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RAPID COMMUNICATION

IMMUNOCHEMICAL DETECTION OF ACETAMINOPHEN-BOUND LIVER PROTEINS

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The widely used analgesic, acetaminophen (paracetamol), when taken in overdose may cause fatal hepatic centrilobular necrosis. Mechanistic studies have linked acetaminophen hepatotoxicity to the formation of an electrophilic metabolite which can covalently bind to hepatic macromolecules (1-3). To better understand the significance of such binding to the initiation and progression of acetaminophen hepatotoxicity, it is important to identify the macromolecules to which the electrophile binds. To date, neither the exact nature of the initiating events nor the specific proteins to which the electrophile binds have been elucidated. To determine the specificity of the covalent binding associated with administration of an hepatotoxic dose of acetaminophen, an affinity-purified antibody directed against protein-bound acetaminophen was used to detect electrophoretically resolved proteins of liver fractions taken from acetaminophen-treated mice. Our findings represent the first immunochemical resolution of acetaminophen-bound liver proteins from animals exposed *in vivo*, and they demonstrate that the binding to individual proteins is not random but is highly selective.

Antibodies to acetaminophen-bound proteins. An antigen constructed by linking acetaminophen to keyhole limpet hemocyanin through diazotized *p*-aminobenzoic acid was injected subcutaneously into rabbits in complete Freund's adjuvant. After a booster, serum was collected, and acetaminophen-specific antibodies were isolated using an affinity column of acetaminophen coupled to epoxy-activated Sepharose 6B and were shown to detect protein-bound acetaminophen by solid phase ELISA (Fig. 1A). The affinity-purified antibody was further characterized by ELISA competition assays which demonstrated its selectivity for the N-acetyl region of the acetaminophen moiety of drug-protein conjugates. Complete details of antibody production, purification, and specificity will be published elsewhere.

Immunochemical demonstration of selective binding to centrilobular hepatocytes. To assess the binding of the drug *in vivo*, two doses (300 and 600 mg/kg, p.o.) of acetaminophen were selected to provide exposures near or somewhat above the apparent threshold for hepatic necrosis in 4-month-old fasted Crl:CD-1 male mice. Both doses have been shown to cause about 80% depletion of hepatic glutathione within 2 hr after dosing; yet, only 600 mg/kg caused hepatic necrosis, significant acetaminophen binding, and marked elevation of plasma sorbitol dehydrogenase activity over the ensuing 18 hr (4). Mice were sacrificed after administering 600 mg/kg of acetaminophen *in vivo*, and sections of livers (5 μm) were treated with Triton X-100 to remove any unbound drug and permit antibody penetration, were exposed to affinity-purified rabbit antibodies, and were counterstained with fluorescein-conjugated goat anti-rabbit IgG. By indirect immunofluorescence we were able to demonstrate that, 2 hr after drug administration, the bound acetaminophen was selectively localized in centrilobular liver

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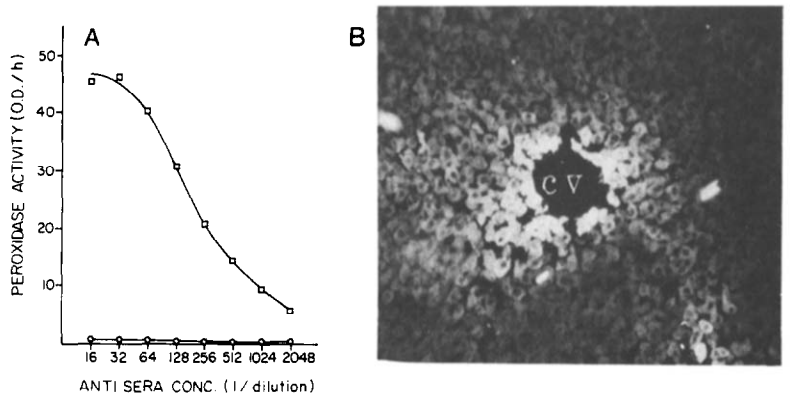

