IMMUNOHISTOCHEMICAL DEMONSTRATION OF CYTOCHROME b_5 AND HEMOPEXIN IN RAT LIVER PARENCHYMAL CELLS USING HORSERADISH PEROXIDASE*

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Received October 23,1974

The membrane-bound hemoprotein, cytochrome b_5, and the serum protein, hemopexin, were demonstrated in rat liver parenchymal cells using an immunohistochemical approach. Other liver cells contained neither protein. The immunological "sandwich" technique employs a complex of horseradish peroxidase with anti-horseradish peroxidase sequentially coupled to other antibodies including those to rat cytochrome b_5 and hemopexin.

The hepatic microsomal mixed-function oxidase system catalizes the hydroxylation and N-demethylation of a wide variety of endogenous and exogenous lipid-soluble compounds (1). The activity of this system depends on the flow of reducing equivalents from reduced pyridine nucleotides to the substrate-oxidase complex. The components of the microsomal electron transport system are cytochrome P-450, cytochrome b_5 and their pyridine nucleotide specific reductases. Reconstitution of a system capable of hydroxylating drugs has been achieved (2-4) by recombining three partly purified components of the microsomal electron transport system, i.e. cytochrome P-450, NADPH-cytochrome c reductase and a lipid fraction. Considerable evidence is available showing that the activity of the cytochrome P-450 system is stimulated by electrons donated by the NADH cytochrome b_5-reductase system (5-9), but the extent

*This work was supported by USPHS Grants #HD-04445 from the Institute of Child Health and Human Development, #HE-08660 from the National Heart Institute, #AM-16737 from the Institute of Arthritis and Metabolic Diseases (UME), and #NP-134 from the American Cancer Society (JO).

to which cytochrome b_5 and its reductase participate in microsomal drug metabolism is still unclear. Nevertheless, the function of cytochrome b_5 and its reductase is firmly established in the oxidative conversion of stearyl-CoA to oleyl-CoA, a reaction requiring both oxygen and NADH (10,11). Recently, using hybrid antibodies labeled with ferritin, Remacle *et al.* (12) demonstrated by electron microscopy that cytochrome b_5 was almost exclusively associated with the smooth endoplasmic reticulum.

Hemopexin binds heme in an equimolar ratio with a Kd $<< 10^{-8}$M (13-15) and is synthesized exclusively by the liver in the human (16), Rhesus monkey (17) and rabbit (17). The hepatocytes are also the site of uptake of this protein during plasma heme clearance (18). Hemopexin is induced by heme (19) and by agents which induce drug-metabolizing proteins (20-22), a finding that may relate to the ability of hemopexin to bind porphyrins (13,14,23), the metabolism of which is related to drug metabolism (24,25).

We report on the localization of cytochrome b_5 in formalin-fixed paraffin-embedded sections of rat liver using an immunohistochemical technique, employing HRP.* Similarly, the serum protein hemopexin is found within hepatocytes.

MATERIALS AND METHODS

Cytochrome b_5 was purified from rat liver by a method described previously (26). The amino acid analysis indicated that this preparation contained *ca.* 150 amino acid residues, and a blocked NH_2-terminus. The hemopexin was purified from rat serum (27). HRP type VI was purchased from Sigma. Antisera were developed in goats unless otherwise stated. The antibody containing fractions (IgG) were isolated by column chromatography with Whatman DE52 (28). The anti-cytochrome b_5 antiserum was absorbed with lyophilized rat serum before chromatography. All antisera showed a single precipitation line by double diffusion in agar over a 100-fold dilution of

*Horseradish peroxidase, HRP; gamma globulin, IgG; phosphate buffered saline, PBS.

antigen and antiserum. HRP was complexed with goat anti-HRP after the IgG of the antiserum was isolated (29). Donkey anti-goat IgG was purchased from Miles Laboratories.

