Epitope-relatedness and phenotyping of hepatic cytochromes *P*-450 with monoclonal antibodies

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The epitope-specific cytochrome *P*-450 content of animal livers was analysed by radioimmunoassay using a panel of seven monoclonal antibodies (MAbs) made to a 3-methylcholanthrene-induced rat liver cytochrome *P*-450. Competitive radioimmunoassays utilizing a reference radiolabelled MAb and a series of unlabelled MAbs indicated that there are at least three distinct classes of MAbs to different epitopes on cytochrome *P*-450. In addition, a direct radioimmunoassay employing a radiolabelled second antibody detected MAB-specific cytochromes *P*-450 in livers from different animals. This radioimmunoassay detected large elevations in the levels of these cytochromes *P*-450 in the livers of 3-methylcholanthrene-treated rats and C57BL/6 mice compared with untreated rats, 3-methylcholanthrene-treated DBA/2 mice or guinea pigs. The two complementary radioimmunoassay methods are sensitive, efficient, and easily applicable for screening large number of tissue samples for MAB-defined cytochrome *P*-450 phenotype.

INTRODUCTION

The cytochromes *P*-450 of the microsomal mixed function oxidase system metabolize a variety of xenobiotics such as drugs and carcinogens as well as endobiotics such as steroids and prostaglandins [1–4]. The individual isoenzymes may exhibit broad and yet unique substrate and product specificity. The multiplicity of cytochromes *P*-450, which has been demonstrated by a variety of biochemical and immunological methods [2], is responsible for their collectively broad spectrum of substrate and product specificity and stereoselectivity. The metabolism of substrates by the cytochromes *P*-450 detoxifies these compounds but often also generates toxic, mutagenic or carcinogenic metabolites. The partitioning of metabolic pathways between conversion to reactive harmful metabolites and detoxication may depend on the phenotype, i.e. the qualitative and quantitative distribution of cytochromes *P*-450. Sensitive and rapid methods for the identification and characterization of individual cytochrome isoenzymes may be quite useful for studies in carcinogenesis, pharmacology, toxicology and environmental sciences.

We have previously reported the isolation and properties of several panels of MAbs to different forms of cytochrome *P*-450 [5–8]. Unlike polyclonal antibodies, MAbs are chemically defined, homogeneous reagents that target specific epitopes on the antigen [9]. They are easily obtained in the quantity desired and, when coupled with conventional characterization methods, are precise probes for specific cytochromes *P*-450. MAbs that inhibit cytochrome *P*-450-dependent catalytic activity have helped identify the contribution of specific classes and individual cytochromes *P*-450 to drug- and carcinogen-metabolizing activities in tissues from animals [10] as well as humans [11,12]. The potential of MAbs for immunopurification [13–15], RIA [15,16] and other analyses

[17,18] of cytochromes *P*-450 of animal tissues has also been demonstrated.

A classification scheme for cytochromes *P*-450 that is based on their epitope content is useful for phenotyping various tissues, species, strains and individuals. The cytochrome *P*-450 distribution in a sample may then be described by the presence or absence of specific epitope-containing cytochromes *P*-450 as recognized by a given panel of MAbs. In the present report we present data demonstrating the feasibility of using MAB-directed RIA as a sensitive probe for epitope-specific cytochrome *P*-450. The competitive RIA method was used both to detect epitope-specific cytochrome *P*-450 in liver from MC-treated rats, and to characterize the MAbs with respect to their epitope specificity. We also report development of a direct RIA method using a radio-labelled rat MAb to mouse IgG as a second antibody to detect the binding of individual MAbs to liver microsomes from MC-induced rats, C57BL/6 and DBA/2 mice, and guinea pigs. Both RIA methods are sensitive and efficient and are applicable to screening studies for cytochrome *P*-450 phenotype.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (fraction V) and MC were from Sigma. PBS was from Biofluid (Rockville, MD, U.S.A.) [³⁵S]Methionine (sp. radioactivity 800 Ci/mmol), NaB³H₄ (sp. radioactivity 64 Ci/mmol), and Aquasol were purchased from New England Nuclear. Other chemicals were reagent grade.

