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MONOCLONAL ANTIBODIES AGAINST HUMAN LIVER CYTOCHROME P-450

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Abstract—Monoclonal hydribomas which produce antibodies against human liver microsomal cytochrome P-450 were developed. Three similar hybridomas produced antibodies which recognized an epitope specific to a family of human P-450 isozymes ($P\text{-}450_5$). This epitope was also present on cytochrome P-450 PCN-E (pregnenolone-16α-carbonitrile induced) from rat liver microsomes, but this isozyme differed from the human $P\text{-}450_5$ by its molecular weight. These antibodies enabled us to quantify cytochrome $P\text{-}450_5$ in human liver microsomes and to demonstrate an important quantitative polymorphism in the human liver monooxygenase system.

Cytochrome P-450,‡ the catalytic moiety of the monooxygenase which oxidizes a large variety of substrates, exists in a number of different isozymes which display various but overlapping substrate specificities, and differ in some of their physicochemical properties [1, 2].

Despite an increasing number of recently published *in vitro* studies on human drug metabolizing enzymes [3–6], limited information is available on human liver P-450 multiplicity.

Immunological methods and more particularly, those requiring specific monoclonal antibodies, might constitute powerful tools for the characterization and quantification of individual P-450s [1, 2, 7–9]. A cytochrome P-450, partially purified from human liver, was used to immunize Balb/c mice. Specific monoclonal antibodies were obtained and shown to be very useful to characterize human and rat cytochrome P-450s, and to quantify single isozymes in microsomal preparations.

MATERIALS AND METHODS

Chemicals. All reagents and solvents were of the highest grade commercially available. Cell culture dishes, plates and T-flasks were obtained from Falcon; the culture medium was acquired from Gibco Europe (Scotland); nitrocellulose sheets were purchased from Schleicher and Schüll (F.R.G.); and chemicals for electrophoresis were obtained from Bio-rad (Richmond, CA).

Animals. New Zealand female rabbits were used to produce polyclonal antibodies. Balb/c female mice (Iffa Credo, France) were used to obtain spleen cells and to produce large amounts of monoclonal antibodies in ascitic fluid. Sprague–Dawley male rats (200–250 g) were used for the preparation of microsomes.

Human liver. Livers were obtained from kidney transplant donors. They were removed within 30 min after circulatory arrest, and were immediately frozen in dry ice and then stored at −80°.

Preparation of microsomes. Microsomes were prepared as previously described [4]. The same methodology was used for human and rat liver microsomes.

Purification of rat cytochrome P-450s. Purified rat liver microsomal cytochrome P-450s were a generous gift from Professor F. P. Guengerich. PB-B, βNF-B, ISF-G, and PCN-E were the major forms induced by phenobarbital-β-naphthoflavone, isosafrole and pregnenolone-16α-carbonitrile, respectively. UT-A was the major form of cytochrome P-450 found in untreated rat liver microsomes. PB-C was a form induced by phenobarbital. This nomenclature was used by Guengerich [1].

Purification of human cytochrome P-450s, epoxide hydrolase and NADPH cytochrome c *reductase.* Cytochrome $P\text{-}450_{1,\ 2,\ 4,\ 5,\ 6\ \text{and}\ 8}$ were the human liver P-450 forms purified by Wang *et al.* [5]. NADPH cytochrome reductase and epoxide hydrolase were purified from human liver microsomes as previously described [10]. The cytochrome P-450 used to immunize mice in order to obtain monoclonal antibodies was purified as previously described [11]. Microsomes were solubilized and then flowed through an Octyl Sepharose 4B column [11]. Fractions containing cytochrome P-450 were pooled, dialysed and submitted to DEAE cellulose chromatography. The fraction which was not retained on the column was used to immunize mice as described below. This fraction was not pure as shown by SDS polyacrylamide gel electrophoresis and probably con-

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‡ Abbreviations used: SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; P-450, cytochrome P-450; ELISA, enzyme-linked immunosorbent assay.

tained cytochrome P-450$_5$ and P-450$_8$; it was used in the first screening of hybridomas which produced antibodies by the ELISA test described below.

Production of polyclonal antibodies. Polyclonal antibodies were produced in female New Zealand rabbits as previously described [1].

