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COMMENTARY

MONOCLONAL ANTIBODIES FOR STUDIES ON XENOBIOTIC AND ENDOBIOTIC* METABOLISM

CYTOCHROMES P-450 AS PARADIGM

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Xenobiotics including a vast array of drugs, carcinogens, mutagens, pesticides, and other chemicals which are both man-made and natural products are metabolized by mammalian and lower organisms by different classes of enzyme systems [1–5]. These include the microsomal cytochromes P-450 containing mixed-function oxidases, flavoprotein-linked reductases, esterases catalyzing the hydrolysis of esters and amides, hydrolases catalyzing the hydrolysis of epoxides, and various transferases catalyzing synthetic reactions which conjugate xenobiotic substrates or their metabolites with glucuronic acid, sulfate, glutathione, acetyl CoA or methyl donors [5]. In many cases, the enzymes involved in xenobiotic metabolism may exist in multiple isozymic forms which may direct the flow of substrates into alternate metabolic pathways. The cytochromes P-450 are a paradigm for multi-isozymic systems in which the distribution of isozymes may govern substrate processing into different products and thus into alternate metabolic pathways [3–6]. The cytochromes P-450 containing mixed-function oxidase system is the metabolic interface between xenobiotics and the host organism and is thus the key enzyme system responsible for the primary metabolism of drugs and carcinogens. This multi-isozymic system is also responsible for the metabolism of certain classes of endobiotics which include steroids, fatty acids, bile acids and prostaglandins [4].

Cytochrome P-450 catalyzed metabolism may be either beneficial or hazardous, leading to either detoxified metabolites that may be conjugated and safely excreted or to metabolites that are toxic, mutagenic, or carcinogenic [3–8]. There are at least fifteen to twenty forms of basal and inducible cytochromes P-450 [4, 6]. Although the precise number of forms of cytochromes P-450 is unknown, their multiplicity has been demonstrated by purification, kinetic, genetic, inhibitor and immunological studies [4, 6]. Enzyme multiplicity and considerable overlapping stereo- and regio-selectivity for different substrates and products have greatly limited our understanding of the precise role of individual cytochromes P-450 in the metabolism, activation and detoxification of different cytochrome P-450 substrates. This has, in turn, prevented our understanding the relationship between cytochrome P-450 phenotype in tissues and individuals and their responsiveness and sensitivity to drugs and carcinogens. Our knowledge is also limited on the role of individual cytochromes P-450 in endobiotic metabolism to related physiological variation in individuals. Monoclonal antibodies (MAbs) specific for different individual or classes of cytochromes P-450 are precise and powerful new approaches to these problems.

Monoclonal antibodies

MAbs are produced by the "hybridoma" technology described by Kohler and Milstein [8]. They are pure chemically defined reagents that recognize a single antigenic determinant or epitope (antigenic determinant and epitope are used interchangeably to describe the antigenic site on a macromolecule) [9]. The hybridoma technology grew out of the clonal selection hypothesis of Burnet and colleagues [10] which states that each antibody forming cell or B-lymphocyte and its progeny is committed to the production of a single type of antibody molecule which recognizes and binds to a single antigenic determinant. An animal can produce millions of antibodies in response to the various antigens present in nature. Only a small fraction of β-lymphocytes respond to any given antigen. These replicate and differentiate into antibody secreting cells. Since most antigens have many determinants, the B-cells collectively produce a large number of antibodies recognizing different epitopes on the antigen. Conventional antisera are thus polyclonal and heterogeneous. Furthermore, the polyclonal antibody produced by the same antigen will vary with each immunization. The hybridoma technology [8, 11] is a method in which myeloma tumor cells are fused with the B-lymphocytes of the spleens of mice immunized with a particular antigen. The fused cells or "hybridomas" combine the characteristics of the B-lymphocytes which individually are committed to the production of a specific antibody and the immor-

* We propose the use of "endobiotic" from the Greek meaning "inner" or "from within" to describe endogenous substrates as contrasted to "xenobiotic" for foreign compounds.

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tal property of the myeloma tumor cells. The individual hybridomas are cloned and screened for the production of the desired monoclonal antibody. The individual clone and its progeny produce large amounts of an identical antibody to a single epitope. The selected clone is usually subcloned two to three times to assure the monoclonality of the cell line. These hybridomas can then be grown *in vitro* in a defined medium where they produce the chemically defined monoclonal antibody. The hybridomas can also be injected into the peritoneal cavity of appropriate recipient mice where they grow as ascites tumors and produce large quantities of a specific monoclonal antibody. In our laboratory, the entire procedure of immunization, hybridization, selection, subcloning three times and growth in cell culture or as ascites fluid requires approximately 7 months [12–15]. The MAb producing hybridomas are essentially immortal and can be either frozen and stored or grown in culture indefinitely to produce MAbs. Depending on the use and application, the tissue culture fluid or ascites fluid containing MAbs can be easily purified in a simple procedure to yield highly purified monoclonal antibodies. The monoclonal antibodies can be directed to conformational or sequential epitopes of the antigen protein. Conformational determinants are defined as those depending on the native spatial conformation of the protein while sequential epitopes depend only on the sequence of amino acids. In each case, the antigenic determinants are topographic, i.e. composed of structures on the protein surface. MAbs to different types of epitopes may help elucidate the topography of the antigens and identify sites that are immunodominant, i.e. those to which most of the immune response is directed [16].

