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COMMENTARY

IMMUNOHISTOCHEMISTRY OF DRUG-METABOLIZING ENZYMES

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The mammalian drug-metabolizing enzymes metabolize a wide range of endogenous and xenobiotic compounds [1]. Since this can result in either the activation or the detoxification of chemicals, the responses of the body to, for example, steroid hormones, therapeutic and recreational drugs, environmental and dietary chemicals, carcinogens and other toxic agents can be greatly influenced by the activities of these enzymes [2, 3]. It has long been recognized that the majority of drug-metabolizing enzymes are located primarily, although not exclusively, in the liver, and that the gross metabolic profiles of drugs as measured in blood, urine and feces are largely a consequence of their hepatic metabolism. It is, however, increasingly clear that the anatomical distribution of drug-metabolizing enzymes, both in specific tissues and in specific cells within those tissues, can be a major influence on the tissue- and cell-specificity of chemical toxicity and pharmacological efficacy. The purpose of this commentary is to review how information on the tissue distribution and cell-specific expression (cellular localization) of drug-metabolizing enzymes has been provided by the application of immunohistochemistry. Four groups of Phase 1 and Phase 2 drug-metabolizing enzymes will be mainly considered—the P450s§, EH, GSTs and UDPGTs—reflecting the balance of information in the literature. Enzymes identified immunohistochemically will normally be designated by the name of the protein against which the antibody used was raised. The cytochromes P450 are designated where possible using a recommended sequence-based nomenclature [4]. In examples where the information in the cited references is insufficient to identify individual P450 forms, subfamily designations are used. GSTs, UDPGTs and EH have been named as in the references cited.

Immunohistochemistry can be defined as the antibody-based staining of specific cell and tissue components (antigens) and their localization in cells and tissue sections by microscopy. Immunohistochemistry has the necessary combination of analytical sensitivity, biochemical specificity and spatial resolution to identify individual cells containing specific isoenzymic forms of drug-metabolizing enzymes. The technique is especially valuable in tissues with a low overall content of drug-metabolizing enzymes and for use with small tissue specimens, e.g. needle biopsies, or when the particular cell type that contains the drug-metabolizing enzymes constitutes only a minor proportion of the total cell complement, e.g. certain tumours. In these cases, there will often be insufficient tissue for alternative gross biochemical studies such as immunoblotting or the measurement of drug metabolism activities.

The drug-metabolizing enzymes have been studied extensively by a variety of biochemical and molecular biological techniques using isolated cells or subcellular fractions of whole tissue homogenates. With these techniques, however, it is difficult to obtain information regarding the distribution of the enzymes within tissues and cells. Biochemical studies can provide only average values for enzyme levels amongst all the different cells in a sample and, most importantly, cannot generally show whether an enzyme is present specifically in a particular cell type. In contrast, immunohistochemical studies enable identification of the precise cellular localizations of enzymes, permitting the correlation of drug-metabolizing enzyme expression with tissue morphology and cell-specific pathological changes within a tissue. A major limitation, however, is that whereas biochemical studies can measure enzyme activities, immunohistochemistry can only provide information on enzyme protein, an unknown proportion of which may be inactive. To remedy this, substrates are being developed that will generate insoluble, highly coloured, or fluorescent metabolites, to allow the histological measurement of drug-metabolizing enzyme activities in tissue sections. A further limitation of immunohistochemistry is that it cannot distinguish between different immunologically cross-reactive proteins, whereas biochemically these can often be differentiated according to their molecular weight using immunoblotting. The

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§ Abbreviations: BNF, β-naphthoflavone; P450, cytochrome P450; GST, glutathione *S*-transferase; EH, microsomal epoxide hydrolase; PB, phenobarbitone; and UDPGT, uridine diphosphoglucuronosyl transferase.

overriding advantage of immunohistochemistry, however, is its ability to localize enzymes to individual cells in heterogeneous samples. This is crucial, since all tissues are almost invariably a complex mixture of different cell types, both structurally and functionally. Moreover, structurally identical cells often have different functional capabilities. The application of immunohistochemistry to the study of drug-metabolizing enzymes is, therefore, complementary to biochemical methods and provides specific information that is difficult or impossible to obtain by other analytical techniques.