Preparation of microsomes of various tissues and species

The animals were treated by intraperitoneal injection for 3 days with MC (30 mg/kg per day in corn oil). The

Abbreviations used: MAb, monoclonal antibody; RIA, radioimmunoassay; MC, 3-methylcholanthrene; PB, phenobarbital; MC-*P*-450, major forms of MC-induced rat liver cytochrome *P*-450; PBS, phosphate-buffered saline (5 mM-sodium phosphate, 1.5 mM-potassium phosphate, 0.15 M-NaCl, pH 7.4).

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livers of male Sprague-Dawley rats (200–300 g), guinea pigs (180–200 g) and genetically defined mouse strains C57BL/6 and DBA/2 (15–20 g) were excised and immediately processed to prepare the 'microsomal' pellets as previously described [15]. The microsomes were resuspended in 0.25 M-sucrose and stored at -70°C until used. Storage did not affect the results. The protein concentration was determined by the method of Lowry *et al.* [19] with bovine serum albumin as a standard.

Preparation and labelling of MABs

Mouse MABs against MC-induced rat liver *P*-450 were prepared as previously described [7]. The MABs used were 1-2-10, 1-7-1, 1-8-1, 1-25-3, 1-31-2, 1-36-1, and 1-56-2. These MABs bind only to MC-*P*-450 but not to PB-*P*-450. Rat MAB 187.1 to mouse immunoglobulin (κ light chain and γ heavy chain) was kindly supplied by Dr. M. Scharff (Albert Einstein College of Medicine, New York, NY, U.S.A.). Anti-lysozyme MAB HyHEL-9 [20], obtained from Dr. S. Smith-Gill (National Cancer Institute), was used as a control for nonspecific binding. MABs were metabolically labelled with [^{35}S]methionine by the method of Yelton & Scharff [21]. The specific radioactivities of ^{35}S -labelled MABs were 1×10^5 c.p.m./ μg of protein. [^3H]MAB 187.1 was prepared by reductive methylation with NaB^3H_4 [22] to a specific radioactivity of 1×10^6 c.p.m./ μg of protein. Experiments performed with MABs that were radiolabelled by both methods, with either ^{35}S or ^3H , yielded similar results.

Radioimmunoassays

Two types of RIA were performed which are termed competitive and direct. The solid-phase competitive RIA was carried out as described [15] by measuring the inhibition of binding of radiolabelled MAB to antigen. The wells of microtitre plates (96 well, polystyrene; Immulon 1 from Dynatech) were precoated with liver microsomes from MC-treated rats ($1 \mu\text{g}$ of protein/well) by overnight incubation at 4°C . After washing and blocking the non-specific sites with 3% bovine serum albumin in PBS (with 0.2% NaN_3) and additional washing, a mixture of the reference [^3H]MAB and the competing unlabelled MAB was transferred into the wells and incubated for 3 h at room temperature. The binding of [^3H]MABs to the wells was then determined after aspiration and several washes.

Direct RIA was performed with a double-antibody technique. Wells were first coated overnight with the microsomes. The unlabelled mouse MAB was then added to the wells and incubated for 3 h. After several washes and a further 3 h incubation with radiolabelled MAB 187.1, the wells were washed and the amounts of bound radiolabelled MAB 187.1 were measured.