Pure monoclonal antibodies from impure antigens (cytochromes P-450): Hybridoma cloning as purification system

Since each B-lymphocyte of mouse spleen is committed to the production of a specific MAb, cloning the hybridoma cells from which the B-lymphocytes are made makes the mouse spleen an instrument for the selection of epitope specific MAbs. The purification of antigens can then be simply accomplished via their binding to specific monoclonal antibodies. Thus, each clone produces MAbs to a single epitope. The epitope can be on a single protein or several different proteins. When these MAbs are coupled to Sepharose columns, the resulting immunoadsorbent specifically binds the epitope containing antigen. This represents a powerful new approach to the purification of proteins and other natural products, particularly those present in low concentrations (see below). This aspect of the hybridoma technology also indicates that completely purified antigen preparations are unnecessary and, in fact, their use may be counterproductive if one is interested in obtaining MAbs to proteins that may be minor deviants of the primary protein in the antigen preparation. Entirely unfractionated preparations, such as solubilized microsomes, may not be useful since the fraction of hybridomas committed to a single epitope of a protein present in low concentration may be very small and an impractically large number of hybridomas may need to be screened to detect and isolate the desired MAb producing hybridoma.

Library of monoclonal antibodies to cytochromes P-450

The properties of cytochromes P-450 make them extraordinarily suitable for investigation with monoclonal antibodies. The cytochromes P-450 are present in multiple forms with some at very low concentrations, their substrate and product specificity are often overlapping, and the levels of any unique cytochrome P-450 are under genetic control and may change in response to environmental, hormonal or nutritional influences. We are developing a library of monoclonal antibodies to different forms of cytochrome P-450. In 1980, we reported the first preparation of MAbs to a cytochrome P-450 [12], and we have since reported on the preparation of additional MAbs to different forms of cytochromes P-450 [13–15]. Since the first report, we and several other laboratories have prepared additional MAbs to cytochromes, P-450 [17–21]. Our library now contains panels of different monoclonal antibodies to seven different forms of cytochrome P-450, and the production of additional MAbs is in process. The MAbs may be of different Ig types. We have isolated MAbs to cytochromes P-450 of the IgG, IgM, IgG_{2a} and IgG_{2b} type [12–15]. These MAbs to cytochromes P-450 can be broadly classified in three categories. First, there are MAbs that bind a specific cytochrome P-450 but do not immunoprecipitate the cytochrome P-450 protein nor inhibit its enzymatic activity. A second category of MAbs binds and immunoprecipitates the cytochrome P-450 but does not inhibit its catalytic activity. A third class of MAbs shows all three activities, i.e. binds, immunoprecipitates, and inhibits the catalytic activity of the cytochrome P-450. Each of these types of MAbs may be useful for specific purposes (see below). It is clear that MAbs in the different classes are directed to different epitopes on the cytochrome P-450 since their binding by the MAb has different functional effects. Within a class, the epitope relatedness of two different MAbs can easily be determined by measuring the ability of one MAb to compete with the second MAb for a binding site on the cytochrome P-450. A total lack of competition indicates unrelatedness of the epitopes on the cytochromes P-450 which the two MAbs target. Different degrees of epitope overlap or interaction between epitopes are indicated by the extent of competition of the MAbs for their target epitopes [20,*].

Monoclonal antibody-based taxonomy of cytochromes P450

Current practice has resulted in a classification of cytochromes P-450 numbered sequentially in the order of isolation, as basal forms present in untreated animals or forms which are induced by treatment of the animal with different inducers such as phenobarbital, methylcholanthrene, pregnenolone-16α-carbonitrile and isosafrole. Migration on sodium dodecyl sulfate (SDS) gels has also been used as

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the basis of a classification system. These systems, however, are somewhat faulty since inducers often may induce more than one form of cytochrome P-450, some of which may also be present in untreated animals. Migration as a criterion for classification is inadequate since different forms of cytochromes P-450 may have similar molecular weights and identical electrophoretic mobility. The specificity of the MAb resides in its precise ability to recognize and bind a specific epitope on the surface of the cytochrome P-450. Thus, a monoclonal antibody recognizes only a single species of cytochrome P-450 if the epitope is unique to that species of P-450. In this case, the MAb would be specific for only a single isozymic form of cytochrome P-450. If the MAb recognizes an epitope that is present in more than one form of cytochrome P-450, that MAb will bind all the forms of cytochrome P-450 containing the epitope. Thus, on theoretical grounds, a comprehensive MAb library may contain MAbs that recognize single unique forms of cytochrome P-450, MAbs that recognize two or more forms, and possibly MAbs that recognize all forms of cytochromes P-450 if the MAb-directed epitope is common to all of them. Thus, a library of MAbs can be used to classify cytochromes P-450 as unique forms or in classes defined by their specific epitope content. We have obtained MAbs that recognize and immunopurify a cytochrome P-450 which migrates as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and other MAbs that recognize more than one species of cytochrome P-450. Many of the MAbs that we have isolated by immunization with purified rat cytochromes P-450 also react with cytochromes P-450 from other species including guinea pig, mouse, hamster and human, which clearly demonstrate the presence of common epitopes in homologous cytochromes P-450 in different species. Thus, the identification of different cytochromes P-450 with a library of MAbs is an alternate classification system for cytochromes P-450. With this taxonomic system the MAbs would classify those cytochromes P-450 that have unique or common epitopes. Such a system possesses the advantage of potentially high resolution, which would be limited only by the volumes of MAbs in the MAb library.

