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Immunohistochemical Localization and Quantification of the 3-(Cystein-S-yl)-acetaminophen Protein Adduct in Acetaminophen Hepatotoxicity

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Acetaminophen overdose causes severe hepatotoxicity in humans and laboratory animals, presumably by metabolism to N-acetyl-p-benzoquinone imine and binding to cysteine groups as 3-(cystein-S-yl)acetaminophen-protein adduct. Antiserum specific for the adduct was used immunohistochemically to demonstrate the formation, distribution, and concentration of this specific adduct in livers of treated mice and was correlated with cell injury as a function of dose and time. Within the liver lobule, immunohistochemically demonstrable adduct occurred in a temporally progressive, central-to-peripheral pattern. There was concordance between immunohistochemical staining and quantification of the adduct in hepatic 10,000g supernate, using a quantitative particle concentration fluorescence immunoassay. Findings include: 1) immunochemically detectable adduct before the appearance of centrilobular necrosis, 2) distinctive lobular zones of adduct localization with subsequent depletion during the progression of toxicity, 3) drug–protein binding in hepatocytes at subhepatotoxic doses and before depletion of total hepatic glutathione, 4) immunohistochemical evidence of drug binding in the nucleus, and 5) adduct in metabolically active and dividing hepatocytes and in macrophagelike cells in the regenerating liver. (Am J Pathol 1991, 138:359–371)

Acetaminophen (n-acetyl-p-aminophenol, APAP, paracetamol) accounts for approximately one third of the market in the United States for over-the-counter analgesic/antipyretic remedies. At therapeutic doses it is a very safe drug. At high doses it produces fulminating hepatic necrosis and has been reported to be nephrotoxic in some overdose victims.[1–3] Although APAP-induced hepatotoxicity has been studied extensively in animals, and there is much information available concerning the metabolic events that lead to the toxicity, the mechanisms that cause cell death have not been determined. A fraction of APAP is metabolized to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is the two-electron oxidation product of APAP, by the microsomal cytochrome P-450 mixed-function oxidase system.[4–9] After a therapeutic dose the reactive metabolite is detoxified by reaction with the cysteine-containing tripeptide glutathione (GSH) to form 3-(glutathion-S-yl)APAP. In contrast, an overdose of APAP depletes the GSH reserves and toxicity presumably occurs by the covalent binding of reactive metabolite to critical cellular proteins.[10–13] Other mechanisms of APAP-induced cytotoxicity may be important, however. For example, it is known that NAPQI is an oxidizing agent[5,7] and may induce oxidative stress. Thus, peroxidative mechanisms[14] may play a role in APAP toxicity. Although the relative importance of oxidative stress, peroxidative damage, and covalent binding remain to be determined, covalent binding of APAP to protein has emerged as the most reliable biomarker of APAP toxicity. In animals, most APAP binds by the reaction of NAPQI with cysteinyl sulfhydryl groups producing the corresponding 3-(cystein-S-yl)APAP (3-Cys-A)-protein adduct.[15,16]

We recently developed an antiserum specific for this major APAP-protein adduct.[17,18] The antiserum is a new tool to study the relation between formation of the 3-Cys-A protein adduct and APAP-induced hepatotoxicity. With a

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competitive enzyme-linked immunosorbent assay (ELISA), we demonstrated that 3-Cys-A adduct accumulates in the liver (10,000*g* supernate), reaches a maximum at approximately 2 hours, and declines by approximately 80% in the next 6 hours. Correlated with this decline, 3-Cys-A adduct appears in serum concomitant with the appearance of alanine aminotransferase (ALT) in serum. We thus hypothesized that the adduct was of hepatic origin, derived from injured hepatocytes during the development of hepatotoxicity. We postulated the adduct in serum was a specific biomarker of the hepatotoxicity and proposed that it could be used to study APAP hepatotoxicity in humans.[19] Subsequently, we analyzed various hepatic cell fractions and specific proteins within each fraction for the presence of this adduct. The most highly drug-modified proteins occurred in the plasma membrane fraction.[20]

Here we report the use of this extensively characterized antiserum to detect the 3-Cys-A protein adduct in liver of APAP-treated mice. The hepatic localization and persistence of the 3-Cys-A protein adduct has been measured as a function of dose and time, and correlated with an immunochemical quantitative technique and other indices of hepatotoxicity.

Methods

Reagents

Acetaminophen, GSH, and metallothionein were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol and sodium azide were procured from Eastman Kodak Co. (Rochester, NY). Dialysis tubing with a molecular weight cutoff of 2000 daltons was obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). Fetal calf serum was a product of Gibco (Grand Island, NY). Fluorescein-conjugated mouse anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). 3-(2-Pyridyldithio)propionic acid N-hydroxy succinimide ester was obtained from Pierce Chemical Co. (Rockford, IL). Amino-substituted polystyrene particles and 96-well assay plates were purchased from Pandex Division of Baxter Laboratories, Inc. (Mundelein, IL).

Animals and Dosing

Male B6C3F1 mice were obtained at 4 weeks of age from the NCTR specific-pathogen-free breeding colony. Animals were maintained under conventional conditions in a room on a 12-hour light–dark cycle provided with high-efficiency particulate-filtered air at a rate of 15 changes per hour at 23° ± 4°C and 50% ± 10% relative humidity. Animals were housed (4 to a cage) in clear plastic cages with hardwood bedding, and provided type 5010 M laboratory chow (Ralston-Purina, St. Louis, MO). Mice were 12 weeks old and had an average weight of 27 g when they were dosed. Food was removed from the feeders (at 5:00 P.M.) 15 hours before dosing. Acetaminophen was dissolved in 40°C pyrogen-free saline (Travenol Laboratories Inc., Deerfield, IL) at concentrations such that 0.025 ml/g body weight injected intraperitoneally delivered the desired dose. The actual doses and killing times are in the appropriate figure legends.

Sample Preparation

Blood samples were obtained from the retro-orbital plexus of CO_2-anesthetized mice. The blood was allowed to clot at room temperature and serum was separated, using Shure Sep II (General Diagnostics, Morris Plains, NJ) and stored at −70°C until analysis. Animals were killed, under CO_2 anesthesia, by cervical dislocation. Segments of the liver were fixed for histologic examination and the remainder was homogenized with a Teflon-coated tissue grinder using a 5:1 vol/wt ratio of 0.01 mol/l (molar) sodium phosphate buffer, pH 7.2, containing 8.5% wt/vol NaCl (phosphate-buffered saline [PBS]). Homogenates were centrifuged at 10,000*g* for 10 minutes at 4°C and the supernates were stored at −70°C. Before quantification of the 3-Cys-A protein adduct, noncovalently bound APAP and APAP-GSH conjugates were removed from serum samples and liver supernates by dialysis three times against 5 liters of PBS. The efficiency of dialysis to remove unbound APAP and 3-(glutathion-*S*-yl)APAP from serum and liver proteins was reported previously.[19]

Immunochemical Quantification of the 3-Cys-A Protein Adduct

Preparation of the 3-(N-acetylcystein-*S*-yl)APAP-hemocyanin immunogen, rabbit antiserum specific for 3-Cys-A (previous designation, Rab-541), and the structurally related competitive inhibitors, N-acetylcysteine-APAP and GSH-S-transferase APAP, have been described.[17,18]

Solid-phase 3-(L-cystein-*S*-yl)APAP metallothionein assay particles were prepared by covalently coupling APAP-derivatized metallothionein to amino-polystyrene particles. [^{3}H-ring]NAPQI (1 mCi/mmol) was synthesized by the method of Dahlin and Nelson.[21] For the synthesis of 3-(L-cystein-*S*-yl)-APAP-metallothionein, 2.6 μmole of NAPQI was added to 1 mg/ml metallothionein in distilled

water at 4°C and the mixture was allowed to react for 5 minutes on ice. The reaction was stopped by the addition of 15 μl of 1 mol/l GSH. 3-(Glutathion-*S*-yl)APAP conjugates were removed by dialysis against PBS.