RESULTS

Competitive RIA using MABs made to a MC-induced rat liver *P*-450

The cytochrome *P*-450 content of liver microsomes from MC-treated rats was examined by RIA with a series of MABs to the major form of MC-induced rat liver cytochrome *P*-450 [7]. The relatedness of the epitopes to which the MABs bind were examined by competitive RIA. Binding of a reference radiolabelled MAB to wells

Fig. 1. Competition of [^3H]MAB 1-7-1 binding (a) and [^3H]MAB 1-36-1 binding (b) to microsomes from liver of MC-treated rats by unlabelled MABs

Anti-lysozyme MAB HyHEL-9 (□), MAB 1-2-10 (■), MAB 1-7-1 (●), MAB 1-8-1 (○), MAB 1-25-3 (■), MAB 1-31-2 (△), MAB 1-36-1 (▲) and MAB 1-56-2 (▲) are shown. The 100% binding level (1000 c.p.m./well) was obtained in the absence of competing antigens. Each point represents the average of duplicate determinations.

coated with microsomes was measured in the presence of each of a series of MABs. The degree of inhibition of binding of the radiolabelled MAB by the individual MAB indicates whether the two competing MABs bind to entirely independent epitopes or to epitopes that are identical or overlapping. The binding inhibition curve obtained with a radiolabelled MAB in the presence of the identical unlabelled MAB is a useful standard curve, since both labelled and unlabelled MABs are directed toward the same epitope. The extent to which each different MAB inhibits binding of the MAB relative to this standard

curve depends on the relatedness of the epitopic sites which are specific for each of the pair of MABs.

A competitive inhibition curve using wells coated with MC-treated rat liver microsomes and added [3 H]MAB 1-7-1 as the reference MAB is shown in Fig. 1(a). With unlabelled MAB 1-7-1 as competitor, significant inhibition of binding is observed, with 20% of maximal binding occurring at 1 μ g of MAB. MAB 1-8-1 also decreases binding, but to a lesser extent; with 1 μ g only 54% of maximal binding is observed. None of the MABs 1-2-10, 1-25-3, 1-31-2, 1-36-1, and 1-56-2, nor anti-lysozyme MAB HyHEL-9, inhibit [3 H]MAB 1-7-1 binding. These results indicate that of the seven MABs examined, only MAB 1-8-1 recognizes an epitope overlapping or interactive with the epitope recognized by MAB 1-7-1. However, since different curves were obtained with MABs 1-7-1 and 1-8-1, the epitopes on cytochrome *P*-450 to which these MABs bind are not identical. The remaining five MABs to MC-*P*-450 are entirely non-competitive with MAB 1-7-1, indicating that they are directed toward target epitopes that are nonoverlapping and noninteracting with the MAB 1-7-1-specific epitope.

The epitope specificity of different MABs relative to MAB 1-36-1 was also measured by competitive RIA using [3 H]MAB 1-36-1 as the reference MAB (Fig. 1b). The unlabelled MAB 1-36-1, as expected, effectively inhibited the binding of the identical labelled MAB. The MABs 1-56-2 and 1-31-2 also significantly inhibited the binding of [3 H]MAB 1-36-1, indicating that these two MABs target epitopes that overlap or interact with the MAB 1-36-1-specific epitope. The MABs 1-2-10, 1-7-1, 1-8-1, 1-25-3 and anti-lysozyme MAB HyHEL-9 do not inhibit the binding of [3 H]MAB 1-36-1, demonstrating that they are directed toward epitopes that are different from, and noninteracting with, the epitope recognized by MAB 1-36-1.

Thus, the two reference radiolabelled MABs, 1-7-1 and 1-36-1, have been successfully used to identify at least three distinct categories of epitopes on rat liver cytochrome *P*-450: one recognized by MABs 1-7-1 and 1-8-1; a second by MABs 1-36-1, 1-56-2 and 1-31-2; and a third by MABs 1-2-10 and 1-25-3, which bind neither of the epitopes on *P*-450 that are recognized by the MABs of the first two groups. A MAB may be classified by its ability to compete successfully with other MABs for binding to a standard cytochrome *P*-450-containing preparation. MABs within a group target epitopes that either overlap or interact allosterically with the epitope recognized by the respective reference MAB, whereas intergroup pairs of MABs target distinct, noninteracting epitopes on the cytochromes *P*-450.