TECHNICAL CONSIDERATIONS

The successful application of immunohistochemistry to the study of drug-metabolizing enzymes depends on a variety of factors. It is vital that the method of tissue preservation is optimal both for the maintenance of the antigenicity of the different drug-metabolizing enzymes and for the structural preservation of the tissue. Fixation with embedding is superior to cryostat sectioning for the preservation of morphology, but certain antibodies may be ineffective with fixed tissue, as antigens can be variably denatured by fixation. A variety of fixatives has been used in drug-metabolizing enzyme studies, including formaldehyde [5], parabenzoquinone [6], Bouin-Allen solution [7], acetone [8] and methanol [9]. Formaldehyde fixation is reportedly superior to Bouin-Allen's fixative for the discrimination of regional differences in P450 staining in liver [7], while acetone provides a better retention of P450 antigenicity than parabenzoquinone or methanol [9]. The optimum fixative for the detection of a specific antigen often has to be determined empirically, although the introduction of heat-based antigen retrieval methods [10] has allowed antigenicity to be recovered from fixed tissue that previously showed no reactivity. However, in our experience these methods have failed to recover immunoreactivity for specific forms of P450.

It is essential that the antigen specificity of the primary antibody is well characterized. For example, a crude preparation of a polyclonal antibody to the PB-induced P450 form, CYP2B1, stained the livers of both untreated and PB-treated rats, whereas after immunopurification designed to remove antibodies recognizing constitutive and other forms of P450, the same antibody stained only the PB-induced livers [11]. It is also important to know the staining efficacy of the primary antibody for the particular sample being studied. Thus, a monoclonal antibody to the polycyclic aromatic hydrocarbon-induced P450 form, CYP1A1, stained liver from BNF-treated rats at a 1:500 dilution, but stained liver from untreated rats only when used at a higher strength (1:10 dilution) [12]. The concentration of primary antibody used can similarly influence the pattern of staining: when used at low concentration, an antibody to the ethanol-induced P450 form, CYP2E1, gave an exclusively centrilobular staining pattern in livers of ethanol-treated rats and alcoholic humans but no staining with untreated rats or abstinent humans, whereas at 3–6 times higher concentrations the same antibody gave panlobular staining in the alcohol-induced rats and centrilobular staining in the control rats and humans [8].

Various visualization methods have been employed. Immunoenzyme-based methods are the most commonly used detection methods. An unlabelled primary antibody (specific for the drug-metabolizing enzyme) is visualized with an anti-serotype secondary antibody conjugated to an enzyme, usually peroxidase or alkaline phosphatase, and sites of antibody binding are revealed by using a chromogenic substrate (e.g. 3,3′-diaminobenzidine is the most commonly used peroxidase substrate) that produces a coloured, insoluble, final reaction product that can be visualized by conventional bright field microscopy [13]. The PAP (peroxidase-anti-peroxidase) and APAAP (alkaline phosphatase anti-alkaline phosphatase) techniques, which introduce a third antibody amplification step before the chromogenic substrate, can provide higher sensitivity. Other methods used include biotin-streptavidin (an anti-serotype secondary antibody coupled to biotin followed by streptavidin-peroxidase conjugate and peroxidase substrate) [7], immunogold–silver staining (which may be more sensitive than peroxidase- or phosphatase-based methods) [9], indirect fluorescence (an anti-serotype secondary antibody conjugated with a fluorophore) [6], and immunogold electron microscopy [11]. Fluorescent methods offer an alternative to immunoenzyme methods and are particularly useful for double-labelling of different antigens in the same section; they are also essential for analysis using confocal laser microscopy. Quantitative immunohistochemistry of P450 has been reported using immunoperoxidase with image analysis, showing a correlation between immunostained CYP3A protein and CYP3A-dependent *N*-demethylase activity [7], and immunogold electron microscopy [11].

DRUG-METABOLIZING ENZYME DISTRIBUTION IN LIVER

Immunohistochemical studies have been crucial in identifying the cellular localization and regional distribution of drug-metabolizing enzymes within the liver. These studies have also proved valuable in determining the effects of inducing agents on the hepatic distributions of individual forms of drug-metabolizing enzymes. The basic morphological subdivisions of the liver are either the lobule or, much preferably, since it takes into account hepatic blood flow, the acinus, which is considered to be the functional unit of the liver.