Amino-substituted polystyrene assay particles (5 ml of 5% wt/vol, 0.89 μ) were packed by centrifugation at 3000*g* for 20 minutes. The packed beads were resuspended in 0.1 mol/l PO_4, 0.1 mol/l NaCl buffer, pH 7.3, followed by addition of 12 mg of 3-(2-pyridyldithio)propionic acid N-hydroxy succinimide ester dissolved in ethanol. The reaction mixture was rocked gently at room temperature for 2 hours. Subsequently, the beads were washed twice in 20% ethanol followed by centrifugation at 3000*g* and once in 0.1 mol/l phosphate buffer, pH 7.0, followed by centrifugation at 3000*g*. Acetaminophen-derivatized metallothionein (40 μg protein per milliliter) was added to the washed pyridine disulfide beads resuspended in 0.1 mol/l phosphate buffer, pH 7.0. This mixture was incubated overnight at room temperature on a rocker. The coupled beads were then washed three times with 0.15 mol/l PBS, pH 7.4, washed once in 2 mmol/l (millimolar) phosphate buffer, pH 5.5, and resuspended in the same buffer (Figure 1, top).

3-(Cystein-S-yl)APAP protein adduct levels were quantified immunochemically as described previously[17,19] with modifications to adapt the assay to a particle concentration fluorescence immunoassay (PCFIA) format (Figure 1, bottom). A limiting amount of rabbit anti 3-Cys-A antibody was incubated with either 3-(*N*-acetyl-L-cystein-*S*-yl)APAP standard or an unknown (mouse liver fraction or serum) for 45 minutes at 37°C. All dilutions were prepared in 16 mmol/l phosphate buffer, pH 7.4, 140 mmol/l NaCL, 5 mmol/l KCL (isotonic buffered saline, IBS) containing 4% vol/vol fetal bovine serum (FBS). Equal volumes (25 μl) of inhibitor-antibody mixture and 0.127% wt/vol solution of metallothionein-APAP assay particles were added to the wells of specially designed assay plates (Baxter). Plates were incubated at 37°C for 30 minutes and then washed three times with 0.05% vol/vol NP-40 in IBS (wash buffer). Subsequently, 25 μl of fluorescein-labeled mouse anti-rabbit IgG (diluted 1:250) was added to each well and incubated at room temperature for 30 minutes. Finally, the plates were washed five times with wash buffer and the remaining fluorescence was quantified (485 nm excitation, 535 nm emission) using a Fluorescence Concentration Analyzer (Baxter). Inhibition by unknown samples was compared with a standard curve prepared using radiolabeled [^{3}H]3-(*N*-acetyl-L-cystein-*S*-yl)APAP. The radiolabel was used to verify the concentration of bound APAP in the standard. The values obtained were corrected for differences in the relative inhibitory potency of 3-(*N*-acetyl-L-cystein-*S*-yl)-APAP and 3-(cystein-*S*-yl)APAP protein adduct (120 fmol/well and 2300 fmol/well,

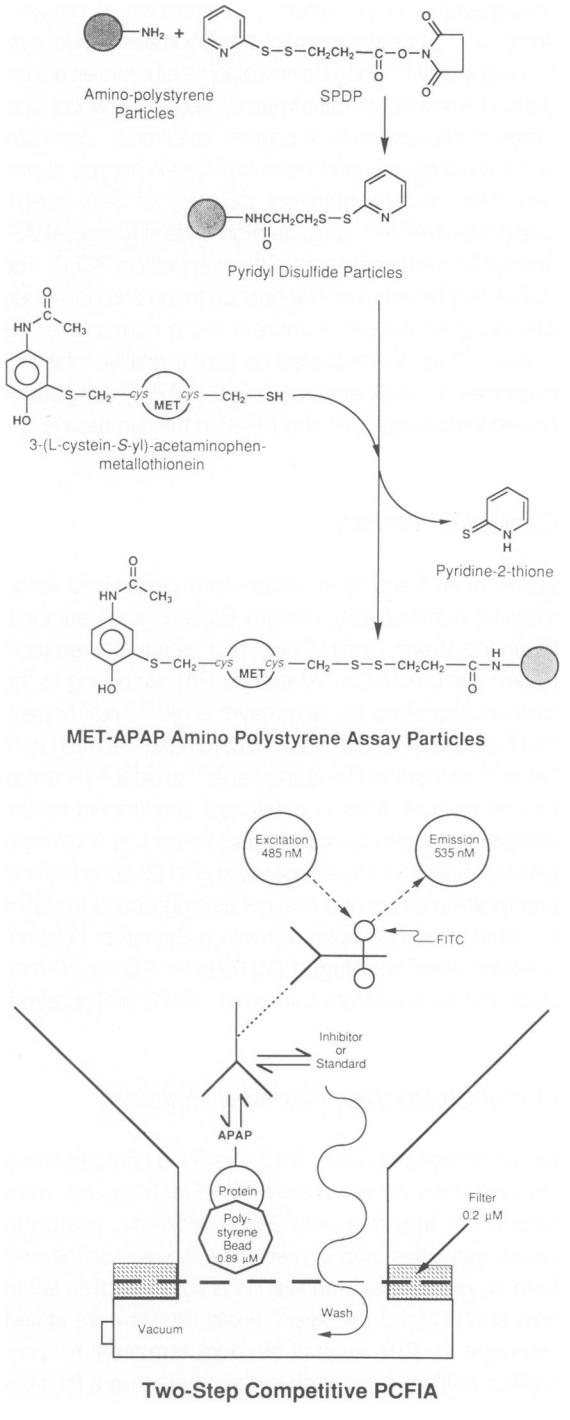

Figure 1. *Development of a particle concentration fluorescence immunoassay (PCFIA) for the 3-(cystein-S-yl)acetaminophen-protein adduct, and the preparation of the covalently bound solid-phase assay antigen, 3-(cystein-S-yl)acetaminophen-metallothionein covalently bound to 0.89-μm amino polystyrene particles (Met-APAP modified polystyrene assay particles) are described in Methods. In this PCFIA assay, the 50% inhibitory concentrations were 269 fmole/well for N-acetyl-cysteine acetaminophen, 3055 fmole/well for glutathione-S-transferase acetaminophen, and 4.3 × 10^6 fmole/well for acetaminophen.*

respectively.[18] The protein concentration of dialyzed samples was determined by the Coomassie blue dye-binding assay,[22] using bovine plasma albumin as a standard. Unknowns samples were diluted to a final concentration of approximately 4 μg/well, assayed in duplicate, and expressed as nanomoles of 3-Cys-A per mg of protein. The relative inhibitory potency of 3-(N-acetylcystein-S-yl)APAP, 3-(glutathion-S-transferase)APAP, and APAP were compared in the competitive PCFIA. The PCFIA recognized a similar epitope to the one described previously for the same antisera in the competitive A-B ELISA[17,18] as demonstrated by similar relative inhibitory potencies for 3-(N-acetylcystein-S-yl)APAP, 3-(glutathion-S-transferase)APAP and APAP in the two assays.