Tissue phenotyping of monoclonal antibody-directed immunoassay

The MAbs, being chemically defined reagents with extraordinarily high specificity for unique epitopes, can be the basis for both qualitative detection and quantitative measurement of epitope defined individual or classes of cytochrome P-450. The type of immunoassay can be tailored to the particular application and can utilize either radiolabeled MAbs or cytochromes P-450 or can be of the ELISA type utilizing as a detection system an appropriate enzyme linked anti-IgG. In addition, we have utilized for radioimmunoassay a ^{35}S- or ^{3}H-labeled monoclonal antibody to IgG. With this detection system one may screen numerous unlabeled MAbs for their binding to cytochromes P-450. In the direct binding method, the unknown proteins or tissue preparations are coated on microtiter wells, incubated with the labeled MAb, and the binding of the MAb is measured. When utilizing unlabeled MAbs, their binding can be measured with an ELISA assay or with a second anti-Ig radiolabeled MAb. Thus, with a large panel of MAbs, the presence in a tissue of specific MAb defined cytochromes P-450 can be detected. We have also developed competitive assays for quantitative measurements of MAb specific cytochromes P-450 in tissue samples [22, 23]. These assays have utilized either purified cytochromes P-450 or standardized preparations of microsomes containing the desired cytochromes P-450 for coatings on the microtiter plates. The labeled MAbs are incubated with different amounts of tissue containing unknown amounts of the MAb defined cytochrome P-450. The cytochromes P-450 in the unknown sample effectively compete for and titrate the MAb, thereby reducing the amount of MAb that binds to the coated plates. The amount of inhibition of binding is a measure of the content of the MAb specific cytochrome P-450 in the unknown samples. There have been developed a variety of immunoassays, and the appropriate assay can be chosen for the individual problem being investigated. With these MAb-directed immunoassays, a tissue, organ, or individual can be phenotyped for MAb defined cytochrome P-450 content.

We have examined MAb specific cytochrome P-450 content in a variety of rat and mouse tissues, utilizing MAbs that are specific for different single or classes of cytochromes P-450. An example of this approach can be illustrated with the use of MAb 1-7-1 which recognizes two forms of a 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450 and of MAb 1-31-2 which recognizes only one of the two forms. We have analyzed the binding inhibition curves for different tissues with MAb 1-7-1. The amount of tissue required for a 50% inhibition, i.e. the I_{50} (μg), of MAb 1-7-1 binding is as follows: rat liver, 0.042; $C_{57}Bl/6$ mouse liver, 0.050; guinea pig liver, 0.21; DBA/2 mouse liver, 0.23; hamster liver, 0.78; rat lung, 1.02; and rat kidney, 1.04. Thus, with this assay, as little as 0.04 μg of liver microsomes from MC-treated rats gave a 50% inhibition. The greater levels of rat kidney and lung required for inhibition indicate that they have much less of the MAb 1-7-1 specific cytochrome P-450. When using MAb 1-31-2, which recognizes only one of the two cytochromes P-450 recognized by MAb 1-7-1, lower levels of cytochrome P-450 were detected. The livers of DBA/2 mice, hamster liver and rat lung did not contain detectable amounts of cytochromes P-450 with the MAb 1-31-2 specific epitope.

Phenotyping of a tissue for specific cytochromes P-450 has not been possible with ordinary methodologies. Polyclonal antibodies are not sufficiently specific, and phenotyping by measuring the enzyme activity of a tissue does not distinguish between the multiple forms of cytochrome P-450 that may contribute to total tissue enzyme activity. Further, enzyme based assays require relatively large amounts of tissue and are quite limited in the number of samples that can reasonably be assayed. The MAb-directed immunoassay permits the easy assay of several hundred samples in a day by a single technician. Another important factor supporting the use of the

MAb-based immunoassay is that the stability of the epitope available for MAb binding is likely to be far greater than that of the cytochrome P-450 dependent enzyme activity which is often quite labile. We have compared the stability of the MAb-sensitive epitope in rat liver using the MAb binding assay, with the stability of cytochrome P-450 dependent aryl hydrocarbon hydroxylase (AHH) activity. We find that prolonged incubation at 0° or 21° greatly reduces the AHH activity, whereas there is virtually no diminution in the immunoassay detected binding of the MAb. Thus, the MAb-based immunoassay, being sensitive, precise for individual and classes of cytochrome P-450 and sufficiently stable to differences in tissue processing, appears to be an ideal assay for population or field studies in biochemical epidemiology. The goal of such studies is to understand the relationship of cytochrome P-450 phenotype to drug and carcinogen sensitivity and to detect polymorphisms of the cytochromes P-450 in the human population.

Monoclonal antibody-directed immunopurification of cytochromes P-450

A major goal toward a full understanding of cytochrome P-450 structure and function requires the isolation and purification of the multiple forms of cytochrome P-450. The classical procedures are tedious and often laborious. Although successful purification of cytochromes P-450 have been reported from many laboratories [4, 6], the task has been generally slow and difficult and often results in low yields of the cytochrome P-450. This has been especially true for closely related and minor forms of cytochromes P-450 and those present at low levels in extrahepatic tissues such as lung, kidney, intestine, and pancreas, and in low availability human tissues such as placenta and lymphocytes. In extrahepatic tissues, there have been only rare reports of successful purification of cytochromes P-450 [4, 6].