Immunochemical techniques. ELISA test and immunorevelation after transfer to nitrocellulose sheets from SDS PAGE ("western blots") were performed as previously described [1, 12]. For both techniques, when using polyclonal antibodies, antigens were revealed by swine antirabbit peroxidase-conjugated immunoglobulin, whereas with monoclonal antibodies, rabbit antimouse peroxidase-conjugated immunoglobulin was used. Antiserum or ascitic fluid was diluted 1/100 when used to immunoreveal western blots, and 1/1000 to 1/5000 when used for the ELISA test. For monoclonal antibodies screening, pure culture supernatants were used. For each ELISA test, positive (homologous polyclonal antibody) and negative (isotonic NaCl) controls were used. For quantitative analysis, a standard curve was obtained with pure cytochrome P-450$_5$ and the area containing spots (pure P-450$_5$ or human liver microsomes) was measured by densitometry (PHI6F, Vernon, France).

Monoclonal antibody production. Monoclonal antibodies were produced according to de Fazekas *et al.* [13]. Female Balb/c mice were immunized by i.p. injection with 50 μg of cytochrome P-450 diluted in 200 μl isotonic NaCl and mixed with 200 μl complete Freund adjuvant. Animals were boosted with 10 μg of cytochrome P-450 three weeks later, and three days prior to fusion. Blood samples were analysed by the ELISA test to verify the degree of immunization of the animals.

Spleen cells were fused with myeloma cells and hybrids were cultured as described by de Fazekas *et al.* [13]. Production of antibodies in the culture medium was verified by ELISA as soon as cell growth

