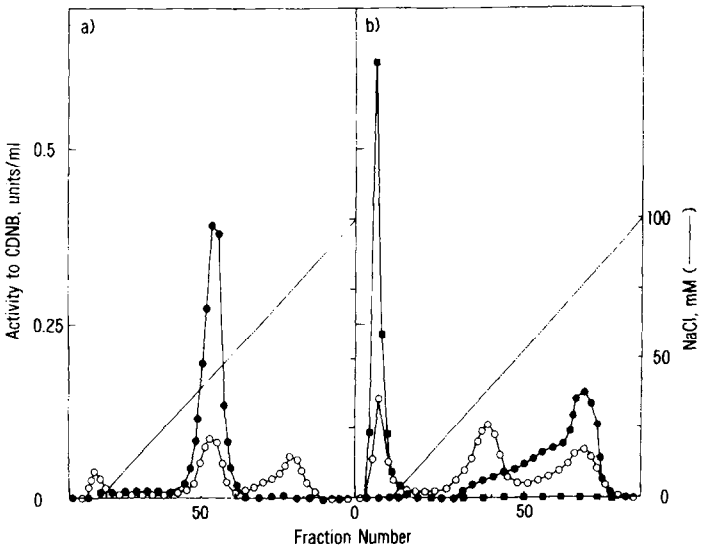

Fig.6. Elution patterns of native and guanidine-teated GSTs on CM-Sephadex C-50 column chromatography. Each sample was applied to a column (2.7 x 6.5 cm) equilibrated with 10 mM Na phosphate buffer (pH 6.7). In Fig. 6a, ● and ○ show elution patterns of GST-C (375 μg) before and after guanidine hydrochloride treatment, respectively. In Fig. 6b, ■ and ● show patterns of GST-C_2 and GST-A alone after guanidine hydrochloride treatment, respectively, and ○ shows the pattern of a mixture of GST-C_2 (750 μg) and GST-A (750 μg) after the treatment.

In addition, a faint precipitin line was observed between C_2 and anti-A IgG (Fig. 5c) and between A and anti-C_2 (Fig. 5d), suggesting that the subunit of A may differ from that of C_2 and C may have a common subunit of A and C_2, though A and C_2 have some common antigenicities.

To identify the subunits of GST-C, purified GST-C was treated with guanidine hydrochloride, renatured, and the recombined enzymes were separated by CM-Sephadex C-50 column chromatography (Fig. 6a). By this treatment, GST-C was separated into three peaks, and the main peak was eluted at the position of the native GST-C and other two peaks were eluted at the positions of GST-C_2 and GST-A. The new forms were also identified immunologically as GST-C_2 and GST-A, respectively (data not shown). After this treatment of GST-C_2 or GST-A alone, these eluted mostly at the position of the native form, whereas the mixture of these two

forms showed a new form intermediate between the two forms after the same treatment (Fig. 6b). These results clearly indicate that GST-C is a hybrid between GST-A and GST-C_2.

DISCUSSION

It was observed that both activity and the protein content of GST-A were markedly increased in livers bearing preneoplastic foci or HNs and also in individual HNs induced by chemical carcinogen(s). The induction of GSTs, especially of the molecular forms of the B-group, have been well-documented (3, 4). Lig, a major form in rat liver, has been investigated as a basic azo-dye binding protein (13-16). Guthenberg et al. (17) reported that activities of GST-A, GST-B and GST-C as well as respective protein contents increase after treatment of rats with trans-stilbene oxide. GST-A was, however, not separated from GST-C immunologically. Bannikov et al. (18) demonstrated immunologically that a basic azo-dye binding protein is present in the highly differentiated hepatomas but absent in poorly differentiated ones.

We have also confirmed the subunit relationship proposed by Mannervik and Jensson (5) among GST-A and its immunologically related forms, GST-C and GST-C_2. Habig et al. (19) first reported that GST-A and GST-C are immunologically indistinguishable.

Recently it has been reported that the nonidentical Ya and Yc subunits of GST-B are translated from different mRNAs transcribed from different genes (20-22). However, it remains to be shown whether GST-A and GST-C_2 or the two subunits of GST-C are derived from the same or different genes.

As pointed out by Mannervik and Jensson, the molecular forms of GST in rat liver cytosol can be divided into two groups according to their catalytic, immunologic and subunit properties. One is the B group including AA, B and Lig and the other is AC

group including A, C and C_2. There are immunochemical cross-reactivities among three forms in each group, but not between the two groups. Thus, the new nomenclature of GST molecular forms in rat liver cytosol proposed by Mannervik and Jensson may be reasonable, as it reflects the subunit composition of the respective forms, e.g. GST-A as A_2.

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