Vol. 119, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1984 Pages 744-750

PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST PHENOBARBITAL-INDUCIBLE CYTOCHROME P-450

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Received January 25, 1984

Monoclonal antibodies against cytochrome P-450 were prepared from phenobarbital-induced rat liver microsomes. The immunoglobulin classes and subclasses, as well as the binding capacity to cytochrome P-450, of the different antibodies were characterized. Their specificity was verified by various techniques and seemed to correspond to a single form of cytochrome P-450, the major phenobarbital-inducible form. However, the antibodies were unable to inhibit completely the monooxygenase activities investigated. These antibodies may constitute very specific and powerful analytical tools for characterizing and quantifying cytochrome P-450 isoenzymes.

The catalytic moeity of the microsomal monooxygenase activity, the cytochrome P-450, exists in a number of different isoenzymes which display various but overlapping substrate specificities. They also differ in their physicochemical properties such as molecular weight, amino acid sequence and immunoreactivity (1-3).

Immunological methods have become a necessary tool for the study of cytochrome P-450 multiplicity. They are particularly helpful in characterizing and quantifying individual isoenzymes (4-6). Antibodies can also be used for the purification of cytochrome P-450 (7-9). Numerous antibodies have been raised in rabbits, sheep or goats against purified and semipurified cytochrome P-450 fractions (4,10,11). Although the use of monoclonal antibodies has largely increased within the past several years, only a few applications have been published in the field of cytochrome P-450 (12,13).

Monoclonal antibodies being perfectly homogenous and specific for a single epitope constitute a precise and powerful tool (14). They can also give valuable information as to the structure of proteins, and the homology or heterogeneity of several cytochrome P-450 preparations (15). They can also be

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0006-291X/84 $1.50

used to compare isoenzymes from different organs or species. Another advantage is that the production of monoclonal antibodies does not require the use of pure antigens.

These diverse advantages inherent to monoclonal antibodies for the study of cytochrome P-450 multiplicity prompted us to prepare antibodies against a cytochrome P-450 fraction purified from phenobarbital-induced rat liver microsomes.

MATERIAL AND METHODS

Material

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

Cytochrome P-450

Cholate-solubilized microsomes from phenobarbital-induced rat livers were chromatographed on octyl-sepharose. The cytochrome P-450 peak was subsequently chromatographed on DEAE-cellulose (DE 52) according to Warner et al. (16). The fraction eluted with the NaCl gradient was used as the antigen. This fraction contained the phenobarbital-induced isoenzyme, had a specific activity of 6 nmol/mg of protein and showed a major band accompanied by two minor bands in the 40-60 Kd region when visualized by sodium dodecylsulfate polyacrylamide gel electrophoresis.

Preparation of the antibodies

Three female Balb/c mice were immunized by i.p. injection of 50 µg cytochrome P-450 diluted in 200 µl isotonic NaCl and mixed with 200 µl complete Freund adjuvant. The animals were boosted with 10 µg of cytochrome P-450 three weeks later and three days prior to fusion. Blood samples were analyzed by the Elisa test to verify the degree of immunization of the animals

Spleens were fused with myeloma cells and hybrids cultivated as described by Fazekas et al. (17). Production of antibodies in the culture medium was verified as soon as cell growth 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 again cloned. 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 2,000 x g and the supernatant stored at -80°C until use or further purification.

Immunoglobulins were purified from ascitic fluid by DEAE-affigel blue chromatography according to Bruck et al. (18).

Screening

Screening for antibody-secreting cells was performed by an enzyme-linked immunosorbent assay (Elisa) as described elsewhere (19).

Electrophoresis and electroblotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and protein transfer on a nitrocellulose sheet by electroblotting has been described by Guengerich (5). Purified cytochrome P-450 fractions obtained from Professor F.P. Guengerich were identified according to his nomenclature (5). βNF-B, PCN-E, ISF-G and PB-B, respectively, were the main fractions isolated from β-naphthoflavone-, pregnenolone carbonitrile-, isosafrole-, and phenobarbital-treated rat liver microsomes. PB-D and PB-C were two minor forms isolated from phenobarbital-induced rat liver.

Monooxygenase activities

The various monooxygenase activities were determined according to published methods (benzopyrene hydroxylase (20), steroid-16α-hydroxylase (20), aldrin epoxidase (22) ethoxycoumarin deethylase (23)). The effect of the antibodies on these enzyme activities was investigated as follows: the antibodies were preincubated with the microsomes in the incubation medium for 15 min at 37°C. The enzymatic reaction was then started by the addition of the cofactors (NADPH regenerating system). Control samples were microsomes preincubated with a corresponding amount of inactive antibodies (IgG prepared against a different protein).

RESULTS AND DISCUSSION

Antibodies

Three antibody-producing clones (2A2, 2C2, 4D6) were selected and grown in mice. Immunoglobulins were purified from 2A2 by chromatography on DEAE affigel blue. The nature of the antibodies was analyzed by an Elisa test using specific rabbit antibodies against the various classes and light chains of mouse immunoglobulins. The results (table 1) show that 2A2 and 4D6 are primarily composed of γ_1 immunoglobulins with a κ light chain, and 2C2 of a γ_2 immunoglobulin with a κ light chain; as 2C2 and 4D6 are not totally purified immunoglobulins, they are contaminated with some unspecific antibodies present in the ascitic fluid.