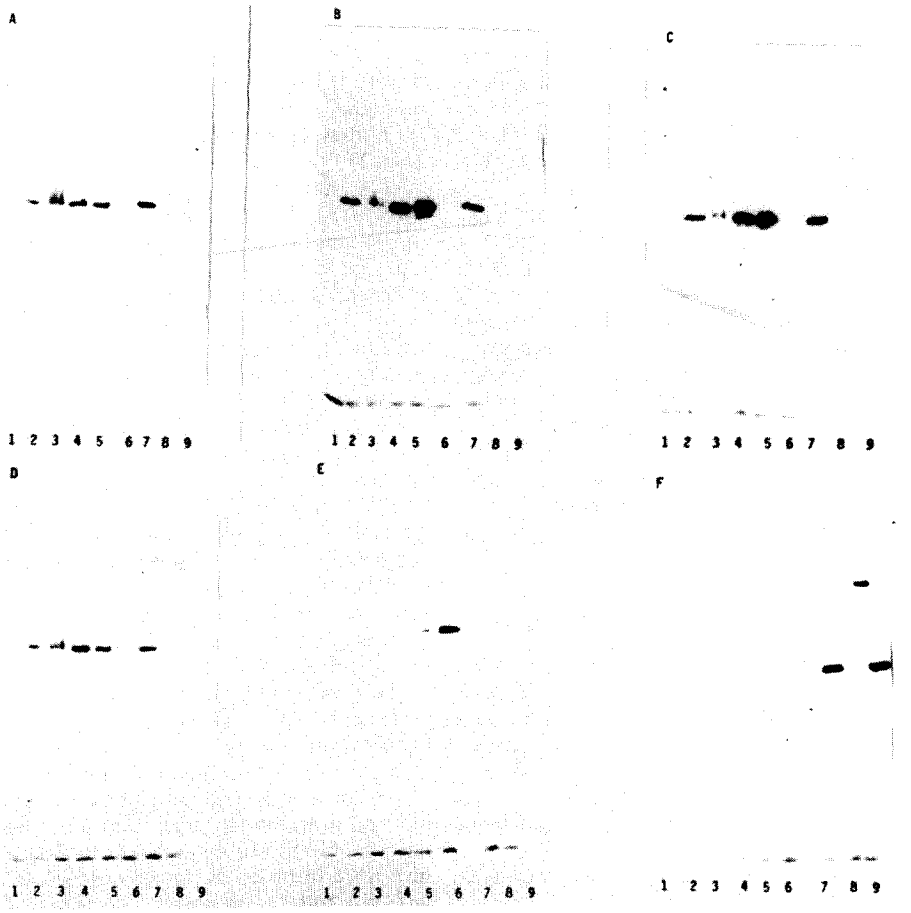

Fig. 1. Immunochemical staining of sodium dodecyl sulfate polyacrylamide gel electrophoretograms after transfer to nitrocellulose. Human liver proteins used as antigens and submitted to electrophoresis were: P-450$_1$ (1.8 pmoles) in lane 1, P-450$_2$ (1.5 pmoles) in lane 2, P-450$_4$ (8.7 pmoles) in lane 3, P-450$_5$ (2.2 pmoles) in lane 4, P-450$_6$ (1.5 pmoles) in lane 5, P-450$_8$ (7.2 pmoles) in lane 6, human liver microsomal proteins (4 μg) in lane 7, NADPH cytochrome *c* reductase (0.25 μg) in lane 8, and epoxide hydrolase (0.8 μg) in lane 9. The nitrocellulose sheets were revealed with the following antibodies used at 1/100 dilution: (A) ascitic fluid from clone 13-7-7; (B) ascitic fluid from clone 13-7-8; (C) ascitic fluid from clone 13-1-13; (D) rabbit serum anti-human P-450$_5$; (E) rabbit serum anti-human P-450$_8$; and (F) rabbit serum anti-epoxide hydrolase.

was adequate. Positive cultures were cloned by diluting to single cell density. A few days later, antibody production was again verified and positive cultures were cloned again. Productive clones were grown in T-flasks and finally implanted intraperitoneally in Balb/c mice (10^7 cells/mouse) which had been primed with pristane (0.5 ml i.p./mouse, 3–10 days prior to injection of the cells). Ten to sixteen days later, ascitic fluid was collected, centrifuged for 5 min at 2000 *g* and the supernatant stored at −80° until use. Immunoglobulins were purified from ascitic fluid by DEAE-affigel blue chromatography according to Bruck *et al.* [4].

Assays. Cytochrome P-450 was determined according to Omura and Sato [15] and proteins according to Lowry *et al.* [16], using bovine serum albumin as standard.

RESULTS

Selection of clones

The hybridomas were selected via a two-step procedure. They were first screened for the production of antibodies reacting with the partially purified human liver P-450 that was not retained on DE52 cellulose as described by Beaune *et al.* [11] and used to immunize the mice. In a second step, they were screened for the production of antibodies capable of recognizing one of the extensively purified P-450 isozymes, $P\text{-}450_5$. Three hybridomas considered as monoclonal after being cloned twice by limiting dilution were finally selected: 13-1-13, 13-7-7 and 13-7-8.

Specificity of the monoclonal antibodies

The antibodies produced behaved quite similarly on western blots (Fig. 1). All of them recognized human liver P-450 isozymes 2, 4, 5 and 6 (isozyme 6 was not pure but was contaminated by isozymes 5 and 8), and reacted as a single band (Figs 1A, B and C) with solubilized human liver microsomes, but did not recognize epoxide hydrolase, cytochrome *c* reductase or isozymes 1 or 8. For the comparison, the same samples were revealed with polyclonal antibodies raised in rabbits against isozymes 5, 8, and epoxide hydrolase (Figs 1D, E and F, respectively). The antibody raised in rabbits against human liver epoxide hydrolase only recognized the epoxide hydrolase (Fig. 1F) and in human liver microsomes, a single band which migrated similarly to epoxide hydrolase (lane 7). An unexplained positive reaction was also observed with purified NADPH cytochrome *c* reductase (lane 8). The anti-$P\text{-}450_8$ (Fig. 1E) only reacted with isozyme 8 (lane 6) and to a lesser extent