The MAbs offer extraordinary promise for the purification of the multiple forms of cytochrome P-450. MAbs covalently linked to Sepharose are useful immunosorbents for the purification of individual and classes of cytochromes P-450 [23–25]. The MAb-based purification is rapid, precise and far more efficient than conventional purification. In a simple one-step procedure, solubilized microsomes or other tissue preparations containing cytochromes P-450 are incubated with the Sepharose-MAb. Nonspecific and weakly binding materials are removed by extensive washing. This can be done with the Sepharose-MAb used as a column, batchwise washing, or by filtration washing. After thorough removal of the weakly absorbed material, the MAb-bound cytochrome(s) P-450 is eluted with appropriate eluants. The nature of the eluant depends on the tightness of the Sepharose-MAb to cytochrome P-450 binding. We have successfully used two MAbs made to an MC-induced rat liver cytochrome P-450, MAb 1-7-1 and MAb 1-31-2 [23], and an MAb made to a phenobarbital (PB)-induced rat liver cytochrome P-450, MAb 2-66-3 [25]. The MAb 1-7-1 recognizes two forms of MC-induced cytochrome P-450, while the MAb 1-31-2 recognizes only one of these two forms. Using these two Sepharose-linked MAbs either individually or in tandem, eight cytochromes P-450 have been isolated by a simple one- or two-step immunopurification [23]. These cytochromes P-450 were isolated from the livers of MC-treated rats, C_{57}Bl mice, DBA/2 mice, guinea pigs, and hamsters and from rat lung. The apparent molecular weight of these forms ranges between 53K and 57K. The Sepharose-MAb 1-7-1 was bound to two forms of cytochrome P-450 from rat liver (56K and 57K) and two forms from C_{57}Bl mouse liver (56 and 57K). The Sepharose-MAb 1-7-1 also binds a single cytochrome P-450 in rat lung (57K), DBA mouse liver (57K), hamster liver (57K) and guinea pig liver (53K). The Sepharose-MAb 1-31-2 binds only the 57K form in rat liver, C_{57}Bl mouse liver, and rat lung. Immunopurification with Sepharose-MAbs has also been accomplished for rabbit cytochrome P-450 form 1 [19]. In this study three Sepharose-MAbs immunoadsorbed cytochromes P-450 form 1; one of them also immunoadsorbed cytochrome P-450 3b which indicates that these two cytochromes P-450 possess a common MAb recognized epitope. The individual cytochromes P-450 eluted from MAb-Sepharose columns are of high purity [23]. They migrate as a single band on SDS-PAGE gels, and they can be analyzed for structural features such as amino acid content, N-terminal amino acid sequences, and peptide mapping. We have used the simple Sepharose-MAb immunopurification to obtain two forms of cytochrome P-450 from rat liver (56K and 57K), two forms from C_{57}Bl mice (56K and 57K), one form from DBA/2 mice (57K), and one form from guinea pigs (53K). We have been able to successfully sequence the N-terminal amino acids and obtain peptide maps of each of these forms and compare their structural relationship [23, 26]. MAbs isolated by Sepharose-MAb purification are thus quite pure and suitable for primary structure analysis. Thus, with the appropriate Sepharose-MAbs, individual or classes of cytochromes P-450 are easily and rapidly obtained from virtually any cytochrome P-450 containing source and can be easily applied to a phylogenetic study of cytochromes P-450. The technique can also be modified for rapid phenotyping of tissues in conjunction with immunoassays for individual or classes of cytochromes P-450.

Immunoaffinity purified cytochromes P-450 have not been enzymatically characterized since the current techniques use conditions for elution that inactivate enzyme activity. An important goal that is likely to be accomplished soon is the development of conditions of elution that do not inactivate the cytochrome P-450 enzymatic activity. Another useful approach utilizes non-enzyme inhibiting MAbs. When these MAbs are covalently linked to Sepharose, the Sepharose-MAb binds to cytochromes P-450 to form complexes which, when reconstituted with cofactors and cytochrome P-450 reductase, will exhibit cytochrome P-450 enzyme activity [18]. This technique has promise for the easy isolation and use of specific cytochromes P-450 for the production of specific metabolites of xenobiotics or endobiotics that are formed by cytochromes P-450 catalyzed reactions.

Monoclonal antibody-based "reaction phenotyping"

The cytochromes P-450 collectively catalyze the oxidation of literally thousands of xenobiotic and endobiotic substrates including a large variety of different classes of chemicals. One of the essential questions concerning cytochrome P-450 function relates to our ability to measure the contribution of unique forms or classes of cytochromes P-450 to the total metabolism of an individual substrate in different tissues and individuals. Available methods do not permit such a measurement, and current understanding of the substrate and product specificity of unique forms of cytochrome P-450 is derived only after prior purification and isolation of the unique cytochrome P-450. The purified cytochrome P-450 is then reconstituted with cofactors and enzymes of the mixed-function oxidase complex, and its substrate and product specificity is measured. The latter methods have yielded useful information on cytochrome P-450 specificity but do not add much to our understanding of the contribution of the particular cytochrome P-450 to total tissue metabolism. This approach also has severe limitations imposed by the requirement for purification of the cytochrome P-450, a difficult task especially for the minor forms and those in extrahepatic tissues. Furthermore, the reconstituted system may not adequately reflect the *in situ* activity of the cytochromes P-450 located in the endoplasmic reticulum. Monoclonal antibodies that completely inhibit the enzyme activity of the epitope containing cytochrome P-450 to which they bind are extraordinarily useful for "reaction phenotyping". "Reaction phenotyping" is the identification and quantification of the contribution of individual or classes of cytochromes P-450 to the total metabolism of a specific cytochrome P-450 substrate in a tissue. The inhibitory MAb is added to the tissue preparation at saturating levels, and the amount of inhibition of the reaction is a measure of the minimum contribution of the MAb-sensitive cytochrome P-450 to the total reaction of the tissue. This reflects only the minimum contribution of the sensitive cytochromes P-450 and not always the maximum. This is the case since in some instances the MAb-sensitive epitope in the cytochrome P-450 may be bound or inserted in the membrane structure and unavailable to the MAb. If this is the case, a solubilization of the microsomes is required prior to MAb-inhibition measurements. After solubilization, the inhibition by the MAb measures the total contribution of the MAb-sensitive cytochrome P-450 to the total reaction.

"Reaction phenotyping" of cytochromes P-450 can be applied to analysis of a variety of reactions which may be direct or indirect results of cytochrome P-450 activity. These include: (1) substrate disappearance, (2) substrate and product specificity with respect to the formation of different metabolites, and (3) indirect action phenomena which are a result of cytochrome P-450 catalyzed formation of reactive metabolites. The formation of these reactive metabolites may result in (a) metabolite binding to micromolecules such as protein and DNA, (b) cell toxicity, (c) mutagenicity, and (d) carcinogenicity. The addition of an inhibitory MAb to any of the above assay systems will in each case qualitatively and quantitatively define the contribution of the MAb epitope specific cytochrome P-450 to the total activity measured.