*P*450

Immunohistochemical studies of hepatic P450 expression have shown that some forms of P450 are not uniformly expressed throughout the liver acinus, whereas other forms of P450 have a more uniform distribution. However, it is important to remember the effect of antibody concentration on the perceived distribution, as discussed above. Tissue preparation may also influence the apparent distribution of a particular antigen. It is also noticeable that attempts to demonstrate immunohistochemically the induction

of P450 are most successful when the induced P450 form is not expressed constitutively in the same cells.

Immunohistochemical studies of liver sections show that several major hepatic P450 forms are present exclusively in hepatocytes, with no immunoreactivity in Kupffer, sinusoidal, bile duct epithelial or vascular endothelial cells [6, 13, 14]. Within hepatocytes, the pattern of P450 immunoreactivity is cytoplasmic, reflecting the endoplasmic reticulum location of P450, with no immunostaining of the nucleus [6, 13].

In the livers of untreated rats most forms of P450 show a non-uniform distribution, being located predominantly in perivenular hepatocytes (centrilobular cells, or zone 3 of the acinus), e.g. CYP1A2, CYP2A1, CYP2B1, CYP2E1 and CYP3A1 [5–9, 15]. CYP2A1 may be more broadly distributed (perivenular and midzonal) than CYP2B1, CYP2E1 and CYP3A1 (restricted to a layer of 8 cells nearest the central vein) [5]. However, CYP2B1 has also been reported to stain weakly in midzonal cells [16] or even throughout the acinus [6, 9]. In contrast, CYP1A1 is much more uniformly distributed across the hepatic acinus in untreated rats, although still staining most intensely in perivenular hepatocytes [6, 12, 16]. CYP2C, however, has a panacinar distribution in livers of untreated rats [5]. These clear immunohistochemical visualizations of the inducible P450 forms, CYP1A1 and CYP2B1, in non-induced rat liver contrast with their absence or very low levels of staining on immunoblots of non-induced rat liver microsomes. This highlights both a potential strength and a weakness of immunohistochemistry. A protein that is expressed in only a few cells of the liver would, in microsomes prepared from the whole liver, probably be diluted by the proteins of the other cells to below the level of detection of biochemical methods, yet the protein might nevertheless be detected immunohistochemically because each cell is stained individually. However, it is also possible that the immunohistochemical detection of apparently constitutive CYP1A1 and CYP2B1 may be due to the antibodies cross-reacting with abundant constitutive forms. Unfortunately, the valuable check for this eventuality, the determination of the molecular weight of the immunoreactive protein, which is inherent in sodium dodecyl sulfate–polyacrylamide gel electrophoresis with immunoblotting, is not available to immunohistochemistry.

The apparent effects of induction *in vivo* on the distribution of CYP1A1, CYP2B1, CYP2E1 and CYP3A depend on both the dose of inducer administered and the concentration of antibody used for immunohistochemistry. A low dose of inducer and/or a low concentration of antibody shows induction occurring preferentially in perivenular hepatocytes, whereas with high dose induction and/or a high antibody concentration the induction appears in all hepatic zones or even preferentially in acinar zones 1 and 2 (the periportal and midzonal regions, in which case the induction appears as a change from a zonal to a more uniform distribution) [5, 7–9, 11, 12, 15–19]. These differences, and also the discrepancies noted above between immunohistochemistry and immunoblotting, may be due to the antibodies used cross-reacting between CYP1A1 and CYP1A2 or between members of the CYP2A, CYP2B and CYP2C subfamilies.