Direct RIA for detection and phenotyping of cytochrome *P*-450 in animal tissues

The competitive type of RIA can measure the amount of epitope-specific cytochromes *P*-450 and can determine the degree of epitope relatedness of the cytochrome *P*-450 for different MABs. A direct noncompetitive RIA can quantitatively detect individual epitopes without requiring a reference labelled MAB. In the direct RIA, the unknown sample is coated on the wells and separately incubated with each of a series of MABs. The degree of binding by each MAB to the well-coated material is then assessed by incubation with 35 S- or 3 H-labelled MAB 187.1, a rat MAB specific for mouse IgG, followed

Fig. 2. Direct RIA of cytochromes *P*-450 in rat liver microsomes with rat MAB 187.1 to mouse MABs

The relative binding of the following MABs to microsomes from MC-treated rat liver (20 μ g of protein/well) were detected with 23000 c.p.m./well of [35 S]MAB 187.1: anti-lysozyme MAB HyHEL-9 ($\square$), MAB 1-7-1 ($\bullet$), MAB 1-8-1 ($\blacktriangle$), MAB 1-31-2 ($\triangle$), MAB 1-36-1 ($\circ$) and MAB 1-56-2 ($\blacktriangle$). Each point represents the average of duplicate determinations.

by measurement of the amount of radiolabel bound to the well. In this manner, a panel of unlabelled MABs can be used to screen a tissue efficiently and rapidly for a large number of MAB-specific cytochromes *P*-450.

The results of direct RIA of liver microsomes from MC-induced rats are shown in Fig. 2. Binding of the rat anti-(mouse IgG) [35 S]MAB 187.1 to different mouse anti-(rat liver cytochrome *P*-450) MABs was measured at different levels of the mouse MABs to determine the optimum conditions for this RIA. The amount of bound [35 S]MAB 187.1 measures the amount of mouse MAB bound to the well, which in turn reflects the amounts of MAB epitope-specific cytochromes *P*-450 present in these microsomes. The greatest degree of binding was observed with MABs 1-7-1 and 1-8-1, followed by 1-36-1. The binding levels of MAB 1-31-2 and 1-56-2 were low but significant, since they were higher than that of the control anti-lysozyme MAB HyHEL-9.

The degree of binding of [35 S]MAB 187.1 (Fig. 2) to the microsome-coated wells may not be directly proportional to the level of different MAB epitope-specific cytochromes *P*-450 due to a possible variability in the affinity of MAB 187.1 for individual mouse MABs. To examine this possibility, equal amounts of each mouse MAB were coated onto microtitre wells, and the binding of [3 H]MAB 187.1 radiolabelled with high specific activity was measured. As shown in Fig. 3, the affinities of MAB 187.1 for MABs 1-7-1, 1-8-1, 1-31-2, 1-56-2, and anti-lysozyme HyHEL-9, although not identical, are quite similar and thus the binding curves in Fig. 2 for these MABs reflect the relative levels of their MAB epitope-specific cytochromes *P*-450. The binding of rat anti-(mouse IgG) MAB 187.1 to MABs 1-2-10 and

Fig. 3. Interaction of MAb 187.1 with different mouse MABs

Wells were coated with various mouse MABs (10 μ g of protein/well) including anti-lysozyme MAB HyHEL-9 ($\square$), MAB 1-2-10 ($\blacksquare$), MAB 1-7-1 ($\bullet$), MAB 1-8-1 ($\circ$), MAB 1-31-2 ($\triangle$), MAB 1-36-1 ($\blacktriangle$) and MAB 1-56-2 ($\blacktriangle$). Different amounts of [3 H]MAb 187.1 were then added to each well and the amounts of bound radioactivity were determined. Each point represents the average of duplicate determinations.

1-36-1, however, is far less efficient. This results in the more gradual increase in binding relative to that of MABs 1-7-1 and 1-8-1 as shown in Fig. 2. The relative level of the MAB 1-36-1 epitope-specific cytochromes *P*-450 is therefore underestimated in the direct comparison of the curves in Fig. 2. On the other hand, MABs 1-31-2 and 1-56-2 bind MAB 187.1 with the same affinity as

MABs 1-7-1 and 1-8-1, and the lower binding observed with the former MABs in Fig. 2 therefore represents a lower level of cytochromes *P*-450 containing their target epitopes.