Fig. 1A. Indirect ELISA of affinity-purified antibody against acetaminophen-bound protein. Bovine serum albumin (BSA) was derivatized by adding 0.88 μmole of N-acetylbenzoquinone imine (NAPQI), which was synthesized by the method of Streeter et al. (5), to 10 ml (1 mg/ml) of BSA in phosphate-buffered saline (PBS) at pH 7.4 and dialyzed overnight. Microtiter plates were coated with 10 ul of a 10 μg/ml solution of NAPQI-derivatized BSA (-□-) or native BSA (-○-) for 1 hr at 37° and treated according to the ELISA method of Coleman et al. (6), except that 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) was substituted as the chromogen. Peroxidase activity was monitored at 405 nm on a computer-interfaced Artek plate reader and expressed as extinction/hr.

Fig. 1B. Localization by indirect immunofluorescence of acetaminophen-bound proteins in liver sections from treated CD-1 mice. Two hours after acetaminophen administration (600 mg/kg, p.o., in 50% propylene glycol), livers were perfused with PBS, fixed with 4% formalin in PBS, frozen, and sliced into 5 μm sections. The sections were washed for 1 hr in PBS containing 0.3% Triton X-100 to remove free acetaminophen; they were incubated first in 3% non-immune goat serum and then with affinity-purified antibodies (1:100) for 3 hr at room temperature. The bound antibody was detected by fluorescein-conjugated goat anti-rabbit IgG (1:60). Central vein is denoted C.V. Magnification was 300X.

cells (Fig. 1B). This provided immunochemical confirmation of the autoradiographic localization of bound [^{3}H]-acetaminophen initially reported by Jollow et al. (1). No immunofluorescence was detected in cells of the periportal zone nor in liver sections from control mice. In addition, acetaminophen-bound proteins were detected as early as 30 min following drug administration and prior to glutathione depletion. This may signal the greater sensitivity of the immunofluorescence approach compared to earlier studies which used autoradiography after administration of isotopically labeled acetaminophen.

Electrophoretic resolution of acetaminophen-protein conjugates. The utility of the immunochemical approach for determining the specificity of in vivo arylation of liver proteins by the acetaminophen metabolite was effectively demonstrated by Western blotting. Detection of acetaminophen-bound proteins was evaluated in three subcellular fractions from livers of treated mice killed 2, 4 or 8 hr after administration of acetaminophen (600 mg/kg, p.o.). Proteins from each fraction were first resolved on 10% SDS-polyacrylamide gels by electrophoresis under reducing conditions, transblotted to nitrocellulose for incubation with antibody, and subsequently reacted with ^{125}I-labeled conjugated goat anti-rabbit IgG. The resulting autoradiographs demonstrate that in vivo covalent binding of acetaminophen to proteins was highly selective (Fig. 2); not all protein bands detected by Coomassie brilliant blue were stained immunochemically. Furthermore, comparison of intensities between the protein bands detected by Coomassie blue and those stained immunochemically indicated that the greatest accumulation of acetaminophen was not reflective of the relative amounts of the different proteins. Thus, in some cases, protein bands which represented only a small fraction of total protein on the gel contained relatively large amounts of acetaminophen (Fig. 2; lanes CB vs 2, 4, and 8). The absence of antibody reactive bands in all control lanes (lanes c) demonstrated that there was negligible non-specific binding of the purified rabbit antibody.