Clinical Chemistry

Levels of ALT activity in serum were determined as an index of hepatotoxicity using a Baker Encore autoanalyzer and Baker CentrifiChem ALT optimized reagents (Baker Instrument Co., Allentown, PA) according to the method described by Bergmeyer et al.[23] Total hepatic GSH levels were determined according to the method of Tietze[24] as modified by Gandy et al[25] to adapt the assay for use on a Multistat III centrifugal spectrophotometric analyzer. Samples were prepared by adding 5 volumes (wt/vol) of ice-cold 1% sulfosalicylic acid (to denature and precipitate proteins and prevent autoxidation of the GSH to GSSG) and homogenized with a Brinkman Polytron. Samples were centrifuged (10,000*g* at 4°C for 20 minutes) and the supernates stored at −70°C until assayed.

Immunohistochemistry and Histology

Rabbit antiserum specific for 3-Cys-A was prepared and characterized as described.[17,18] Fresh tissues were trimmed to approximately 2-mm thickness, placed in plastic cassettes, and immersed in ice-cold saline until fixed by microwave irradiation in a 700-watt oven set to hold at 60°C for 2 minutes.[26] Fixed tissues were stored overnight in 70% ethanol at room temperature, processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Localized APAP-protein adduct was detected using a modification of the unlabeled antibody enzyme method described by Sternberger.[27] Drug-protein antigens were stained by sequential incubations with 1) rabbit anti-3-Cys-A (diluted 1:350 in PBS containing 1% FBS, 20 minutes), 2) sheep anti-rabbit linking antiserum, 20 minutes, 3) rabbit peroxidase-antiperoxidase (PAP), 20 minutes, and 4) 3,3′-diaminobenzidine substrate (containing 0.03% H_2O_2, 10 minutes). Slides were washed twice with PBS between each incubation and all incubations were at room temperature. Sections were counterstained with Mayer's hematoxylin. Reagents for immunohistochemical detection of rabbit anti 3-Cys-A were components of an immunoperoxidase staining kit (Cambridge Research Laboratory, Cambridge, MA). Non–covalently bound APAP and APAP-GSH conjugates are soluble in polar solvents and were removed before immunostaining by the large relative volumes of saline and graded alcohols used to treat the blocks and thin sections during fixation and routine tissue processing. The efficiency of these procedures to remove APAP-GSH was demonstrated by the absence of immunostain in liver from mice killed 2 hours after a hepatotoxic dose of APAP (400 mg/kg) that were 'rescued' by treatment 15 minutes after dosing with N-acetyl-cysteine (1200 mg/kg). N-acetyl-cysteine acts as an antidote by supporting glutathione production so that the reactive metabolite reacts with sulfhydryls on glutathione in lieu of sulfhydryls on tissue macromolecules. Except as noted, the numbers used in descriptions of the deposition of immunostain reaction product are subjective estimates determined by comparison, at 40 × magnification, of sections from animals in each treatment group, to the maximal intensity present at 2 hours after a 400 mg/kg dose. The subjective estimates of adduct quantity localized in tissue sections were corroborated by quantitative immunochemical measures of adduct in liver homogenates by the PCFIA. Saline-treated controls had no hepatocyte injury and no adduct formation (Figure 2a, b). No reaction product was present in sections from adduct-positive animals when normal preimmune serum was substituted for anti 3-Cys-A antibody (Figure 2c).

Acetaminophen-related hepatotoxicity was inferred from the morphologic changes in sections of liver fixed by microwave irradiation, processed routinely, and stained with hematoxylin and eosin (H&E) using standard techniques.

Results

In the livers of APAP-treated animals, only hepatocytes appeared to be affected; the extent and the degree of injury was uniform throughout the liver for each dose and time, but zonal within lobules. Only centrilobular and midzonal hepatocytes were demonstrably injured, whereas periportal hepatocytes appeared unaffected. Although areas of injury invariably correspond with areas of adduct localization, adduct localization was not always associated with cellular injury.

Time Course

The localization and relative content of the 3-Cys-A protein adduct in liver fixed by microwave irradiation was

determined using antibody specific for the adduct and a PAP detection system that deposited a microscopically visible, brown 3,3′-diaminobenzidine reaction product at the sites of antibody binding. The temporal relationships between the development of hepatic necrosis and the formation of APAP-protein adduct were examined in mice dosed with a hepatotoxic dose (400 mg/kg) of APAP, and killed at 0.25, 0.5, 1, 2, 4, 6, and 8 hours after dosing. The livers of mice evaluated 15 minutes after 400 mg/kg APAP were morphologically normal (Figure 2d), but 3-Cys-A adduct was demonstrable in the cytoplasm of centrilobular hepatocytes nearest the central vein (Figure 2e). These cells were stained with an intensity approximately 30% of that present at 1 to 2 hours after 400 mg/kg, and adduct staining at 15 minutes decreased in intensity toward the lobule periphery. This decrease was very consistent among lobules. It was notable that the total area of the stained zone was actually similar to the maximal adducted region present at later times, except the adduct in the peripheral region (lobule midzone) was very sparse and could be detected only at higher magnification (100 to 250×). The content of 3-Cys-A adduct in the midzonal region was estimated to be one half of that present in centrilobular cells.

At 30 minutes, the intensity of adduct stain was approximately twice that present at 15 minutes. The amount present at the periphery of the stained zones had increased disproportionately to the central cells, diminishing the radial gradient that was present earlier, so the adduct-containing zones, visible at low magnification, were slightly larger than at 15 minutes. With the H&E stain, the hepatocytes appeared altered but necrosis was not present. In the region corresponding to the 3-Cys-A adduct localization, the hepatocytes were mildly to moderately enlarged, slightly more eosinophilic than periportal hepatocytes, and presented a finely granular 'ground-glass' appearance.

One hour after treatment, the adduct-containing areas were approximately 15% to 20% larger than at 30 minutes, and the staining intensity had increased and was essentially uniform over the adducted area (Figure 2f). In some animals, the ring of hepatocytes bordering the central vein stained more intensely than the surrounding adducted area (Figure 2f). At 2 hours, the adduct-containing centrilobular region was unchanged (and maximal) in size. There was a discernible but variable decrease in the immunochemical stain intensity in the central portion of the stained zone associated with disintegration of the necrotic hepatocytes. The hepatocytes in the entire adduct-containing area were highly vacuolated and shrunken, and the sinusoids were dilated and prominent (Figure 2g). By 4 hours, approximately one half of the cells in the adduct-containing region (predominantly those nearer the central veins) were ghost cells, anuclear and retaining less than 25% of the stained adduct present earlier. The cells in the periphery of the adducted area were now severely vacuolated and contained nuclei in various stages of degeneration. These cells retained 50% to 75% of the maximal adduct content, based on stain intensity. At 6 hours (Figure 2h) and 8 hours, there was progressive loss of stain intensity as the coagulated necrotic hepatocytes disintegrated, beginning with those nearest the central veins.