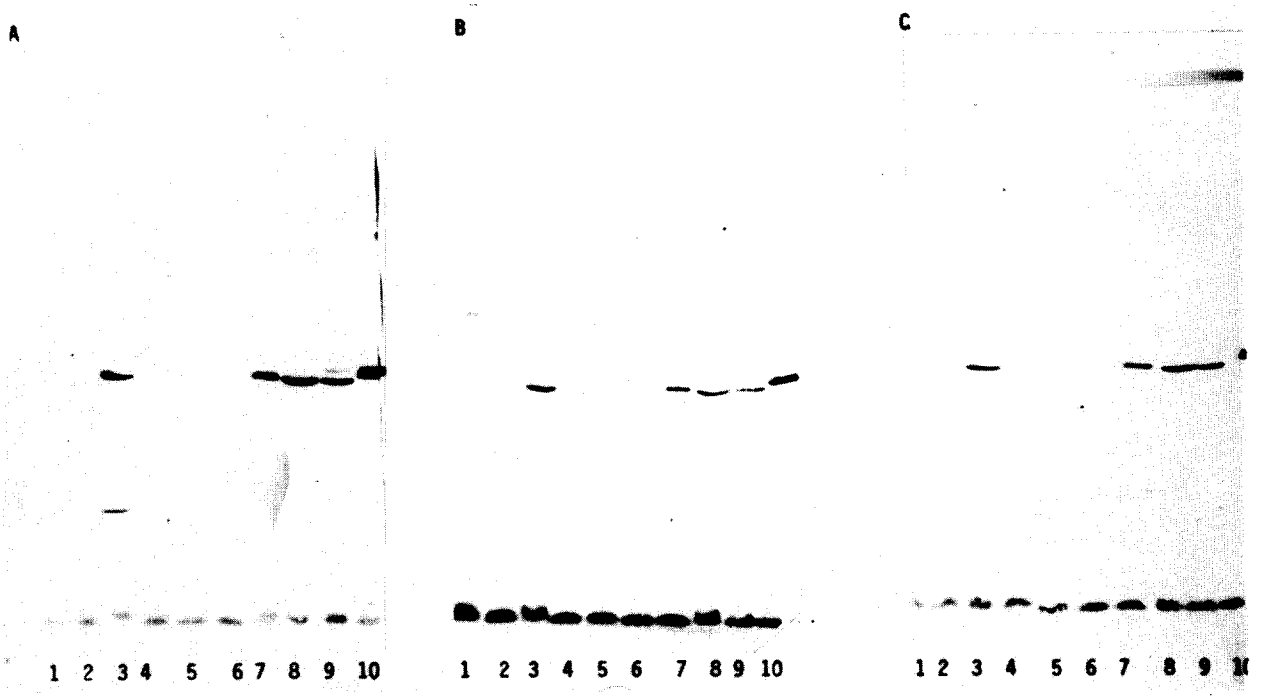

Fig. 2. Immunochemical staining of sodium dodecyl sulfate polyacrylamide gel electrophoretograms after transfer to nitrocellulose. Rat liver proteins used as antigens and submitted to electrophoresis were: P-450 PB-B (1.6 pmoles) in lane 1, P-450 PB-D (3.0 pmoles) in lane 2, microsomal proteins (2 μg) from β-naphthoflavone-treated rats in lane 3, P-450 βNF-B (3.0 pmoles) in lane 4, P-450 PB-C (2.4 pmoles) in lane 5, P-450 UT-A (7 pmoles) in lane 6, P-450 PCN-E (4.2 pmoles) in lane 7, liver microsomal proteins (3 μg) from phenobarbital-treated rats in lane 8, liver microsomal proteins (3 μg) from untreated rats in lane 9, and human liver microsomal proteins (2.8 μg) in lane 10. The nitrocellulose sheets were revealed with the following antibodies used at 1/100 dilution: (A) ascitic fluid from clone 13-7-7; (B) ascitic fluid from clone 13-7-8; and (C) ascitic fluid from clone 13-1-13.

with fraction 6 (lane 5) which contained isozymes 8 and 5. Only a faint band was recognized in microsomes (lane 7). Anti-P-450$_5$ recognized the same bands as the monoclonal antibodies (Fig. 1D).

Cross reaction with rat liver P-450s

The three monoclonal antibodies did not react with the various rat liver P-450s tested (Fig. 2) except for P-450 PCN-E (lane 7), the cytochrome-induced by pregnenolone-16α-carbonitrile. In microsomes, they only recognized a single band (lanes 3, 8 and 9) which migrated at a level very close to that of P-450 PCN-E. In human liver microsomes (lane 10), they revealed a band having a higher molecular weight.