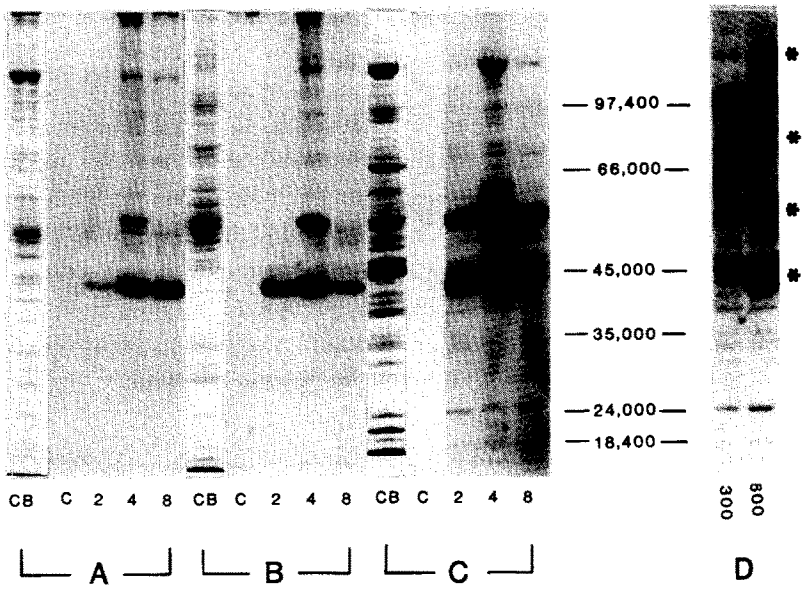

Fig. 2. Detection of acetaminophen-bound proteins in subcellular fractions from acetaminophen-treated CD-1 mice. Subcellular fractions are designated (A) 8,000 g pellet; (B) 105,000 g pellet; and (C) 105,000 g supernatant. All lanes are from 10% SDS-PAGE run under reducing conditions. Lanes CB are profiles from 4-hr treated extracts stained with Coomassie brilliant blue. Lanes C, 2, 4, and 8 are, respectively, Western blot profiles from extracts of untreated control mice, and from 2, 4 and 8 hr treated mice (600 mg/kg, p.o.). (D) Western blots of 105,000 g supernatants from 300 and 600 mg/kg treated mice 2 hr after dosing. The relative migrations of M_r standards are indicated. Livers from control and treated mice were perfused in PBS, homogenized in .25M sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$ at pH 7.4, and fractionated by centrifugation at 8,000 g for 20 min followed by 105,000 g for 1 hr. Samples were diluted 1:1 in 2X gel-buffer containing 2% SDS, 5% mercaptoethanol, 20% glycerol and 0.25% bromophenol blue in Tris-HCl (pH 7.4) and boiled for 5 min. After centrifugation, the supernatants were electrophoresed on 10% SDS-polyacrylamide gels at 30 ug protein/lane. The protein bands were transblotted electrophoretically to nitrocellulose membranes, blocked with 3% bovine serum albumin in Tris-buffered saline, incubated with the affinity-purified antibody, and detected by autoradiography after the addition of ^{125}I-labeled goat anti-rabbit IgG. Following incubation, the nitrocellulose membranes were dried and exposed to Kodak XAR-5 film at -70° with an intensifying screen. Transblotted gels were stained with Coomassie brilliant blue to determine the quality of transfer; only those blots that had complete transfer were utilized further.

Two predominant protein bands with apparent M_r of 42-44 kD and 56-58 kD accounted for the majority of the acetaminophen-bound proteins detected by Western blot profiles from treated mice (lanes 2, 4, and 8). The 42-44 kD band was prominent as early as 2 hr in both the microsomal and cytosolic fractions (Fig. 2; lanes B2, C2). It is intriguing that proteins of M_r 40-45 kD have been shown to bind a number of drugs and carcinogens (7-9). For example, glutathione transferases, which are abundant soluble enzymes implicated in binding xenobiotics, have similar M_r values under non-denaturing conditions but would be expected to dissociate into two equal subunits with detergents (8). However, the 42-44 kD acetaminophen-bound proteins in this study were detected in gels run in the presence of SDS and did not coincide with any intense Coomassie staining. The 56-58kD protein band was seen in all subcellular fractions but initially appeared only in the 105,000 g soluble fraction (Fig. 2, lane C2). Cytochrome P-450 isozymes, which activate acetaminophen, have an M_r of 52-58 kD but they would not be expected to be detected in cytosol. Figure 2 also indicates that, based on the relative intensities of the stained protein bands, acetaminophen binding was greater at 4 hr than at 2 hr and, for most bands, decreased by 8 hr after drug administration (compare lanes 2, 4, and 8). Since it is unlikely that acetaminophen once covalently bound to macromolecules would subsequently be released, the observed decrease in bound