With respect to morphology, H&E stain at 1 hour showed the cells in the adducted zone to be distinctively vacuolated and increased in eosinophilia. Individual cells were necrotic, characterized by absence of a nucleus or having a pyknotic or fragmented nucleus. Periportal areas appeared unaffected. At 2 hours, most centrilobular hepatocytes were obviously necrotic, containing anuclear coagulated cytoplasm or multiple large vacuoles (Figure 4a). At 4 hours, most of the necrotic hepatocytes were still discernible albeit shrunken, with lysis or leaching of cytoplasm. This area corresponded with the midzonal pallor evident in the sections stained immunochemically for the presence of the 3-Cys-A adduct. The maximum extent of injury was evident in 4 to 6 hours, when hepatocellular necrosis encompassed the entire centrilobular and midzonal portion of the liver lobule (Figure 2g).

Except for the most peripheral row of adduct-stained hepatocytes in each lobule, all adduct-containing cells showed toxic injury; they contained one to several large vacuoles and many of the centralmost cells were anuclear or had a pyknotic or fragmented nucleus (Figure 2g, h). At the periphery of the adduct-containing zone there was a nearly complete ring of adduct-containing hepatocytes that appeared normal morphologically. These surviving peripheral cells had approximately 25% to 50% of the reaction product contained by adjacent necrotic cells. Figure 2f shows the normal fine cytoplasmic vacuolation of these surviving peripheral adduct-containing cells, in sharp contrast to the adjacent dying cells, and also shows that their nuclei are free of adduct, while those of the adjacent dying cells contain adduct. The zone containing adduct visualized with the immunochemical stain appeared slightly larger than the necrotic zone apparent in the H&E stain, because the peripheral adduct-containing cells were normal in the latter preparation. Periportal areas were spared, as indicated by the absence of 3-Cys-A adduct and cell injury.

At 6 and 8 hours, more than 75% of hepatocytes were necrotic in a uniform pattern of centrilobular and midzonal necrosis that spared the periportal hepatocytes. At 8 hours there was marked dilatation of hepatic sinusoids in the adduct-positive areas, with continued shrinkage of necrotic hepatocytes. Periportal sinusoids were normal. The necrotic areas were more clearly circumscribed and

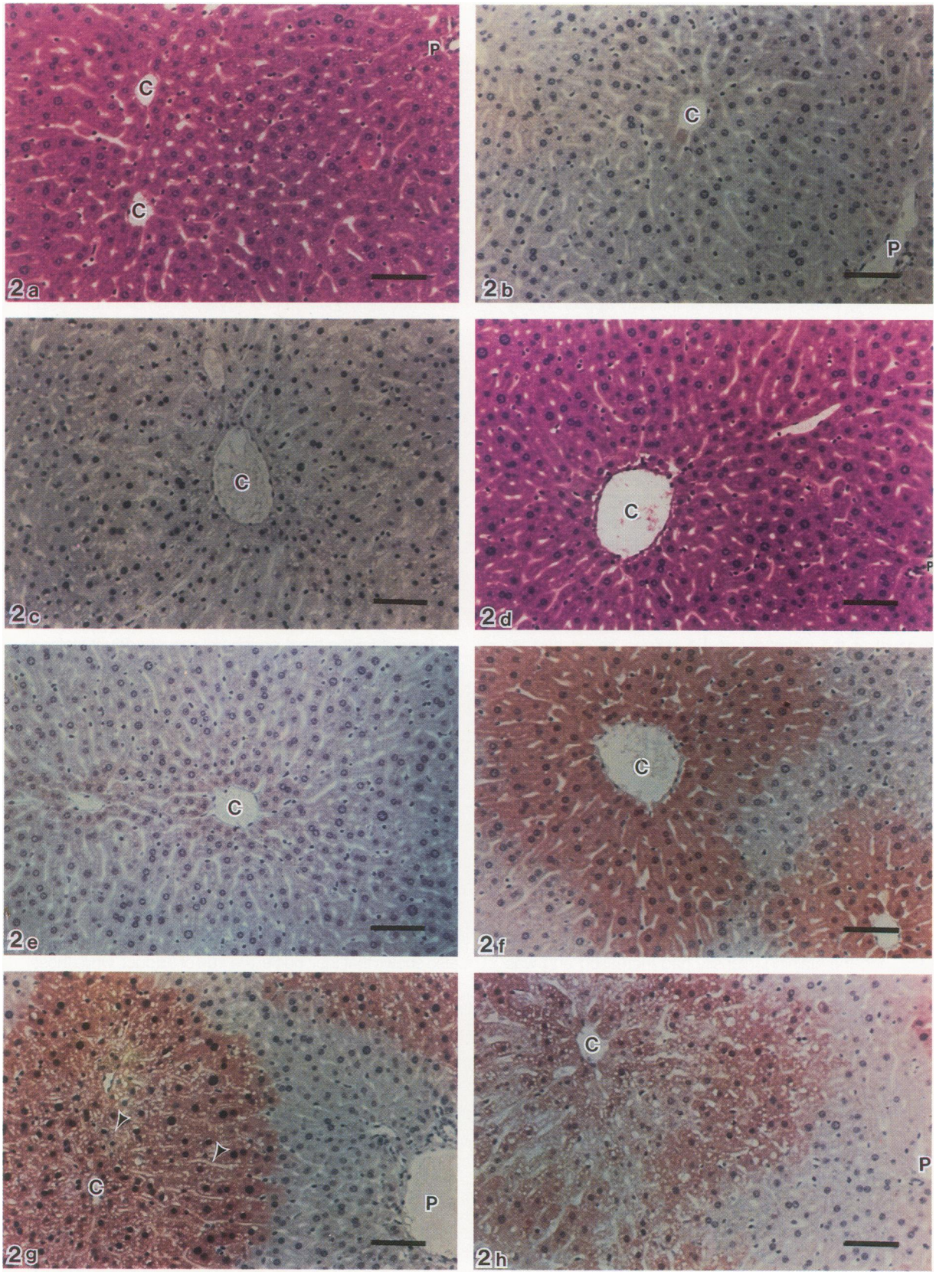
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Figure 2. *Mouse liver sections at various times after 400 mg/kg acetaminophen IP. C = central vein, P = portal area. Bars = 75 μm.* **a**: *Treatment control, 2 hours after normal saline IP. H&E stain. Normal liver.* **b**: *Same animal as Figure 2a. Immunohistochemical stain for the 3-Cys-A protein adduct is negative.* **c**: *Control demonstrating the absence of nonspecific background staining. Stained 2 hours after acetaminophen treatment (same animal as in Figure 2g), but preimmune serum was substituted for omitted primary antibody. No reaction product is present, despite presence of adduct.* **d**: *Fifteen minutes after treatment. H&E stain. The liver is morphologically normal.* **e**: *Fifteen minutes after treatment. Immunohistochemical stain is positive for the 3-Cys-A protein adduct in hepatocytes nearest the central veins.* **f**: *One hour after treatment. Immunohistochemical stain reveals adduct throughout centrilobular and midzonal regions.* **g**: *Two hours after treatment. Immunohistochemical stain is maximal in extent. Centrilobular hepatocytes are vacuolated, shrunken, and necrotic and sinusoids are prominent (arrows).* **h**: *Six hours after treatment. Intensity of immunohistochemical stain is diminished as adduct is lost from disintegrating necrotic cells, beginning centrally.*

gave the impression of being larger at the 8-hour point because of the continued dissolution and disappearance of cells. By 8 hours a small number of neutrophilic leukocytes was present at the periphery of the necrotic zones.