Direct RIA analysis of livers from MC-treated rats, C57BL/6 and DBA/2 mice, and guinea pigs were performed at saturating levels of mouse MABs for interspecies comparison of cytochrome *P*-450 phenotype. For comparison, direct RIA of liver from untreated rats was also performed. The results are displayed as the histogram in Fig. 4 in which the amount of [35 S]MAb 187.1 binding to each individual mouse MAB is represented by a letter designation. Because of the possible variation in the affinity of different MABs to the cytochrome *P*-450 and the MAB 187.1 for different mouse MABs, direct RIA cannot be used for intratissue comparison of MAB epitope-specific *P*-450 content with different MABs. However, interspecies comparison of individual epitope-specific *P*-450s can be made, since the same mouse MAB is employed in each of these measurements. The magnitude of binding as shown in the histograms demonstrates that there are greater amounts of cytochromes *P*-450 recognized by our panel of MABs in MC-treated rats and C57BL/6 mice than in untreated rats or MC-treated DBA/2 mice and guinea pigs. The lower level in DBA/2 mice is consistent with the relative noninducibility of this strain for cytochrome *P*-450-dependent aryl hydrocarbon hydroxylase activity, since all the MABs used in this study were made to an MC-induced *P*-450 which is aryl hydrocarbon hydroxylase-active and presumably present at low levels in DBA/2 mice. The effect of MC induction of enzyme activity and the effect of MAB on their activities have been previously reported [10].

More precise visualization of cytochrome *P*-450

Fig. 4. Phenotyping of MAB-epitope-specific cytochrome *P*-450 in animal liver microsomes with a panel of MABs

Wells coated with liver microsomes from different animals (20 μ g of protein/well) were first incubated with the following mouse MABs (15 μ g of protein/well): A, MAB to lysozyme HyHEL-9; B, MAB 1-7-1; C, MAB 1-8-1; D, MAB 1-31-2; E, MAB 1-36-1; and F, MAB 1-56-2. Each well was subsequently incubated with [35 S]MAb 187.1 (103 000 c.p.m./well), and the relative amounts of bound radioactivity were measured. Each point represents the average of duplicate determinations.

phenotype is accomplished by qualitatively comparing the profiles in Fig. 4. This experiment was performed three separate times and the same profiles were observed for all samples. For example, although similar levels of B and C are observed for MC-treated rats and C57BL/6 mice, the levels of D and F for rats is twice that observed for the C57BL/6 mice. In comparing mouse strains, the levels of B and C are $\frac{1}{5}$ and $\frac{1}{10}$ as great in DBA/2 than in C57BL/6 mice, but the level of D is only 50% less. Also, the levels of D and E are low but comparable in DBA/2 mice and guinea pig, but D is far less than E in MC-treated rats and C57BL/6 mice. In this manner, additional intertissue comparisons can also be made with respect to the content of MAb epitope-specific cytochrome *P*-450. The histograms in Fig. 4 may thus be viewed as a representation of the cytochrome *P*-450 phenotype of these tissues as determined by a panel of MAbs to epitope-specific cytochromes *P*-450.