The titer of the monoclonal antibodies was determined by an Elisa test on plates coated with purified cytochrome P-450 PB-B. It was arbitrarily defined as the amount of immunoglobin (expressed in pg protein in the preparation) required to obtain 50% of the maximum response with 1 fmole of cytochrome P-450. The titer varied from 36.4 to 230 pg/fmol (table 1) and enabled us to dilute the antibodies (1/200 to 1/500) for most applications. We routinely used a 1/250 dilution of the antibody for quantitating the cytochrome PB-B after electroblotting.

Specificity of the antibodies

Two methods, the Elisa test and electroblotting, were used to verify the specificity of the antibody preparations. For the Elisa test, different

Table 1: Characteristics of the three monoclonal antibodies

Antibody	Subclass	Light chain	IgG/total protein (%)	Titer pg/fmol
2A2	IgG_1	κ	28.3	64.5
2C2	IgG_{2a}	κ	9.2	230
4D6	IgG_1	κ	8.9	36.4

IgG content is expressed as a percentage of the total protein content of the preparation. The titer is calculated in Pg of protein necessary to produce a 50% saturation reaction with 1 fmole of cytochrome P-450 PB-B.

Table 2: Specificity of the different antibodies as measured by the ELISA test

P-450 fractions	Antibodies		
	2AC	2C2	4D6
PB-B	1.30	1.10	1.70
PB-C	0	0	0.05
PB-D	0.80	0.60	0.20
βNF-B	0.1	0	0
βNF-ISF-G	0.02	0	0.04
Microsomes PB	1.50	1.20	1.70
UT	0.14	0.19	0.29
βNF	0.16	0.15	0.34

Five hundred fmoles per hole were coated for each fraction. Results are expressed as optical density at 405 nm.

purified cytochrome P-450 fractions and solubilized microsomes were coated (500 fmoles/hole) on microtiter plates. The plates were incubated with the various antibodies and the resulting complex was finally visualized by a colorimetric reaction. The optical density evaluated at 405 nm was a direct measurement of the antibody-antigen reaction (table 2). The three antibodies were specific for cytochrome P-450 PB-B. They did not react with other fractions tested except for PB-D. This is well known that these two cytochrome P-450s share immunological properties (24). The reaction with microsomal proteins seemed proportional to the amount of PB-B present in the preparation; the greatest reaction was obtained with PB microsomes.

These observations were confirmed by the results obtained after electroblotting (figure 1). Different cytochrome P-450 fractions as well as liver microsomes were separated by electrophoresis in the presense of sodium dodecyl sulfate on a polyacrylamide gel and then transferred by electroblotting to a nitrocellulose sheet. The nitrocellulose sheet was then incubated with the monoclonal antibodies. Positive reactions were observed only with isoenzymes PB-B and to a lesser extent, with PB-D. None of the other purified fractions reacted; moreover, only one band was revealed by antibodies in microsomes. This observation confirmed the high specificity of the monoclonal antibodies.

Another but less interesting characteristic of these antibodies was their inability to form an antibody-antigen complex precipitate. Various attempts were made to overcome this difficulty: ouchterlony double diffusion, precipitation in the presence of protein A, large excess of rabbit anti-mouse IgG serum, mouse serum, etc. The lack of precipitation was a limitation to these experiments because it eliminated the possibility of homogenous phase assays.

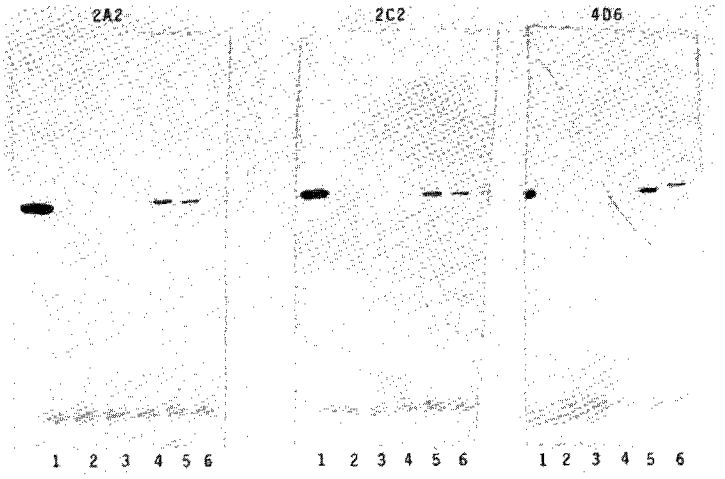

Figure 1: Specificity of the monoclonal antibodies.
A polyacrylamide gel electrophoresis followed by an electroblotting of the purified P-450 fractions was performed. The nitrocellulose sheet was subsequently incubated with the monoclonal antibodies. The nature and amount of cytochrome P-450 in each lane are as follows: 1) 5 pmol PB-B; 2) 4.2 pmol βNF-B; 3) 3 pmol PCN-E; 4) 2.7 pmol PB-C; 5) 2 pmol PB-D; 6) 4 pmol ISF-G.