Liver and serum samples were obtained from the mice killed at the 0.5-, 1-, 2-, 4-, 6-, and 8-hour points. These samples were evaluated to determine hepatic GSH, serum ALT, and 3-Cys-A adduct levels in liver homogenates and serum (Figure 3). Thirty minutes after a hepatotoxic dose (400 mg/kg) of APAP, 3-Cys-A adduct was evident in the centrilobular region by immunohistochemical staining, in 10,000*g* hepatic supernate, but not in serum. This correlated temporally with a 90% depletion of hepatic GSH, no histologic evidence of necrosis, and normal concentrations of ALT in serum (Figure 3). Adduct levels in the 10,000*g* liver supernate increased between 0.5 hours and 4 to 6 hours, whereas the immunohistochemical staining intensity and the width of the affected centrilobular areas became maximal at 2 to 4 hours (Figures 2 and 3). After 6 hours, when the staining intensity was markedly decreased and had developed midzonal pallor, there was a corresponding decrease in the level of the 3-Cys-A adduct in the 10,000*g* supernate, which was inversely correlated with parallel increases of serum adduct and ALT (Figure 3). By 8 hours, hepatic GSH concentrations had recovered slightly.

Dose Response

The relationship between dose and 3-Cys-A adduct localization at a fixed time was also examined. Mice were dosed with 0, 100, 200, 300, or 400 mg/kg of APAP, and killed 2 hours later. At the 400-mg/kg dose, the deposition of reaction product, and thus the localization of 3-Cys-A adduct, was similar in each lobule. Immunohistochemical staining showed abundant antibody binding to 3-Cys-A in the cytoplasm and most nuclei of centrilobular and midzonal hepatocytes. The stain was less intense in the central region of the adducted area, because of greater loss of cytoplasm from those hepatocytes (Figure 2g). With H&E, the necrotic zones corresponded to areas of intense adduct localization in adjacent immunochemically stained sections. The necrotic zones were charac-

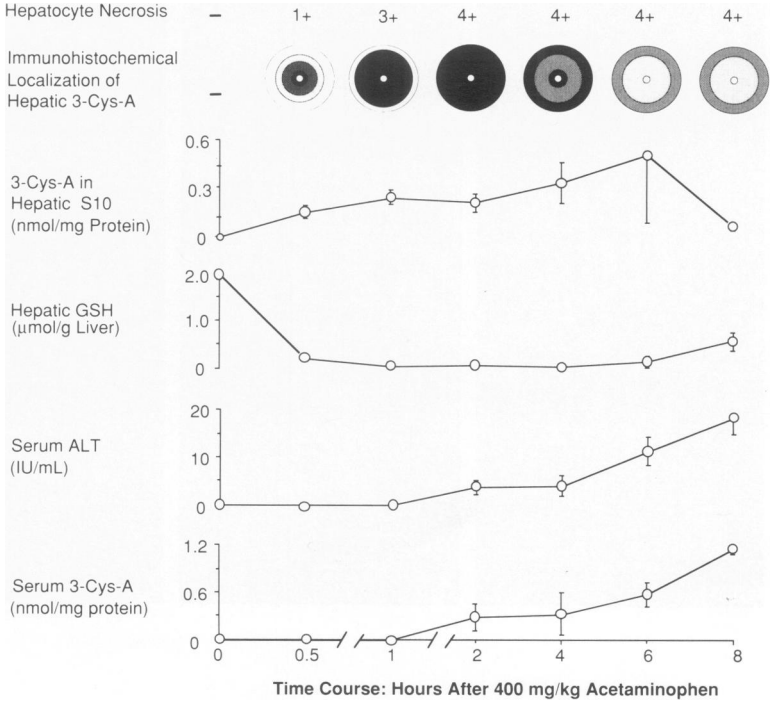

Figure 3. *Time course for acetaminophen-induced hepatotoxicity and the immunochemical localization of 3-(cystein-S-yl)acetaminophen (3-Cys-A) protein adduct in liver. Mice were treated with 400 mg/kg acetaminophen and killed 0.5, 1, 2, 4, 6, and 8 hours after dosing. Controls were saline-treated mice killed 2 hours after dosing. Liver histopathology is symbolically represented where "—" indicates no pathology (control), "1+" indicates minimal necrotic area "2+" indicates moderate necrotic area, "3+" indicates extensive necrotic area, and "4+" indicates very extensive necrotic area. The intensity and relative density of immunohistochemically localized 3-Cys-A protein adduct is represented as "—" indicating no adduct (control), and circular symbols in which diameter and darkness of shading symbolically represent relative differences in the area and intensity of 3-Cys-A binding around representative centrilobular regions. The immunochemical quantitation of the 3-Cys-A protein adduct in hepatic 10,000 g supernate (S10) and in serum, determination of hepatic glutathione concentrations and serum concentrations of ALT are described in Methods. Data points represent the mean ± standard error for three animals per point, except at the 4- and 8-hour times, where there were two animals per point.*