Reaction phenotyping for substrate specificity

This technique has been used to reaction phenotype the contribution of MAb specific cytochromes P-450 to various reactions [27–30]. The distribution of cytochromes P-450 that catalyze AHH and 7-ethoxycoumarin *O*-deethylase (ECD) were studied with MAB 1-7-1 which completely inhibits these activities of a purified 3-methylcholanthrene-induced rat liver cytochrome P-450 [14]. The degree of inhibition by MAb 1-7-1 quantitatively assesses the contribution of antigenically defined cytochromes P-450 in the liver, lung, and kidney microsomes from untreated, MC- and PB-treated rats, mice, guinea pigs, and hamsters [28]. Enzyme sensitivity to MAb 1-7-1 inhibition defines two types of cytochrome P-450 contributing to AHH and ECD. The MAb 1-7-1 sensitive cytochrome P-450 is a major contributor to AHH in rat liver, lung, and kidney of MC-treated rats, $C_{57}Bl/6$ mice, guinea, pigs, and hamsters; this type is also present in lesser amounts in the extrahepatic tissues of the control and PB-treated animals, and in the lungs of relatively "noninducible" DBA/2 mice treated with MC. This form, however, makes little or no contribution to liver aryl hydrocarbon hydroxylase of control or PB-treated animals. ECD is also a function of both the MAb 1-7-1 sensitive and insensitive classes of cytochrome P-450. The ratio of the classes contributing to AHH and ECD differs in the various tissues and species and after inducer treatment.

Liver AHH from MC-induced rats and $C_{57}Bl/6$ mice was inhibited by 75 and 88%, respectively, indicating that MAb 1-7-1 sensitive cytochromes P-450 contribute 75 and 88% of the total tissue activity. With ECD, however, the results were different. In the same tissues the inhibition of ECD was 75 and 40% respectively. Thus, in C57 mice, 60% of the ECD activity is contributed by cytochromes P-450 other than the MAb 1-7-1 sensitive type. In related experiments we found that, in the livers of control and PB-treated rats and $C_{57}Bl/6$ mice, AHH was unaffected by MAb 1-7-1 while ECD was inhibited by only 10–15%. This indicated that in these tissues virtually all of the AHH and 85–90% of the ECD were contributed by an MAb 1-7-1 insensitive form of P-450. Furthermore, in hamster and guinea pig none of the ECD was sensitive to MAb 1-7-1. In DBA/2 mice, although the AHH was induced 5-fold by MC, it was insensitive to MAb 1-7-1, indicating that this induced activity of AHH is the result of an AHH active cytochrome P-450 that is different than the highly induced AHH active cytochrome P-450 in MC-treated $C_{57}Bl/6$ mice. This technique was also applied successfully to measure the contribution of MAb-specific cytochromes P-450 to the metabolism of an endobiotic substrate. In one study, the inhibitory effect of an MAb to rabbit liver cytochrome P-450 3b was measured on the microsomal 16α- and 6β-hydroxylation of progesterone [30]. In this case, 40–70% of the 16α-hydroxylase was inhibited. The same MAb did not inhibit 6β-hydroxylation in microsomes

from III VO/J rabbits but did inhibit similar preparations from New Zealand white rabbits. The MAb also inhibited the increased 16α-hydroxylase activity of III/VO7 microsomes that is observed in the presence of an allosteric effector of the variant form of the cytochrome P-450 3B. Thus, the use of an MAb provides useful information relating the isolated forms of cytochromes P-450 to their contribution to the reactivity of the whole tissue.

Reaction phenotyping for product specificity

The MAbs can be used to identify the contribution of MAb-specific cytochromes P-450 to the formation of different metabolites formed from the same substrate. Thus, the formation of the 9-OH metabolite from acetylaminofluorene by a purified cytochrome P-450 is entirely insensitive to inhibition by an MAb that inhibits the formation of five other metabolites by 50–70% [31]. With microsomes, the 9-OH formation is not inhibited whereas 5-OH formation is inhibited by more than 95%, suggesting that all of the latter activity is due to an MAb-sensitive cytochrome P-450. Similar type studies have been performed with the cytochrome P-450 catalyzed metabolism of benzo[*a*]pyrene. In these studies with microsomes, MAb 1-7-1 (MC-P-450) inhibited the formation of nine of the ten metabolites by 50–100%, whereas the formation of the 1-6 quinone metabolite was stimulated 3-fold in the presence of MAb 1-7-1 [14]. These studies indicate that this metabolite is likely formed through a mechanism not dependent on the MAb 1-7-1 sensitive cytochrome P-450 which is responsible for the formation of the other benzo[*a*] pyrene metabolites.

Reaction phenotyping of human tissues for cytochrome P-450 catalyzed reactions

The reaction phenotyping of human tissues can be achieved by MAbs that are raised to either cytochromes P-450 from humans or MAbs raised to cytochromes P-450 from animal tissues that cross-react with cytochromes P-450 from human tissue. For use in reaction phenotyping of human tissues, the MAbs must be of the enzyme-inhibiting type. Phenotyping by immunoassay can utilize all types of MAbs. We have obtained several hybridomas that produce MAbs that recognize polycyclic hydrocarbon-induced cytochromes P-450 in rat liver that have a common epitope with cytochromes P-450 that are present in several mammalian species including humans [27–29]. These MAbs were used to reaction phenotype several cytochrome P-450 catalyzed activities in different human tissues.