acetaminophen between 4 and 8 hr was likely due to the removal of altered proteins from the hepatocyte by intracellular proteolysis and/or leakage from the cell.

Figure 2 further demonstrates that selective binding occurred even at near threshold doses of acetaminophen. In an initial attempt to determine if any of the protein bands detected at 600 mg/kg might be somewhat more critical in determining the ultimate hepatotoxicity, electrophoretic separation of acetaminophen-bound proteins was also studied in subcellular fractions of livers from mice killed 2 hr after 300 mg acetaminophen/kg. Comparison of cytosolic proteins from mice given either the toxic or nontoxic dose revealed that the immunochemical staining of certain proteins did not become more intense as the dose of acetaminophen was increased from 300 to 600 mg/kg (Fig. 2D). In contrast, only four protein bands (identified in the figure with *) exhibited increased acetaminophen binding at the higher (toxic) dose.

Gillette (10, 11) had cautioned previously that covalent binding should be viewed as an indicator of the formation of reactive metabolites and not necessarily the mediator of the resulting toxicity. Clearly, not all protein binding will be of equal significance to the hepatotoxicity. It is possible that the binding which appeared to remain constant irrespective of dosage may merely serve a limited function in diminishing the concentration of reactive metabolites. By contrast, it is tempting to speculate that those few protein bands which increased in acetaminophen binding at the higher dose may be more critically involved in mediating the hepatotoxic process. These proteins could be either "protective" proteins which contain an abundance of potential arylating sites that reduce the pool of reactive metabolite, or they could be "essential" proteins that get damaged by arylation and alter cellular homeostasis. Further studies will be required to validate the postulated importance of such proteins in the hepatotoxic process. Subsequent elucidation of the role and identity of these proteins should lead to a clearer understanding of the interaction of drugs with cellular macromolecules and of the biochemical events that initiate the cellular perturbations which lead to hepatotoxicity.

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REFERENCES

1. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. Gillette and B. J. Brodie, J. Pharmac. Exp. Ther. 187, 195 (1973).
2. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. Gillette and B. Brodie, J. Pharmac. Exp. Ther. 187, 211 (1973).
3. J. A. Hinson, in Reviews of Biochemical Toxicology (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), Vol. 2, pp. 131-71. Elsevier/North Holland, Inc., New York (1980).
4. G. L. Ginsberg, M. E. Placke, D. S. Wyand and S. D. Cohen, Toxic. appl. Pharmac. 66, 383 (1982).
5. A. J. Streeter, D. C. Dahlin, S. D. Nelson and T. A. Baillie, Chem. Biol. Interact. 48, 349 (1984).
6. J. W. Coleman, J. H. Yeung, M. D. Tingle and B. K. Park, J. Immun. Meth. 88, 37 (1986).
7. J. D. Sun and J. G. Dent, Toxic. appl. Pharmac. 76, 243 (1984).
8. A. M. Sarrif, P. V. Danenberg and C. Heidelberger, Biochem. biophys. Res. Commun. 70, 3 (1976).
9. A. Wendel and P. Cikryt, Res. Commun. Chem. Path. Pharmac. 33, 463 (1981).
10. J. R. Gillette, Biochem. Pharmac. 23, 2927 (1974).
11. J. R. Gillette, Biochem. Pharmac. 23, 2785 (1974).