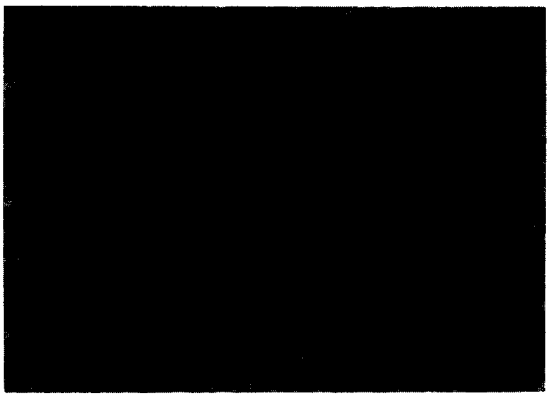

Fig. 1. Immunolocalization of CYP3A in normal human liver. There is a heterogeneous expression of CYP3A within the liver with CYP3A mainly expressed in perivenular hepatocytes. CYP3A immunoreactivity was identified with a monoclonal antibody recognizing all of the human forms of CYP3A. Immunohistochemistry was performed using an immunoperoxidase technique on a formalin-fixed wax-embedded section of liver. Diaminobenzidine was the peroxidase substrate, and the section was counterstained with hematoxylin to allow identification of morphological detail. P = portal tract, and T = terminal hepatic venule.

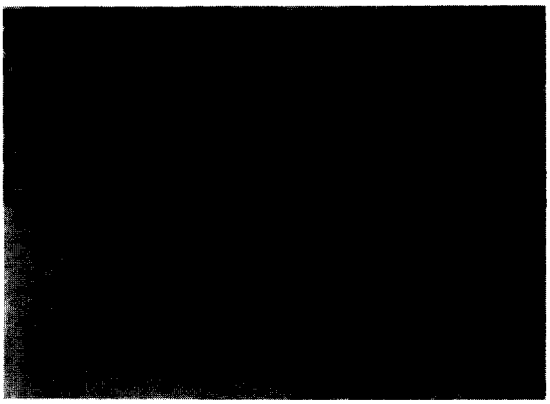

Fig. 2. CYP3A immunohistochemistry in a liver biopsy obtained from a patient who was receiving long-term phenytoin therapy, a known inducer of CYP3A. There is induction of CYP3A expression in periportal hepatocytes, and in contrast to normal liver CYP3A is expressed in all hepatocytes without any zonal variation in P450 expression. The immunohistochemical technique used was identical to that of Fig. 1. P = portal tract, and T = terminal hepatic venule.

In human liver, CYP1A and CYP3A (the latter is shown in Fig. 1) are localized mainly to perivenular hepatocytes, with no immunoreactivity in periportal hepatocytes and variable immunoreactivity in midzonal hepatocytes (CYP3A shows a more

extensive midzonal immunoreactivity than CYP1A) [14, 20]. Similarly, CYP2E1 in human liver has a mainly perivenular distribution and is induced selectively in perivenular hepatocytes by excessive alcohol consumption [8]. In contrast, both CYP2A6 and CYP2C have a uniform distribution of immunoreactivity within the human liver acinus ([21]; Murray GI and Burke MD, unpublished observation). Induction of CYP3A in human liver results in a uniform distribution of CYP3A within the liver acinus (Murray GI and Burke MD, unpublished observation; Fig. 2), indicating that induction of CYP3A occurs especially in periportal hepatocytes.

Immunohistochemistry has also proved to be a valuable technique for assessing the loss of specific forms of P450 and changes in their intra-acinar distribution following the administration of hepatotoxins. This has been demonstrated in liver injury induced by carbon tetrachloride in rats, which was associated with a loss of CYP2A1 selectively in perivenular hepatocytes [22]. The distribution of specific forms of P450 within the liver acinus, measured immunohistochemically, can provide an explanation for the morphological patterns of liver toxicity of xenobiotics, which are activated by P450 metabolism. The zonal pattern of ethanol liver damage in alcoholics may be a consequence of the perivenular distribution of the ethanol-induced P450 form, CYP2E1, and its catalysis of the formation of toxic metabolites such as acetaldehyde and free radicals [8]. Similarly, the pattern of paracetamol hepatotoxicity is probably a consequence of the perivenular distribution of CYP1A2 and CYP2E1, which are responsible for activating the drug [23].