Cytochrome P-450 dependent AHH and ECD in human tissues were differentially inhibited by MAbs that were prepared and completely inhibit the activity of a 3-methylcholanthrene-induced rat liver cytochrome P-450. The AHH and ECD of placentas from individual women who smoked cigarettes were inhibited by the MAbs by 83–90% and 34–74% respectively [27, 29]. Benzo[*a*]anthracene (BaA)-induced AHH and ECD in peripheral lymphocytes from humans were inhibited 18–65% and 30–78% respectively [27]. The enzymes in both control and BaA-induced human cells in culture were inhibited to different extents. Both the AHH and the ECD in control and BaA-induced monocytes and in normal liver were largely unaffected by the MAb [27]. Thus, with human tissue, the MAbs have: (i) identified cytochromes P-450 with a common antigenic site in placenta, lymphocytes, and human cells in culture; (ii) identified two forms of hydrocarbon-induced cytochromes P-450 in human lymphocytes, at least one of which has a common epitope with a cytochrome P-450 of placenta and with a cytochrome P-450 form present in uninduced lymphocytes; (iii) identified two forms of cytochrome P-450 responsible for ECD activity in placenta, one of which is also responsible for AHH activity; (iv) shown that the cytochromes P-450 of liver, basal, and BaA-induced monocytes are different from the MAb-sensitive cytochromes P-450 of placenta and lymphocytes; and (v) quantitated in several human tissues the percentages of control and inducible AHH and ECD that are dependent on the MAb-sensitive P-450. The results obtained with the MAb analysis of human tissue demonstrate the value of MAbs for defining antigenic site relatedness for different enzymatic functions of cytochromes P-450 and for identifying and quantifying the amount of a specific enzyme activity in a tissue dependent on specific cytochrome P-450. The study with human tissues may be a prototype for the use of MAbs for phenotyping and mapping of cytochromes P-450 responsible for specific metabolic reactions in humans and thus may be useful in determining the relationship of cytochrome P-450 phenotype to individual differences in drug metabolism and carcinogen susceptibility.

Population studies of cytochromes P-450 with monoclonal antibodies

The MAbs can be useful for population studies such as required for "biochemical or molecular epidemiology" [32], identifying differences in ethnic populations [33] or pharmacoanthropology [34] or predictive identification of hypersusceptibile individuals [35]. The MAbs add another dimension of analyses to the usual enzymatic assay for cytochrome P-450 catalyzed reactions. The MAbs add the dimension of quantitating the contribution of immunochemically defined forms of cytochromes P-450 to the total reaction. A model study for this type of analysis has been performed with placenta tissue from single and multiple births from women who smoked cigarettes and from non-smokers. These were examined for cytochromes P-450 dependent AHH and ECD and their inhibition by MAb 1-7-1 [29]. The MAb 1-7-1 inhibited the smoking-induced AHH activity of essentially the entire population of placentas by 80–95%. Thus, up to 95% of the AHH in a population of human placentas is catalyzed by a type of cytochrome P-450 that contains an antigenic site recognized by MAb 1-7-1. A second type of cytochrome P-450, which is insensitive to MAb 1-7-1, is responsible for some of the ECD activity in the placentas of nonsmokers. In the placentas from smokers, both types of cytochromes P-450 contribute to ECD activity. Their ratios can be determined by the amount of inhibition by MAb 1-7-1 which ranges from 0 to 70%. The placentas from both dichorionic dizygotic and dichorionic monozygotic twins show extraordinarily high intrapair concordance for both

the absolute amounts of AHH and ECD and their inhibition by MAb 1-7-1 compared with unrelated individuals, indicating that interindividual differences in these parameters of biological activity are not due to random variation or experimental error. These results show that the amount of activity of antigenically unique types of cytochrome P-450 responsible for different drug and carcinogen reactions can be measured in different individuals by the amount of their inhibition by highly specific monoclonal antibodies [27, 29].

Genetic studies utilizing MAbs

The entire range of MAb properties including their usefulness for phenotyping of tissue by direct qualitative or competitive quantitative immunoassay, immunopurification and reaction phenotyping can be utilized for the genetic analysis of cytochrome P-450 regulation and expression. Several studies have utilized the MAbs for the analysis of cytochrome P-450 activity in different strains of mice. In one study, MAb-directed analyses were made of the AHH and ECD activities in the liver, lung, and kidney of control, phenobarbital-treated and methylcholanthrene-treated $C_{57}Bl/6$ and DBA/2 mice. The enzymes inhibiting MAb 1-7-1 (to MC-P-450) and MAb 2-66-3 (to PB-P-450) were able to determine the contribution of the sensitive cytochromes P-450 with the MAb-specific epitope and cytochrome P-450 lacking this epitope to the total tissue enzyme activity. An interesting finding was that 56% of the lung but none of the liver AHH of MC-treated DBA/2 mice was dependent on the MAb-sensitive cytochromes P-450 induced in the MC-inducible $C_{57}Bl/6$ mouse strain [28]. In other genetic studies with $C_{57}Bl/6$ and DBA/2 mice, measurements were made of the contribution of epitope specific cytochromes P-450 recognized by MAb 1-7-1 (MC-P-450) and MAb 2-66-3 (PB-P-450). In this study, the MAbs were used to reaction phenotype the AHH, ECD, ethoxyresorufin *O*-deethylase, aminopyrine demethylase and testosterone 6β-, 7α- and 16α-hydroxylation in control mice and those treated with 3-methylcholanthrene, phenobarbital and pregnenolone-16α-carbonitrile (PCN). This study was able to successfully measure the genetic differences in the cytochromes P-450 of the two strains of mice for each of the above reactions.*

Cytochrome P-450 catalyzed protein and DNA adduct formation, toxicity, mutagenicity, and carcinogenicity

Microsomes and appropriate cofactors incubated with xenobiotics result in the conversion of the xenobiotics to metabolites that are reactive and that may covalently bind to proteins [36, 37] or DNA [38, 39]. This *in vitro* formation of adducts to macromolecules such as protein or DNA may reflect the mechanisms engaged in *in vivo* formation of xenobiotic covalent binding to protein or DNA. These reactions are believed to be responsible for xenobiotic-induced toxicity [37], mutagenicity, and carcinogenicity [40, 41]. The formation of benzo(*a*)pyrene(BP)-DNA adducts [38, 39] may be a paradigm for the numerous adducts of xenobiotic to protein or DNA formed *in vivo*. The MAbs being specifically inhibitory to the activity of individual or classes of cytochromes P-450 will be useful in defining the role of the specific cytochromes P-450 for the formation of individual adducts. Thus, the MAbs may be useful in defining the role of specific cytochromes P-450 to the toxic, mutagenic, or carcinogenic activity of individual xenobiotics.