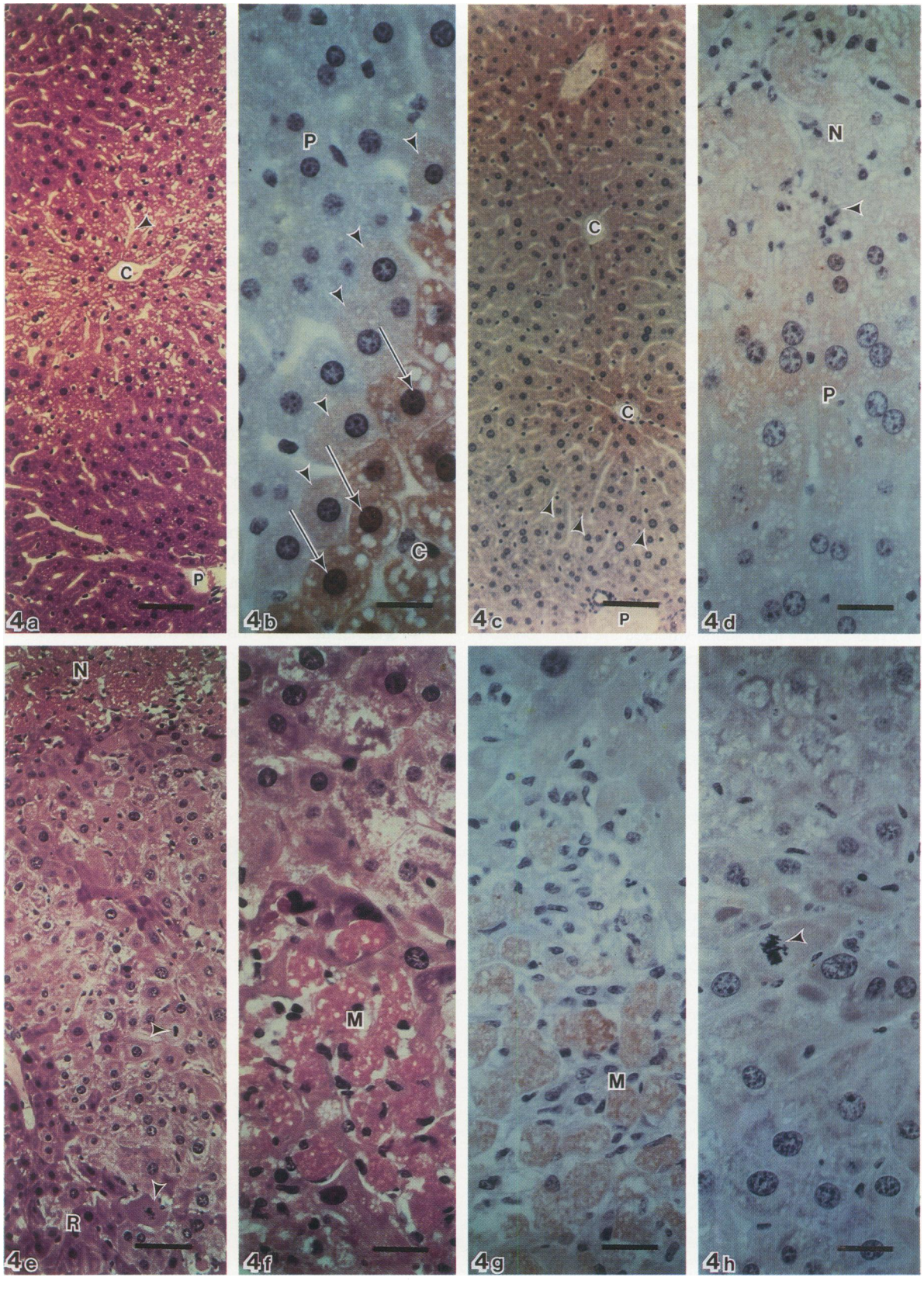
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Figure 4. a: *Mouse liver 2 hours after treatment with 400 mg/kg acetaminophen. H&E stain. Most centrilobular cells are necrotic and sinusoids are dilated (arrow). C = central vein, P = portal area. Bar = 75 μm.* b: *Two hours after treatment with 300 mg/kg acetaminophen. Immunohistochemical stain reveals reduced quantity of adduct in morphologically normal cells (short arrows) at periphery of heavily adducted necrotic region. Note reaction product (red-brown color) in nuclei of dying cells (long arrows). Centrilobular region (C); Periportal region (P). Bar = 19 μm.* c: *Two hours after treatment with 100 mg/kg acetaminophen. Immunohistochemical stain reveals adduct in moderate quantity in centrilobular cells nearest the central vein and in smaller quantity in cells extending to the midzonal region (arrows). C = central vein, P = portal area. Bar = 75 μm.* d: *Twenty-four hours after 200 mg/kg acetaminophen. Immunohistochemical stain reveals adduct in surviving and binucleate hepatocytes deep in periportal region (P). Most adduct has leached from necrotic central cells (N). Note inflammatory cells at junction of necrotic and surviving zones (arrow). Bar = 30 μm.* e: *Seventy-two hours after 200 mg acetaminophen. H&E stain. Regenerating hepatocytes (R), including many in mitosis (arrows), contrast sharply with coagulated necrotic zone (N). Bar = 75 μm.* f: *H&E stain. Macrophages (M) at edge of necrotic zone contain granular debris from disintegrating hepatocytes. Bar = 30 μm.* g: *Immunohistochemical stain, same liver as Figure 4f. The debris in macrophages stains positively for adduct (M). Bar = 30 μm.* h: *Adduct is still clearly present in visible hepatocytes at 72 hours, including dividing cells (mitotic figure at arrow). Bar = 30 μm.*

terized in the H&E sections by severe cytoplasmic vacuolation, increased eosinophilia, coagulative necrosis, and by the nuclear and cytoplasmic loss noted also in the immunohistochemically stained sections. The sinusoids were dilated in the affected zones. In contrast, the periportal hepatocytes were similar to those in saline-treated animals. In representative lobule cross sections, there were approximately 20 concentric rings of hepatocytes, expanding peripherally from the central vein to the portal triad. With the H&E stain, typically the first three or four rings of cells nearest the central vein in the 400-mg/kg group were necrotic. The next three or four rings had normal-appearing nuclei, hepatocytes were severely vacuolated but not shrunken, and sinusoids were less dilated; the next band of three or four rings was less vacuolated although not normal, and the final (periportal) cells were unaffected.

At the 300-mg/kg dose, the major difference from the 400-mg/kg dose was an increased density and a more uniform localization of the adduct across a slightly smaller affected zone. Figure 4b shows the necrotic centrilobular hepatocytes that contain abundant stained adduct in cytoplasm and nuclei, periportal hepatocytes that are normal, and an intervening ring of morphologically normal hepatocytes that contain a reduced amount of adduct. The disruption and vacuolization of cytoplasm and the corresponding sinusoidal dilation in the most central portion of the lobules were not as severe, with less unstained space in the sections. The diameter of the adducted area was less than that at 400 mg/kg, by two or three rings of cells.

At 200 mg/kg, the stain was intense and uniform; adduct localization was very similar to that at 300 mg/kg, with possibly a one- or two-cell decrease in the diameter of the adducted area in each lobule. With the H&E stain, the size of the injured area at 300 and 200 mg/kg appeared similar to that at 400 mg/kg. The necrosis was much more obvious at 400 mg/kg because the degree of vacuolation and shrinkage of cytoplasm increased with dose.

Two hours after the 100-mg/kg dose, the area that was stained immunochemically appeared to be only half (50%) of that stained in sections from animals receiving higher doses and examined at the same magnification (40×). The intensity of the adduct stain was also markedly decreased, and appeared to consist of two concentric bands of three or four cells each. The centralmost band contained about half the reaction product present at higher doses, with the outer band reduced by half again (Figure 4c). At higher magnification (100 to 250×), the reaction product could be seen clearly in areas of less concentrated adduct localization. It was sparsely but uniformly detectable throughout the cytoplasm in most hepatocytes beyond those evident at 40×, to the same midzonal extent affected by the higher doses. Livers obtained 2 hours after the 100-mg/kg dose appeared normal histologically with the H&E stain, except for the presence of a large cytoplasmic vacuole in occasional hepatocytes near the central vein.

The dose-related increase in centrilobular necrosis and in adduct localization 2 hours after dosing was accompanied by a similarly dose-related increase in the quantity of the 3-Cys-A protein adduct measured by PCFIA in the 10,000*g* liver supernate, by depletion of hepatic GSH and by appearance in the serum of the liver-specific transaminase, ALT (Figure 5). The pattern of adduct localization within the liver lobule in relation to these parameters and as a function of dose is presented in Figure 5.