Cell surface localization of P450 and autoimmune liver disease

Immunohistochemical studies based on sections of liver have been unable to address the question of whether P450 is present in the cell surface membrane. However, immunohistochemistry with isolated intact human hepatocytes has identified the presence of several forms of P450 (CYP1A2, CYP2C, CYP2D6, CYP2E1 and CYP3A4) within the cell surface membrane [24]. Moreover, it was shown that CYP1A2, CYP2C and CYP2D6 are located on the external surface of the plasma membrane, where they can act as auto-antigens and may be involved in the mechanism of certain types of drug-induced and autoimmune hepatitis [24]. Others, however, have disputed this, failing to find evidence for CYP2D6 on the external surface of hepatocytes [25].

Fetal liver

The ontogeny of P450 development has been investigated by immunohistochemistry. In fetal rabbit liver, CYP1A1 has a uniform distribution in all hepatocytes, while CYP1A2 is undetectable. CYP1A2 expression appears in late fetal liver, initially confined to a few hepatocytes around the terminal hepatic venules, and by 10 days post-partum shows a uniform distribution throughout the liver acinus [26]. In human fetal liver, both CYP1A and CYP3A7 are distributed uniformly in the acinus, which contrasts with their zonal immunostaining pattern in adults [14, 20]. The transition from the fetal to the adult pattern of immunoreactivity for CYP3A forms in human liver occurs during the first few years of life [21].

EH

The localization and distribution of EH are similar in both rat [27] and human [21, 28] liver, where it is confined to hepatocytes and shows a panacinar distribution.

NADPH–cytochrome P450 reductase

The distribution of NADPH–cytochrome P450 reductase in both rat [16, 29] and human [30] liver has been shown to be zonal with a predominant localization to perivenular hepatocytes, although both midzonal and periportal hepatocytes also express NADPH–cytochrome P450 reductase.

GSTs

In rat liver, GST forms B, C and E are localized to hepatocytes, with slightly more intense immunoreactivity in perivenular hepatocytes [16, 31]. In addition, GST-C and GST-E also display strong immunoreactivity in bile duct epithelium. In human liver, alpha and mu class GSTs are localized in hepatocytes, with the alpha and mu classes showing a uniform and predominantly periportal immunostaining pattern, respectively [32, 33]. Immunoreactivity for both alpha and mu GSTs is present in both the cytoplasm and nuclei of human hepatocytes. In contrast, pi class GST immunostains predominantly in bile ducts and bile ductules, with very little or no immunoreactivity in the cytoplasm of hepatocytes [28, 32]. The microsomal form of GST immunostains in both hepatocytes and biliary epithelium [32, 33]. In mid-trimester human fetal liver, GST-pi immunostains both hepatocytes and bile ducts, whereas at full term it is confined to bile duct epithelium. In contrast, the pattern of GST-alpha immunoreactivity in human fetal liver at different gestational ages is similar to that of adults, being present in all hepatocytes and bile ducts [34].

UDPGTs

Extensive antibody cross-reactivity between different forms of UDPGT presents a particularly difficult problem for the immunohistochemistry of these enzymes. This extensive cross-reactivity has been explained recently by the realization that UDPGT proteins can be formed by alternative gene splicing [35]. In rat liver, the immunoreactivity of *p*-nitrophenol UDPGT is distributed non-uniformly, being strongest in perivenular hepatocytes, whereas 3α- and 17β-hydroxysteroid UDPGT immunostains uniformly across the acinus [36]. Similarly immunohistochemistry with an antibody recognizing UDPGT isoform V shows a panacinar distribution [37]. *p*-Nitrophenol UDPGT immunoreactivity is also present in bile duct epithelium and endothelium of the hepatic artery and portal vein [36]. In human liver, a monoclonal antibody to *p*-nitrophenol UDPGT immunostains all hepatocytes, with no evidence of any zonal distribution [38].

EXTRAHEPATIC TISSUES

Immunohistochemistry is particularly valuable in the study of drug-metabolizing enzymes in extrahepatic tissues, where the enzymes are often confined to a few specific cell types that may be a relatively minor proportion of the total cell population.

P450 in respiratory tract

In the upper respiratory tract of the rat, CYP1A1 is localized to subepithelial glands (Bowman's glands) within the nasal mucosa, with very weak immunoreactivity in the surface respiratory epithelium [39]. CYP2B1 is present in the surface respiratory epithelium, the specialized olfactory epithelium and Bowman's glands [40]. Two forms of P450 that are specifically expressed in the olfactory mucosa, CYP2G1 and an incompletely characterized CYP2A form, have been localized exclusively to Bowman's glands of rat olfactory mucosa [41], while in human nasal mucosa CYP2A10 has been identified in both the surface respiratory epithelium and Bowman's glands [42].