The microsome-catalyzed activation of benzo[*a*] pyrene to metabolites binding to DNA [38, 39] is the system used for the activation of xenobiotics to their mutagenic forms as tested in the Ames mutagen detection system [40, 41]. A large preponderance of all known mutagens requires metabolic activation [40, 41]. In most cases, the activation system is essentially a result of cytochrome P-450 catalysis. Thus, the cytochromes P-450 are the primary biological agents of mutagen activation. Currently, the role of the individual cytochromes P-450 in the activation of the numerous mutagenic agents is virtually unknown. A library of MAbs that inhibit individual and classes of cytochromes P-450 may be useful for defining the role of each type of cytochrome P-450 in the activation of specific mutagens. MAbs have been successfully used for this purpose.* This study examined the effects of specific inhibitory MAbs to a PB-induced P-450 and an MC-induced P-450 on mutagen activation by liver microsomes from $C_{57}Bl/6$ and DBA/2 mice. The study defined the role of the MAb-specific cytochromes P-450 for the activation of acetylaminofluorene (AAF), aflatoxin, *N*-nitrosomorpholine and benzopyrene-7,8-diol to mutagenic metabolites in control, MC-, PB-, and PCN-treated mice. Each MAb defined form of cytochrome P-450 contributed to the mutagenicity of aflatoxin, AAF and BP-diol to different extents in the livers from the differently pretreated rats. In the case of *N*-nitrosomorpholine, there was a large stimulation of mutagenic activity indicating that the MAb-sensitive cytochrome P-450 was likely involved in *N*-nitrosomorpholine detoxification. The inhibition of this cytochrome P-450 by the MAb resulted in the *N*-nitrosomorpholine being more available to those other cytochromes P-450s engaged in its activation to mutagenic forms.

Immunocytochemical localization of cytochromes P-450 in tissues and cells

Immunochemical studies utilizing polyclonal antibodies have localized the distribution of certain cytochromes P-450 in liver and other rat tissues [42, 43]. The use of MAbs offers a far more precise tool for localizing specific epitope containing individual and classes of cytochromes P-450 among various tissues and within the different organelles of the cell. For example, there have been reports on the presence of cytochromes P-450 in the nuclear components [44] and in the mitochondria [45] of hepatocytes. Some concerns about such reports relate to the possibility of contamination of the preparation with small amounts of smooth endoplasmic reticulum (SER) or rough endoplasmic reticulum (RER) during the cell fractionation procedures. MAb-directed immuno-

* F. Heitanen, C. Malaveille, J. B. Bereziat, G. Brun, S. S. Park, H. V. Gelboin and H. Bartsch, manuscript submitted for publication.

fluorescence with electron microscopy obviates these problems. We have utilized MAbs, Protein A-gold conjugates and electron microscopy to localize the epitope containing cytochromes P-450 that are bound by monoclonal antibody MAb 1-7-1.* In this study, the cytochrome P-450 was clearly localized in the nuclear envelope as well as in both the RER and SER. The MAb-specific cytochrome P-450, however, was not found in the mitochondria of hepatocytes.

MAb-directed detection of cytochromes P-450 expressed in vivo *during differentiation, different nutritional conditions, and in cell culture*

The MAbs will prove useful for identifying forms of cytochromes P-450 that are under regulational control by different nutritional and hormonal conditions, during developmental stages, and after inducer exposure. For this purpose, a variety of MAb-based methods can be used: histological by localizing specific cytochromes P-450 by light or electron microscopy linked MAb-directed immunofluorescence, by qualitative or quantitative phenotyping by immunoassay, or by reaction phenotyping utilizing enzyme inhibitory MAbs. MAb-directed studies utilizing immunofluorescence and microscopy have been used successfully for determining the developmental appearance and disappearance of specific enzymes and antigens during fetal development [46] and may similarly be applied to cytochrome P-450 studies.

Enzyme inhibitory MAbs have been used to phenotype the effect of dietary components on the expression of MAb 1-7-1 sensitive cytochromes P-450 active for ECD and AHH in rat intestine. In this study, the appreciable ECD and AHH activities in the small intestine of rats fed cholesterol high diets were found due to an MAb 1-7-1 sensitive cytochrome P-450 [47]. This cytochrome P-450 was found in the small intestine of control and PB-treated rats as well as in MC-treated rats. In contrast, the livers of the same animals showed sensitivity to MAb 1-7-1 inhibition of AHH and ECD only when they were pretreated with MC and not in control or PB-treated rats. Thus, the MAb 1-7-1 detected a cytochrome P-450 in intestine with a common epitope to the MC-induced liver cytochrome P-450 and identified cytochromes P-450 with common epitopes whose presence are diet sensitive.

MAbs have also been used successfully to identify the specific type of cytochrome P-450 that is expressed in various cells grown *in vitro* and exposed to different types of inducers. In one study [48], MAb 1-7-1 (MC-P-450) and MAb 2-66-3 (PB-P-450) were used to study the cytochromes P-450s expressed in nine differentiated or de-differentiated cell lines developed from rat hepatoma cells. The cells were reaction phenotyped for cytochromes P-450 responsible for aldrin epoxidase, ethoxycoumarin deethylase, and aryl hydrocarbon hydroxylase. This analysis showed the extent of expression of the MAb-sensitive cytochromes P-450 in the different cells. Both classes of cytochromes P-450, those induced by MC as well as those induced by PB, were expressed to different extents in the different mouse cell lines.

Structure and function of cytochromes P-450

MAbs have been powerful tools for the study of the antigenic structure of proteins. Four of the most intensively studied proteins have been myoglobin, lysozyme, cytochrome *c* and serum albumin [16]. The MAbs have been useful in elucidating the topography of the antigens, characterizing immunodominant areas, and their native conformation. The immunopurified cytochromes P-450 are suitable for studies by contemporary methodology for the determination of protein structure [49,†]. We have shown (see above) that MAb immunopurified cytochromes P-450 were suitable for N-terminal sequencing and peptide mapping [23, 25, 26, 49,‡]. MAbs exhibiting specific interactions with epitopes on the cytochrome P-450 may also be quite useful in a variety of physical-chemical studies on the mechanism of catalytic action, and for characterizing the binding sites for substrates, cofactors or metabolically linked enzymes such as NADPH-cytochrome P-450 reductase or cytochrome b_5. The MAbs may also be useful for determining the topography of cytochromes P-450 within the microsomal membranes.