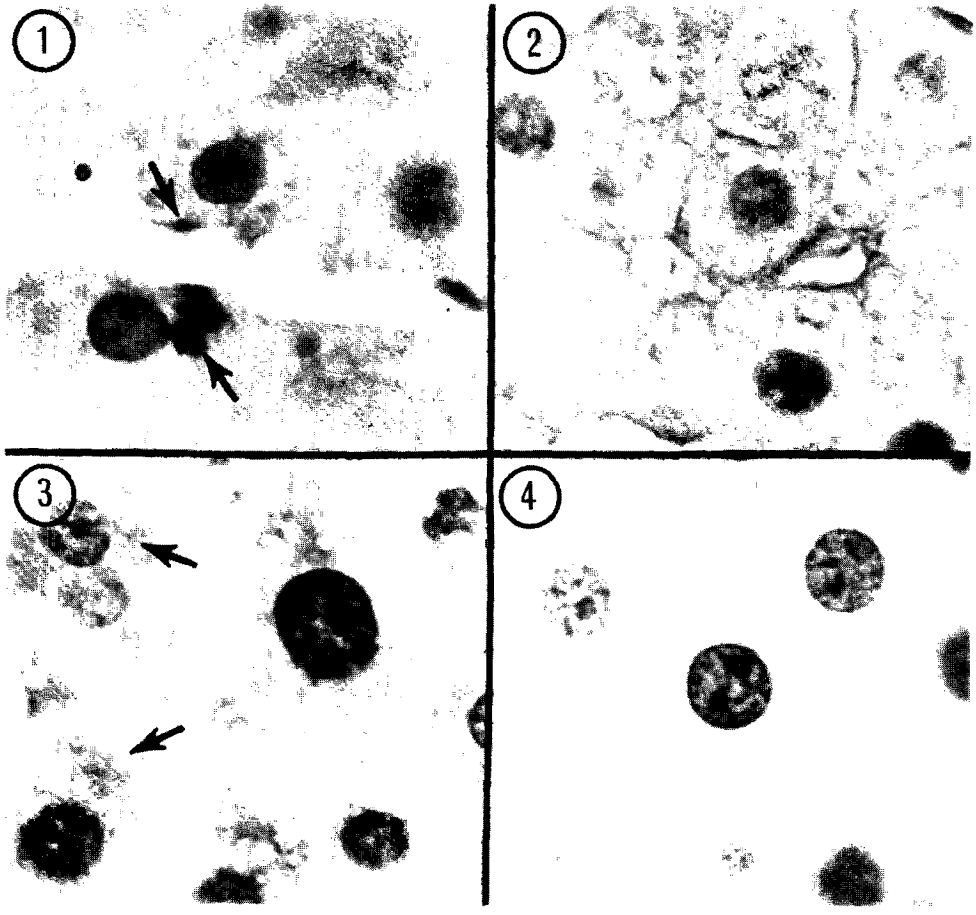
We used Sprague-Dawley rats, 200 to 300 g, whose livers were fixed in 10% buffered formalin for 16 to 24 hours at 4°. Tissue pieces (*ca.* 2 x 2 x 4 mm) were embedded in paraffin and 5μ sections were prepared and mounted on albumin-coated glass slides.** The sections were deparaffinized and treated sequentially with goat anti-rat hemopexin or anti-rat cytochrome b_5, donkey anti-goat IgG and goat anti HRP-HRP complex (29). Each step consisted of a 60 min. incubation at room temperature in a moist box with 1 drop of antiserum per tissue section, followed by a 30 min. wash in PBS at 37°C, and a 5 min. drying period.

Endogenous peroxidase activity was blocked with cold methanol containing 0.01% H_2O_2 for 20 min., followed by a wash with PBS for 10 min. before the addition of the HRP-anti HRP complex (30). Experiments with PBS or normal goat serum in place of the specific antibody proved equally suitable as controls. On completion of the "sandwich" technique, the tissue sections were stained 15 min. for peroxidase (31), washed in H_2O, and counterstained with Mayer's Haemalum. The slides were dehydrated through gradual changes of ethanol to xylene, and were then mounted in a synthetic mounting medium.

RESULTS AND DISCUSSION

Sections of several rat livers were examined by light microscopy for HRP positive areas. Representative tissue areas are shown in the Figure. Both hemopexin and cytochrome b_5 were seen exclusively in hepatocytes (Panels 1 and 3). Kupffer cells and non-parenchymatous liver cells did not stain for HRP. Background staining, obtained for tissue sections treated with PBS or non-immune goat serum was minimal (Panels 2 and 4). Whereas hemopexin molecules formed distinct aggregates, the cytochrome b_5 molecules were

**Performed through courtesy of the Histology Laboratory of the Department of Experimental Pathology, Scripps Clinic and Research Foundation.



Panels 1 and 2: Immunohistochemical demonstration of hemopexin in rat liver. A sinus is seen in Panel 1. Hemopexin is identified in the liver cells by arrows. Panel 2 shows a control section treated identically with exception of omitting the reaction with goat anti-rat hemopexin.

Panels 3 and 4: Immunohistochemical demonstration of cytochrome b_5. Panel 3 shows diffuse staining of cytochrome b_5 (arrows) in the cytoplasm of the hepatocytes. A control section, not treated with goat anti-rat cytochrome b_5, shows no staining in Panel 4. The diffuse staining pattern may reflect the membrane-bound nature of the protein.

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distributed more regularly throughout the hepatocytes. The diffuse staining quality of cytochrome b_5 may be due to its being a membrane-bound protein.

The localization of both cytochrome b_5 and hemopexin in hepatocytes may be of physiological importance. Since both proteins are probably

synthesized in the endoplasmic reticulum, hemopexin may either supply or remove heme from cytochromes such as cytochrome b_5 (32) which depend on the heme moiety for their function.

The immunohistochemical technique for the detection of intracellular proteins in paraffin sections provides an important means for studies concerned with correlating biochemical activity with its localization in normal and pathological tissues. This technique enables us to detect and localize cytochrome b_5 in tissues for which the methodology of preparing microsomes has not been reported. It also permits a comparison of the morphological distribution of cytochrome b_5 and hemopexin in rat liver under various experimental conditions. At present, this method is being employed to demonstrate cytochrome b_5 and hemopexin in paraffin sections of human liver. Since this technique employs paraffin sections, additional microsomal proteins can be localized as their monospecific antibodies are available.

ACKNOWLEDGEMENT

We are deeply grateful for the assistance of Dr. Marie-Jose Sobel, who initiated these experiments.

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