In the lower respiratory tract of the rat, CYP1A, CYP2B and CYP3A immunostain the Clara cells of the bronchial epithelium [9, 11, 43]. Both CYP2B and CYP3A are also present in type 2 alveolar epithelial cells [43]. Similarly, Clara cells throughout the rabbit respiratory tract contain CYP2B and CYP4B [44]. Ultrastructural immunocytochemistry shows that the P450 of Clara cells, whilst predominantly localized to the smooth endoplasmic reticulum, is also in the cell surface membrane, suggesting that P450-mediated metabolism could take place at the air/cell interface [45]. In mouse lung constitutive CYP2B is immunostained in bronchiolar epithelial cells, Clara cells and type 2 alveolar epithelial cells, whereas CYP1A was not detected [46]. In human lung, CYP1A immunostains only in lung tissue from smokers, where it is located in the epithelium of terminal airways and type 2 alveolar epithelial cells, and in some cases vascular endothelial cells also show immunoreactivity [47].

P450 in gut

CYP2B1 [9] and CYP2E1 [48] have been identified constitutively in the absorptive epithelial cells of rat duodenum and jejunum villi, with greater immunoreactivity in jejunum than in duodenum. CYP2E1 immunoreactivity in both duodenum and jejunum is enhanced after induction with ethanol. Ethanol also induces CYP2E1 in the squamous epithelium lining the esophagus, which might help explain the role of alcohol as a risk factor in esophageal cancer [48]. In human small intestine (duodenum, jejunum and ileum), CYP3A has been identified in the columnar absorptive epithelial cells, with a gradient of CYP3A immunoreactivity from the crypts to the villi and the maximum intensity of CYP3A immunostaining occurring in the epithelial cells of the tips of the villi [12]. The identification of a gradient of P450 immunoreactivity indicates the value of immunohistochemistry in determining the cell-specific expression of P450.

P450 in kidney

CYP1A1 [11] has been identified in the proximal tubular cells of rat kidney, whereas CYP2B1 [9] is not detectable. In human kidney CYP3A has been identified in proximal tubular cells (Murray GI and Burke MD, unpublished observation).

P450 in brain

CYP1A1 has been identified in nerve fibers in many regions of rat brain [49], while CYP2E1 has been identified in rat brain oligodendrocytes [50]. The physiological and pharmacological significance of different forms of P450 being localized in different regions and cell types of the brain remains to be established.

P450 in human placenta

The induction of CYP1A in human placenta by smoking has been identified immunohistochemically. CYP1A immunostaining has been detected in the trophoblast cells of placenta from smokers but not non-smokers [51].

EH

The localization of EH in extrahepatic tissues is similar to that of P450. In the rat respiratory tract, EH is present mainly in Clara cells [52], while in kidney EH is present in proximal tubular epithelium [53]. EH immunoreactivity has also been identified in the ductular epithelium of the pancreas [54]. In human extrahepatic tissues, EH has been localized in the epithelium of the breast [55] and colon [56] and in the syncytiotrophoblast of the placenta (Murray GI, unpublished observation).

GSTs

In the rat respiratory tract, the localization of different GSTs is similar to that P450 and EH, with GST-C and GST-E present in Clara cells and ciliated bronchial epithelial cells [52]. In the rat kidney, GST-A is localized to the distal tubules [53], while in the rat pancreas GST-B, GST-C and GST-E are identified in the ductular and acinar epithelium of the exocrine pancreas with no immunoreactivity in the Islets of Langerhans [54]. The distribution of different GSTs has been studied extensively in human tissues by immunohistochemistry. GST-pi and GST-alpha are present in many tissues including all of the major extrahepatic tissues involved in drug metabolism [32, 57–59]; GST-mu, which has a polymorphic expression when expressed, has a localization similar to that of GST-alpha.