Cloning and regulation of cytochrome P-450 genes and cytochrome P-450 peptide processing

We have used MAbs to individual and classes of cytochrome P-450 to identify epitope-specific polypeptides formed by the translation of MC- and PB-induced rat liver mRNAs [50,*]. The MAbs can be powerful tools for cloning of individual cytochromes P-450. MAb-based immunopurification procedures can be used to purify cytochromes P-450 which then can be N-terminal amino acid sequenced and compared to those derived from the analysis of nucleotide sequences of cloned cytochrome P-450 genes. Furthermore, a comparison of sequences generated by the two techniques and of those polypeptides formed as translation products of mRNAs can be very useful in analyzing the synthetic and degradative processing of cytochromes P-450. These may include such steps which yield altered N-terminal amino acid residues [51], glycosidation, or other peptide alterations [52]. Thus, hybridoma technology and DNA recombinant techniques can be important complementary methods for understanding the molecular biology of cytochromes P-450. These approaches can be used to genotype and phenotype differences in the structure and organization of cytochrome P-450 genes in different individuals and to measure their expression under a variety of conditions. This knowledge may lead to better understanding of the physiological and pharmacological implications of different cytochrome P-450 phenotypes and their relationship to hypersensitivity to drugs and carcinogens in human populations.

Implications for ecogenetics, pharmacogenetics and risk assessment

Humans are exposed to a variety of xenobiotics in

* M. D. Snider, R. Brands, Y. Hino, S. S. Park, H. V. Gelboin and J. E. Rothman, manuscript submitted for publication.

† K. C. Cheng, F. K. Friedman, S. S. Park and H. V. Gelboin, unpublished results.

‡ J. Fagan, J. Pastewka, S. S. Park and H. V. Gelboin, manuscript submitted for publication.

foods, in the work place, in the general environment and by drugs administered therapeutically. Many of these xenobiotics are toxic, mutagenic and carcinogenic. There is a large variation in responsiveness of individuals to these xenobiotics [24]. For example, there are large differences in the sensitivity to workers exposed to toxic substances in the work place, as well as highly variable cancer risks to individuals who are heavy smokers of cigarettes. In certain studies there have been conflicting reports on the relationship of AHH inducibility and the incidence of lung cancer in humans [7, 53–55]. The MAbs offer new potential for precise phenotyping of humans for specific forms of cytochromes P-450 and may thus be useful for resolving the question of the relationship of cytochromes P-450 to hypersusceptibility to carcinogens.

Pharmacokinetic studies of drug and carcinogen metabolism in human tissues have described large differences between individuals [1–4]. In some cases there are similarities in the metabolism of certain drugs and carcinogens [56, 57]. Thus, it may be possible to describe a human phenotype by certain patterns or rates of drug metabolism. This, however, is likely to be imprecise in respect to the contribution of specific forms of cytochrome P-450. Recently, an extraordinary polymorphism of human drug metabolism has been reported. Two drugs, debrisoquin [58] and sparteine [59], are deficiently metabolized by 7–9% of the white population. The absence of a particular form of cytochrome P-450 may be responsible for the genetic polymorphism in debrisoquin hydroxylation [60]. *In vivo* metabolic studies, however, express the sum total of a complex of processes that includes absorption, distribution, metabolism and excretion, and thus they may not be very useful for the identification of specific phenotypes related to carcinogen susceptibility.

Monoclonal antibodies may be useful to detect and quantify individual differences in the amount of a carcinogen that has been activated and subsequently bound to DNA. This method of "quantifying" the formation of DNA–carcinogen adducts may prove useful in determining exposure and with certain defined conditions, in assessing the contribution of genetic factors to carcinogen metabolism and activation [32].

The cytochromes P-450 are the primary interface between xenobiotics and higher organisms, and their phenotype is likely to relate to drug and carcinogen sensitivity. Essential questions for the future are whether the cytochrome P-450 phenotype can be precisely defined and whether indeed specific phenotypes are associated with the incidence of cancer induced by chemical carcinogens and with rates of drug metabolism. Several promising approaches are currently being developed. The molecular genetics and regulation of cytochromes P-450 and related enzymes are being studied by gene-cloning methods. Several groups have reported the isolation and characterization of cytochrome P-450 genes [4]. These studies promise important new insights into the structure and regulation of cytochromes P-450. Molecular genetics may successfully identify polymorphisms that relate to individual responsiveness to xenobiotics. This commentary discusses several approaches utilizing monoclonal antibodies specific for individual and classes of cytochrome P-450. These include: (1) tissue phenotyping by direct qualitative or quantitative measurements to specific epitope containing cytochromes P-450 by MAb-directed immunoassay, (2) simple, precise and high yield isolation of cytochromes P-450 by MAb-directed immunopurification, and (3) "reaction phenotyping" by the use of MAbs that inhibit the enzymatic activity of specific forms of cytochrome P-450. The above methods either singly or in combination will permit the development of an atlas of unique cytochromes P-450 responsible for specific xenobiotic and endobiotic reactions in tissues, species and individuals. The MAb-directed methods can determine phylogenetic differences in cytochromes P-450 and assess the changing composition of cytochrome P-450 phenotype during embryonic development, under different hormonal and nutritional conditions, and upon exposure of individuals to natural and synthetic inducers. Epidemiological, toxicological, and pharmacological studies coupled with MAb-directed phenotyping of individuals may then be able to establish the relationship of cytochrome P-450 or related enzyme phenotype and individual sensitivity to xenobiotics including drugs and carcinogens.

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