Measurement of cytochrome P-450$_5$ in human liver microsomes with the monoclonal antibody

Figure 3 shows the results of a western blot performed on microsomes from seven different human liver samples. A single band was recognized by the monoclonal antibody 13-7-8. Identical results were obtained with the two other monoclonal antibodies and with the polyclonal antibody raised in rabbits against cytochrome P-450$_5$. The area and density of the spots were densitometrically evaluated and expressed as a function of the amount of microsomal proteins loaded on the gel. Figure 4 shows that the results obtained with the specific polyclonal antibody correlate well with those obtained with the monoclonal antibody 13-1-13 ($r = 0.92$, $P < 0.1$). Similar correlation coefficients were obtained when both monoclonal antibodies were compared (13-1-13/13-7-7, $r = 0.92$, $P < 0.01$) and when the second monoclonal antibody (13-7-7) was compared to the polyclonal anti-P-450$_5$ ($r = 0.86$, $P < 0.05$). For each determination, five different human liver microsomes were used.

Excellent correlations were also obtained when the quantifications were performed on 12 different microsomal preparations comprised of 8 human fetal livers and 4 human adult livers (T. Cresteil, personal communication).

In a second step, a calibration curve was established using known amounts of purified cytochrome

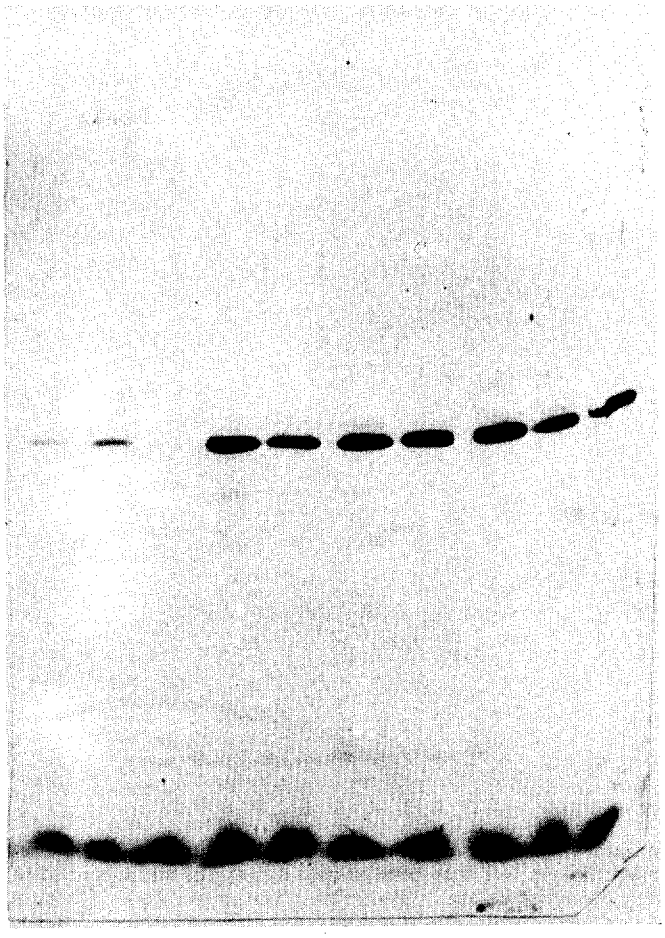

Fig. 3. Immunochemical staining of sodium dodecyl sulfate polyacrylamide gel electrophoretograms after transfer to nitrocellulose. Human liver proteins used as antigens and submitted to electrophoresis were: P-450$_2$ (2 pmoles) in lane 1, P-450$_5$ (2.2 pmoles) in lane 2, P-450$_8$ (7 pmoles) in lane 3, and solubilized microsomal proteins (8 μg) from different liver samples in lanes 4–10. Ascitic fluid from clone 13-7-8 was used as an antibody to reveal the nitrocellulose sheet.

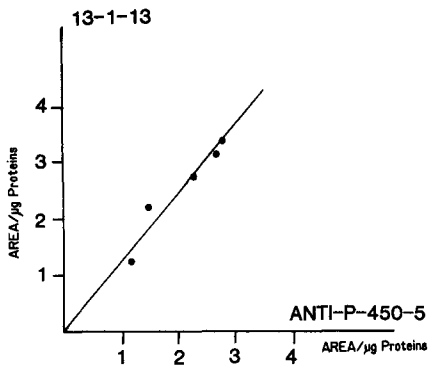

Fig. 4. Correlation between results of immunoquantification obtained with 13-1-13 monoclonal antibody and anti-P-450$_5$ polyclonal antibody. The liver microsomes were identical to those in Fig. 3. Only five livers were measured because the densitometry of two spots was impossible. Area is expressed as arbitrary units.