Late Events

Immunohistochemical localization of the 3-Cys-A adduct was also studied in the recovering liver. For these studies it was necessary to reduce the dose to 200 mg/kg to prevent mortality. At 24 hours after 200 mg/kg, there was massive hepatic necrosis. The centrilobular hepatocytes were coagulated, amorphous, and anuclear. The midzonal cells were anuclear, vacuolated, and greatly attenuated, creating a concentric spongiform band with widely dilated sinusoids. Infiltrating leukocytes and enlarged nuclei of endothelial and Kupffer cells were prominent, especially at the junction of the central and spongiform areas (Figure 4d). The periportal hepatocytes appeared normal. At the interface between the necrotic

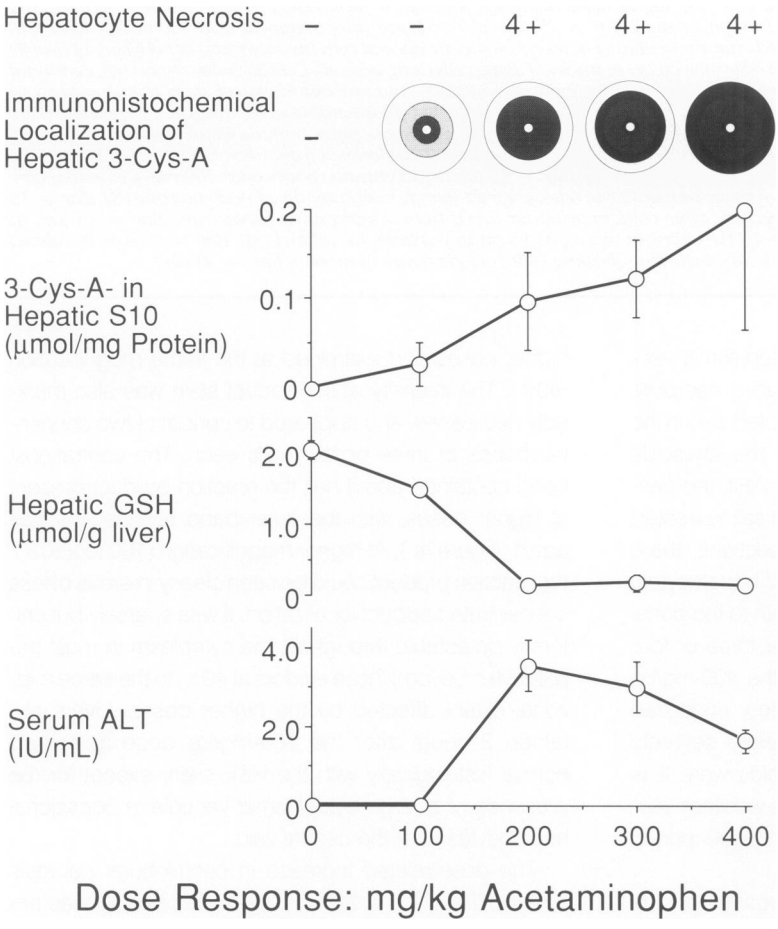

Figure 5. *The dose-response relationship between acetaminophen-induced hepatic necrosis, the localization of the 3-Cys-A protein adduct in liver sections, immunochemical quantitation of the 3-Cys-A protein adduct in hepatic 10,000 g supernate (S10), hepatic glutathione (GSH) concentrations, and serum concentrations of the liver-specific transaminase ALT. Mice were injected with the indicated dose of acetaminophen, and killed 2 hours after dosing. The symbolic representation of the extent of necrosis and immunohistochemically stained 3-Cys-A protein adduct are described in the legend to Figure 3. The procedures for measuring the 3-Cys-A adduct immunochemically in hepatic S10 or serum, measuring hepatic GSH, serum ALT, and sample processing are described in Methods. Data points represent the mean ± standard error for three animals per point.*

zone and the surviving cells, there were numerous binucleate periportal hepatocytes and occasional mitoses, indicating early regeneration. All the necrotic cells at 24 hours contained adduct, although the intensity of the stain was no more than one half of that present 2 hours after the 200-mg/kg dose. The intensity across the affected areas was approximately uniform although the spongiform regions appeared pale because of the sparse cytoplasm. A notable observation was the presence of adduct in surviving cells deep in the periportal region, as well as in surviving binucleate cells (Figure 4d). It was not possible to compare directly the affected regions from tissues obtained 2 hours after the 200-mg/kg dose with those obtained at 24 hours, as the architecture of the lobules was markedly disrupted at the later time.

At 72 hours after 200 mg/kg, there was an intense inflammatory response at the junction of the necrotic and surviving hepatocytes, together with brisk regeneration of hepatocytes along this interface (Figure 4e). The necrotic zone was more densely coagulated than at 24 hours. Unidentifiable spindle and oval cells were among the cells at the interface between zones, and were present as well in the periportal and perilobular regions. Macrophages containing granular eosinophilic material similar to the cytoplasm of necrotic hepatocytes were also numerous (Figure 4f). The necrotic debris at 72 hours still contained immunochemically detectable adduct, and identical stain was present in the cytoplasm of the macrophages (Figure 4e). Adduct also was clearly present in viable and dividing hepatocytes, as noted at 24 hours (Figure 4h).

Discussion

There is considerable interest in the toxicity of APAP because of its wide clinical use and its efficacy to study mechanisms of hepatotoxicity. Binding of the reactive metabolite to protein is generally accepted to be the mechanism of the toxicity; however no information regarding the formation of the 3-Cys-A protein adduct within individual cells as related to the death of these cells is available. The majority of the concepts that have been developed concerning the mechanism of toxicity of APAP[5,6] have used radiolabeled APAP,[10,11,15,16] or more recently, immunochemical analysis[17,19,28] to examine APAP bound to fractions of liver homogenates. Binding was correlated to histologic or clinical chemistry indices of hepatotoxicity. Data concerning localization of bound APAP in the hepatic lobule and in individual cells are

minimal. Jollow et al[11] published an autoradiograph from a liver of a mouse 24 hours after treatment with a hepatotoxic dose of APAP. The data indicated that radiolabel was concentrated in the affected cells; however the radiolabel was not confined exclusively to the affected centrilobular cells. More recently, Bartolone et al,[29] using an antiserum that recognized the N-acetyl portion of APAP but is not specific for the 3-Cys-A protein adduct, showed immunofluorescence in the centrilobular region 2 hours after a 600-mg/kg dose of APAP. Neither study, however, showed the relationship between binding of the 3-Cys-A adduct in individual cells and toxicity or provided resolution necessary to relate APAP binding to the development of necrosis in these cells.

The antiserum used in the present study has primary specificity for antigenic determinants found on APAP bound covalently to cysteine residues that are not found on protein alone or on free APAP. The specificity of this antiserum[18] coupled with the integrity of antigens preserved by microwave irradiation[30] and the sensitivity of the methods have enabled us to examine the formation and localization of the 3-(cystein-*S*-yl)APAP protein adduct during the development of APAP hepatotoxicity with greater detail than was previously possible. Our overall findings are in general agreement with the findings of Jollow et al,[11] Mitchell et al,[4,10] Placke et al,[31] and Bartolone et al[32]: the APAP covalently binds to centrolobular hepatocytes, and these are the affected cells. The current study clearly indicates, however, that the drug derivatives bound in affected cells are 3-Cys-A adduct, and that the hepatocytes that develop large quantities of 3-Cys-A adduct at early times become necrotic at later times.