Several of the immunohistochemical studies of the different classes of GST have shown nuclear staining as well as cytoplasmic staining. It is not clear if nuclear staining represents a true subcellular site of GST expression or if it is an artifact of tissue preparation for immunohistochemical studies, e.g. diffusion of GST from the cytoplasm to nuclei during tissue fixation. Kantor *et al.* [58] have suggested on the basis of immunoblotting of different subcellular fractions that GST-pi is indeed present within the nucleus. Immunohistochemistry of rapidly frozen, freeze-dried or freeze-substituted tissue, which

prevents diffusion, would also help resolve the issue of putative nuclear localization of GSTs.

UDPGTs

In rats, UDPGT has been identified immunohistochemically in the villus cells of jejunum and in kidney proximal convoluted tubule epithelial cells [37]. In humans, UDPGT immunoreactivity is present in the proximal tubules of the kidney, basal (non-keratinized) layers of the epidermis and epithelial cells of the gastrointestinal tract (with a gradient of increasing staining from the crypt to the tip of the villi) [38]. This distribution of UDPGT in human small intestinal villi mirrors that of P450.

TUMOURS

Immunohistochemistry is an ideal technique for determining the localization and distribution of individual forms of drug-metabolizing enzymes in tumours, because tumour samples are usually complex mixtures of tumour cells, extracellular connective tissue, inflammatory cells, blood vessels and often adjacent normal tissue. Moreover, the tumour cells may include both malignant and non-malignant cells. With such a mixture, biochemical methods of analysis with preparations of whole tumour sample homogenates may be misleading, since the localization of drug-metabolizing enzymes may be completely different in the tumour and non-tumour cells. Another advantage of immunohistochemistry is that tumours, especially in their earliest stages and particularly in patient samples, are often very small, with insufficient tissue for some types of biochemical analysis, including immunoblotting and enzyme activity measurements.

Chemically induced rodent liver tumours have been popular for studying drug-metabolizing enzyme expression in tumours. In the earliest stages of diethylnitrosamine-induced preneoplastic foci (enzyme altered foci) in rat liver, CYP2B immunostaining is slightly increased but CYP1A is unaltered or slightly decreased, while in later stages of neoplastic lesions all forms of P450 are decreased [60]. In contrast, EH, GST-B and GST-C are increased consistently [60]. *p*-Nitrophenol-UDPGT is also increased in preneoplastic foci of rat liver [61]. Immunohistochemical analysis of *N*-ethyl-*N*-hydroxyethylnitrosamine-induced preneoplastic foci in kidney shows increases in CYP1A, CYP2B and GST-A, together with an initial increase followed by a decrease in EH [53].

In the analysis of drug-metabolizing enzymes in human tumours, most attention has been directed towards GSTs, perhaps because these enzymes may act as a mechanism of resistance against anti-cancer drugs [62], and biochemical studies show generally increased GST expression [63]. GST-pi has been shown biochemically to be expressed in a wide variety of tumours and has been suggested as a tumour marker [63]. However, immunohistochemical studies have clearly demonstrated that GST-pi in tumour samples is present in both tumour cells and surrounding non-neoplastic cells, including inflammatory cells and stromal cells [55, 56]. Both CYP1A and CYP3A are present in a significant proportion of hepatocellular [28], breast [55] and colon [56] carcinomas, with immunoreactivity being restricted to the tumour cells. P450 has also been identified by immunohistochemistry in animal xenografts of human colon and breast tumours [64].

MONITORING OF ENVIRONMENTAL POLLUTION

Immunohistochemical studies of the induction of drug-metabolizing enzymes in fish appears to be a particularly useful technique for monitoring environmental pollution. CYP1A1 was examined by immunohistochemistry in liver and extrahepatic tissues of scup which had been exposed to environmental pollutants [65, 66]. There was enhanced CYP1A1 immunoreactivity in the liver and in several extrahepatic tissues of fish obtained from polluted water. Assessment of induction by immunohistochemistry was shown to correlate with measurements of CYP1A1 induction based on its characteristic ethoxyresorufin *O*-deethylase activity.