DISCUSSION

The results demonstrate the utility of RIA procedures for both characterizing MAb specificity and for developing profiles of MAb-defined cytochrome *P*-450 content of animal tissues. Seven mouse MAbs made to MC-induced rat liver *P*-450 [7] were used to probe the cytochrome *P*-450 content of livers of MC-treated animals and to study the immunochemical relatedness among the cytochromes *P*-450 of these species. The relationship between the MAbs for their target epitopes was first established by competitive RIA using microsomes from liver of MC-treated rats. The partial inhibition of binding of [³H]MAb 1-7-1 by MAb 1-8-1 indicates that overlapping or interacting epitopes are shared by these MAbs, while the observed non-interference of binding by the other MAbs suggests that the epitope recognized by MAb 1-7-1 is distinct from those recognized by these MAbs. In addition, the binding of [³H]MAb 1-36-1 was partially blocked by MAbs 1-31-2 and 1-56-2, indicating that these two MAbs have epitopes that overlap or interact with the MAb 1-36-1 specific epitope. The latter is also quite different from the epitope recognized by MAbs 1-7-1 and 1-8-1. The non-interference of either [³H]MAb 1-7-1 or [³H]MAb 1-36-1 binding by MAb 1-2-10 or MAb 1-25-3 may indicate that the epitopes recognized by the latter MAbs are quite different from the forementioned ones and that their target epitopes belong to another epitopic class, which is exclusive of the two classes. Thus, based on the competitive RIA, at least three distinct classes of epitopes on MC-*P*-450 in the microsomal fraction were delineated.

In addition to the competitive RIA approach, we have also applied a direct RIA procedure for phenotyping cytochrome *P*-450. The method discussed above defines the content of epitope-specific cytochrome *P*-450 relative to a radiolabelled reference MAb, and when carried out with a large number of such reference MAbs provides much information on the content, in a given tissue, of cytochromes *P*-450 with specific epitopes. A complementary approach which also reveals phenotype is demonstrated by our experiments using a second rat MAb, [³⁵S]MAb 187.1 to mouse MAbs, to detect MAb-specific cytochromes *P*-450. The advantage of this method lies in its simplicity and efficiency. Large numbers of tissue samples can be rapidly screened to yield phenotype profiles such as those in Fig. 4. Although interpretation

of intratissue binding differences among MAbs is difficult because of the variation in affinity of MAb 187.1 for mouse MAbs, this method accurately compares intertissue content of MAb-specific cytochromes *P*-450 since binding of the same MAb to different tissues is compared. Semi-quantitative conclusions can be made when relative binding affinities are evaluated, as in Fig. 3.

Positive MAb binding data indicate that the epitopes are present, located on the antigen surface, and easily accessible to the MAb. Low or no binding may be due to either the absence of a cytochrome *P*-450 with the appropriate epitope or inaccessibility of epitope to MAb owing to steric hindrance by other microsomal components such as membrane or reductase. Accessibility may be improved by solubilization with detergent, although at higher concentration detergent may interfere with MAb binding to cytochrome *P*-450. In either case assessment of [³⁵S]MAb 187.1 binding profiles of different tissues serves to phenotype cytochrome *P*-450 content if the term phenotype is defined to include accessibility of the epitope to an MAb.

One possible disadvantage of using a secondary MAb is the variation in affinity for the primary mouse MAb. For instance, MC-treated rat liver microsomes appear to have a high level of cytochrome *P*-450 recognized by MAb 1-36-1 when assayed by binding of [³H]MAb 1-36-1 to MC-microsomes (Fig. 1b) (relative to [³H]MAb 1-7-1 binding in Fig. 1a), while relatively less binding was observed when the second antibody, [³⁵S]MAb 187.1, was used (Fig. 2). Another disadvantage of direct binding could be variation in binding of microsomal samples to the microtitre well in the first step of the RIA procedure. Despite these disadvantages the direct RIA is most useful for qualitative intertissue phenotyping studies, since binding profiles may be compared. The direct RIA with anti-(mouse IgG) rat MAb 187.1 complements the competitive RIA approach and because of its simplicity is especially useful for screening many samples for epitope-specific cytochromes *P*-450.

Phenotyping studies carried out with a large library of MAbs is a high-resolution method that can distinguish closely related cytochromes *P*-450. The cytochrome *P*-450 content of a given tissue can therefore be classified according to epitope content as measured by different MAb probes. This methodology is readily applicable to a wide range of studies requiring determination of cytochrome *P*-450 in various species, strains, individuals, and tissues under different conditions. The MAb-directed immunoassays are also especially well suited for studies on the biochemical epidemiology of cytochrome *P*-450 phenotype and sensitivity to drugs and carcinogens.

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