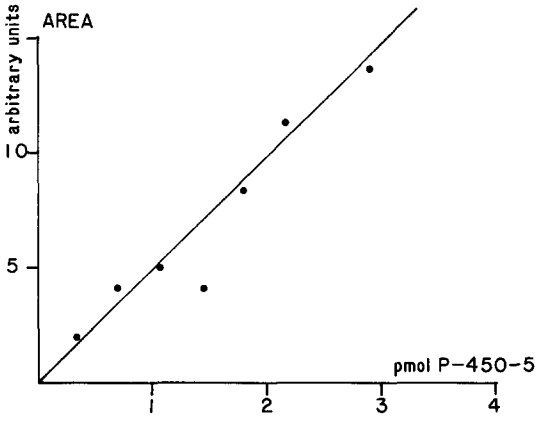

Fig. 5. Calibration curve for the measurement of cytochrome P-450$_5$. The abcissa gives the amount (in pmoles) of pure P-450$_5$ submitted to SDS PAGE and western blot. The ordinate expresses the density in arbitrary units of the spots which were immunorevealed on nitrocellulose, using the monoclonal antibody 13-1-13.

P-450$_5$ (Fig. 5). From this curve, it was then possible to calculate the concentration of cytochrome P-450$_5$ in the various human liver samples (Table 1).

DISCUSSION

By a classical methodology, we prepared and selected three monoclonal hybridomas which produced antibodies against human liver microsomal cytochrome P-450. These antibodies behaved quite similarly (Figs 1 and 2). This was not surprising as they were derived from the same initial well, thus, probably from the same clone. They recognized human liver cytochrome P-450$_{2,\ 4,\ 5\ \text{and}\ 6}$. Isozyme 6 was contaminated by P-450$_5$ [5]. Isozymes 2, 4 and 5 could be considered as members of a family of very close isozymes: polyclonal antibodies against each of these isozymes recognized the other two as well in Ouchterlony double immunodiffusion as in western blots [5].

The monoclonal antibodies did not cross react with isozymes 1 and 8 which represented another cytochrome P-450 family of human liver microsomes [5].

In order to study similarities between rat and human liver microsomal cytochrome P-450s, we studied cross reactions between rat liver cytochrome P-450s and our monoclonal antibodies. Our results indicated that human liver P-450s and rat liver PCN-E cytochrome P-450s shared at least a common epitope which had been maintained during evolution.

Having monoclonal antibodies recognizing a family of human liver cytochrome P-450 as a single band, we used these antibodies for quantitation. We first verified that they gave the same results as polyclonal antibody raised in rabbits against cytochrome P-450$_5$, as demonstrated by Fig. 4. Then using a standard curve of pure cytochrome P-450$_5$, we measured the amount of P-450$_5$ in six human liver microsomes by using either monoclonal antibody 13-1-13 or 13-7-8. We observed a significant quantitative polymorphism

Table 1. Immunoquantification of cytochrome P-450$_5$ in human liver microsomes

Liver sample	Cytochrome P-450$_5$ (nmoles × mg^{-1} proteins)	Total cytochrome P-450 spectrophotometrically determined (nmoles × mg^{-1} proteins)
1	0.24	0.68
3	0.32	0.64
4	0.51	0.52
5	0.54	0.55
6	0.13	0.32
7	0.28	0.38
	M = 0.34 ± 0.15 N = 6	M = 0.51 ± 0.13 N = 6

Spots were measured by densitometry and areas were compared to a standard curve of pure cytochrome P-450$_5$ (Fig. 4). The liver samples were identical to those used for Fig. 3. Monoclonal antibody was 13-1-13.

(1 to 4.2) in these 6 livers (Table 1), indicating that this isozyme could vary as a function of environmental and/or genetic factors. Moreover, in some cases, P-450$_5$ represented almost all of the total cytochrome P-450 determined spectrally; this was due to the fact that by an immunological method, we measured the apoprotein while spectrally, we measured only the heme-linked cytochrome P-450.

These monoclonal antibodies will be a very useful tool for the study of the regulation of human cytochrome P-450s.

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