The major objective of the current work was to relate the appearance of 3-Cys-A adduct in hepatocytes with histologic evidence of APAP hepatotoxicity. In particular we wanted to correlate the dose and time relationships between the formation of the 3-Cys-A protein adduct and the development of hepatic necrosis. Poor correlation may indicate that the necrosis was mediated by an indirect mechanism such as oxygen deprivation of the hepatocytes around the central vein; a positive correlation could indicate that 3-Cys-A protein adduct formation altered hepatocytes directly, resulting in necrosis.

The size of the necrotic area that was demonstrated in each lobule with the H&E stain did not correlate as closely with the adduct content of the liver 10,000*g* supernate, as did the immunohistochemical demonstration of adduct. When a lethal (threshold) amount of adduct is formed, the development of morphologic changes associated with hepatocyte necrosis proceeds at a rate that is presumably independent of the additional (beyond threshold) amount of adduct formed in the cell. Although not determinable at any single dose or time, it is our collective interpretation of the dose response and early time course data that 1) cells in which large amounts of reactive metabolite bind will later die and 2) the subjective estimate of the relative amount (intensity × area) of immunohistochemically stained adduct appears to correlate with the 3-Cys-A adduct per milligram of liver protein quantified by PCFIA in the hepatic 10,000*g* supernate. This hypothesis will be examined systematically by quantitative image analysis in subsequent studies. At later times (beyond 4 hours), when the liver is congested (relative weight data not presented), measurement of adduct in the 10,000*g* supernate of the liver homogenate appears to overestimate adduct content relative to sections processed for immunohistochemical demonstration of adduct. The reason for the temporal difference appears to be the presence of 3-Cys-A adducts in the congestion fluid that are present and measured in the 10,000*g* supernate but are washed out of thin sections during tissue processing before immunohistochemical staining. The areas to be affected are 'marked' by the presence of 3-Cys-A adduct as early as 15 minutes after dosing. Thereafter the intensity increases to a maximum at approximately 4 hours after a dose of 400 mg/kg. Interestingly, even at 4 to 6 hours in animals showing maximal APAP hepatotoxicity and adduct deposition, there are morphologically normal hepatocytes at the periphery of the necrotic areas that contain 25% to 50% of the adduct present in necrotic cells. Although it cannot be demonstrated unequivocally with the current method, it is most likely that the binucleate hepatocytes with sparse adduct observed at 24 hours are the progeny of some of these 'marked' but surviving cells. The interpretation that many of these surviving adduct-laden cells are metabolically active and are undergoing cell replication 24 to 72 hours after APAP is supported by the presence of mitotic figures in adduct-stained hepatocytes and reports that binucleate hepatocytes are transcriptionally active.[33]

In addition to the above finding, the time course studies show that staining intensity in the hepatocytes reaches a maximum at 2 hours after a hepatotoxic dose and subsequently decreases in intensity as the progression of the toxicity develops. This decrease correlates with the appearance of ALT and 3-Cys-A adduct in the serum as we previously showed.[19,20] To determine if acetaminophen toxicity in humans was mediated by a similar mechanism as reported for experimental animals, we recently looked for the occurrence of 3-Cys-A protein adducts by PCFIA in plasma samples from patients who had taken an overdose of acetaminophen. Patients that were treated with antidotal N-acetyl-cysteine within the first few hours after overdose were protected from liver damage, and had no plasma 3-Cys-A. All patients that had liver damage, as indicated by elevated plasma ALT, had 3-Cys-A adducts in their plasma. This is the first direct evidence of a mechanism involving 3-Cys-A adducts in acetaminophen-induced liver toxicity in humans.[34]

From a metabolic perspective, an important finding of this work is the demonstration of immunochemically detectable 3-Cys-A adduct in livers after a dose of APAP that did not substantially deplete total hepatic GSH. Acetaminophen (100 mg/kg) depleted GSH by less than 25% at 2 hours; however the 3-Cys-A adduct was detectable in liver sections and were measured to be 0.05 nmol/mg protein in 10,000*g* supernate. Experiments by Mitchell et al,[10] using radiolabeled APAP, indicated that covalent binding did not occur until total hepatic GSH was depleted by 70%. The difference between our data and Mitchell's data could be a result of the greater sensitivity of the immunologic method or unexplained variability in the susceptibility of mice to APAP hepatotoxicity. This variability may occur using age-, strain-, and sex-matched mice under putatively identical dosing circumstances and may be attributed to a variety of factors that influence fluctuations in P-450 metabolism and GSH reserve. The concentration of GSH is high relative to protein-sulfhydryl groups in the liver, and under conditions of formation of the 3-Cys-A protein adduct, substantial depletion of GSH in affected cells would be expected based solely on mass action. Moreover, because detoxification may be mediated by GSH transferases,[35] substantial depletion of GSH would be expected before the formation of significant amounts of the 3-Cys-A protein adduct. Furthermore total hepatic GSH may not indicate what is occurring in individual affected hepatocytes. Significant depletion of total hepatic GSH may occur only after transport of GSH (perhaps as cysteine residues followed by resynthesis) from the unaffected periportal areas to the centrilobular areas of the liver.

In addition to the concern that APAP overdose may be hepatotoxic or nephrotoxic in humans, the possibility that persistent APAP-protein adduct may occur at nonhepatotoxic doses is important because of the potential immunogenicity of such complexes. Undefined conditions appear to predispose certain individuals to idiosyncratic APAP toxicity that may be immunologically mediated. Although APAP is unlikely to function as an immunogen because of its small size, APAP-protein conjugates, presumably formed by NAPQI binding to sulfhydryl-containing host macromolecules, may be immunogenic. This concept is supported by 1) recent reports of APAP hypersensitivity,[36,37] reports of immunologically mediated APAP-induced thrombocytopenia,[38] and reports of immunologically-mediated APAP-associated hemolysis[39]; 2) the observation that similar APAP conjugates were used to prepare rabbit antiserum;[17] and by 3) the observation reported herein that the 3-Cys-A adduct persists in the recovering liver and in macrophages. Phagocytosis may enhance immunogenicity of drug–protein complexes by presenting antigenic peptides adjacent to host histocompatability molecules. Such bimolecular complexes on the cell surface may be recognized by T cells and initiate events leading to hypersensitivity or other forms of drug-induced immunologic injury.[40]

Continued application of immunoassays specific for the 3-(cystein-*S*-yl)APAP protein adduct will provide experimental approaches for the identification and characterization of the protein structures damaged by the acetaminophen metabolite and provide insight regarding the processes that ultimately lead to acetaminophen-induced cellular necrosis. This insight is a prerequisite for the development of improved treatment interventions for acetaminophen overdose patients. The persistence of APAP adduct in otherwise normal-appearing hepatocytes is analogous to the persistence of acetaldehyde-modified protein epitopes in the liver of alcoholic patients with liver damage[41] and to the halothane adduct in liver after halothane anesthesia.[40] Such adducts could be the basis of subsequent immunologic injury. The emergence of drug metabolite-host macromolecular adducts as a pathologic mechanism in many toxicities suggests that the immunologic approaches described here to quantify and localize adducts may be applicable to other problems in toxicology.

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