QUANTITATIVE IMMUNOHISTOCHEMICAL STUDIES

The information gained in immunohistochemical studies of drug-metabolizing enzymes is essentially qualitative. Quantitation of immunohistochemical data, particularly determining the concentration of antigen, is more difficult, especially at the level of an individual cell. Whilst not quantitative, a simple visual assessment of the relative intensity of immunostaining can give a remarkably useful indication of the amount of immunoreactive protein present, provided that no more than a simple assessment scale is used, e.g. strong, weak or absent.

Microdensitometry and microfluorimetry have been used in attempts to quantify drug-metabolizing enzymes in immunohistochemical studies [6, 16, 19]. This requires carefully performed multiple controls [67]. Factors that must be taken into account when quantifying immunohistochemistry include: (a) the effects of tissue fixation on antigenicity; (b) the effects of variations in section thickness on both antibody binding and the staining reaction; (c) the demonstration of a linear relationship between antigen concentration and the intensity of immunostaining over the range of intensities observed; and (d) the distribution of antigen within each cell. Directly labeled primary antibodies should preferably be used to minimize the variations due to second and third antibody binding in indirect techniques. Recently, NADPH–cytochrome P450 reductase has been measured in rat liver using quantitative immunohistochemistry and video image processing [68], and the factors described above were all considered in the analysis so that the data obtained can be considered accurate.

P450 induction

Quantitative, or even qualitative, immunohistochemistry may be an attractive and economical way of measuring P450 induction during the routine animal testing of new pharmaceuticals, since it can be carried out on the liver and other tissue sections that have probably been obtained for routine pathology. The induction of CYP3A by dexa-

methasone has been quantified immunohistochemically in rat liver sections, using peroxidase staining and computer image analysis, and a correlation obtained with microsomal CYP3A N-demethylation activity [7]. A straightforward method for establishing the relative concentrations of immunoreactive protein in different cell types or for assessing the effects of induction is to use the supra-optimal antibody dilution method [69]. A more sophisticated approach is to use electron microscopy: in a well-controlled study of the induction of CYP2B by phenobarbitone, CYP2B in rat liver sections measured by immunogold electron microscopy cytochemistry correlated well with the CYP2B content in microsomes measured by immunoblot analysis [13]. Alternatively, light microscope based image analysis is also an ideal method for performing quantitative immunohistochemistry [68].

HISTOCHEMICAL MEASUREMENT OF ENZYME ACTIVITIES

Immunohistochemistry measures the protein levels of drug-metabolizing enzymes, but this will not necessarily reflect the activities of the enzymes. There have been relatively few successful attempts to measure the localization and distribution of drug-metabolizing enzyme activities in tissue sections. Indeed, the very first published method, utilizing the intense blue fluorescence and low water-solubility of the hydroxylated metabolites of benzo[*a*]pyrene to demonstrate P450-mediated aryl hydrocarbon hydroxylase activity by fluorescence histochemistry [70], has probably not been bettered. Aniline hydroxylation activity has been measured histochemically [71], while the level of P450 holoprotein has been measured in tissue sections by microspectrophotometry of the reduced CO-binding spectrum [72]. We have measured ethoxyresorufin *O*-deethylase activity in non-frozen, non-fixed liver sections [73], but our attempts to localize the activity have been defeated by the rapid diffusion of the metabolite, resorufin, away from its site of formation as a result of its water-solubility.

CONCLUSIONS

Knowledge of the tissue distribution and cellular localization of drug-metabolizing enzymes is important for defining the roles of individual tissues and cells in the metabolism of xenobiotics. The application of immunohistochemistry, by providing information on the cellular and subcellular localization and tissue distribution of individual drug-metabolizing enzymes and isoenzymes, can make a significant contribution to the understanding of tissue- and cell-specific drug toxicity and efficacy. The identification, by immunohistochemistry, of the cell-specific expression of different drug-metabolizing enzymes can also indicate possible areas of study for the regulation of these enzymes. However, immunohistochemistry does not provide any information on the activities of drug-metabolizing enzymes. This is a significant shortcoming, since the activities of these enzymes may not always correlate with their protein levels for a variety of reasons, including the presence of inactive apoprotein forms of holoprotein enzymes such as P450. There is an urgent need to develop specific metabolic probes suitable for the *in situ* detection and localization of drug-metabolizing enzyme activities.

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