Monooxygenase activity and inhibition by antibodies

Liver microsomes from rats induced by phenobarbital were preincubated with monoclonal antibodies and then routinely incubated with the substrate and necessary cofactors. The enzymatic activities were compared to those of the controls obtained by preincubation of the same microsomes with inactive immunoglobulins. Figure 2 shows the results obtained for four different monooxygenase activities; none were inhibited to more than 50%. Incomplete monooxygenase inhibition by monoclonal antibodies has already been observed by others (25,26). Some monoclonal antibodies have been reported to be inhibitory while not immunoprecipitating (26). It is not presently possible to explain satisfactorily this phenomenon, but we can hypothesize: the antibody could be linked to a part of the cytochrome molecule located relatively far from the catalytic site, and would thus have a limited effect on the enzymatic activity. Another explanation might be the existence of multiple cytochrome P-450 species. The blocking of a P-450 by the antibody does not exclude the catalytic activity of other species which contribute to the monooxygenase activity in question.

In conclusion, monoclonal antibodies prepared against a phenobarbital-inducible cytochrome P-450 have been shown to be very specific and powerful analytical tools. They will be useful for the characterization and quantitation of the corresponding cytochrome P-450 isoenzymes in small samples, such as cell culture and biopsies.

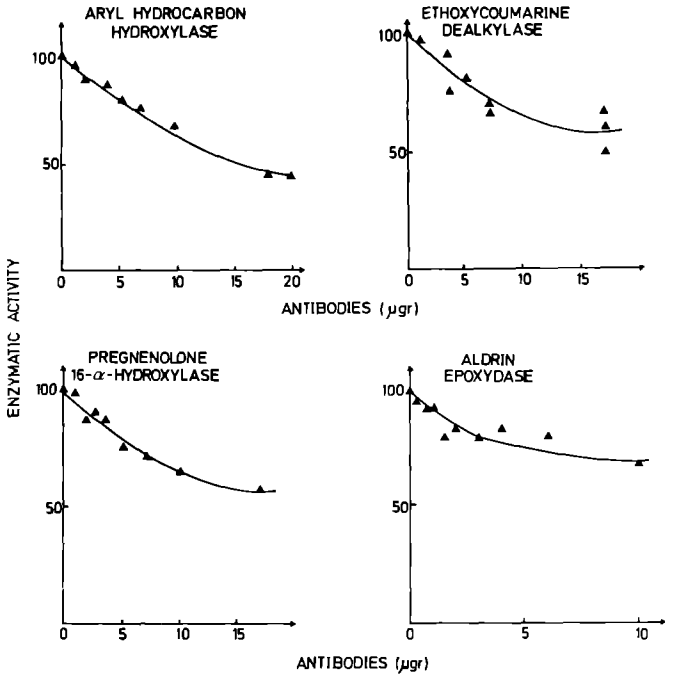

Figure 2: Inhibition of the enzymatic activities by the monoclonal antibody 2A2.
Enzymatic activities are expressed as a percentage of the non inhibited activity, as a fraction of the amount of antibody present in the incubation medium.

ACKNOWLEDGMENTS

The authors thank Professor F.P. Guengerich for the generous gift of purified cytochrome P-450 fractions used as reference material. The authors are grateful to Ms. Janice Lynn Delaval for her assistance in rewriting this manuscript. The authors thank Ms. Martine Verbys for drawing the figures. This work was financially supported by grant n°1072 from the Council for Tobacco Research - U.S.A., Inc. and by grants n° 39001.79, 34522.81, 34523.81 and 35424.81 from the Fonds de la Recherche Scientifique Médicale.

REFERENCES

1. Guengerich, F.P. (1979) Pharm. Thera. 6, 99-121.

2. Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.V. and Kaminsky, L.S. (1982) Biochemistry 21, 6019-6030.

3. Lu, A.Y.H. (1979) Drug Metabolism Reviews 10, 187-208.

4. LeProvost, E., Flinois, J.P., Beaune, P. and Leroux, J.-P. (1981) Biochem. Biophys. Res. Commun. 101, 547-554.

5. Guengerich, F.P., Wang, P. and Davidson, N.K. (1982) Biochemistry 21, 1698-1706.

6. Thomas, P.E., Reik, L.M., Ryan, D.E. and Levin, W. (1981) J. Biol. Chem. 256, 1044-1052.

7. Chen, Y.T., Lang, M.A., Jensen, N.M., Negishi, M., Tukey, R.H., Sidransky, E., Guenther, T.M. and Nebert, D.W. (1982) Eur. J. Biochem. 122, 361-368.

8. Lewis, K.M., Boobis, A.R., Slade, M.B. and Davies, D.S. (1982) Biochem. Pharmacol. 31, 1815-1817.

9. Friedman, F.K., Robinson, R.C., Park, S.S. and Gelboin, H.V. (1983) Biochem. Biophys. Res. Commun. 116, 859-865.

10. Harada, N. and Omura, T. (1981) J. Biochem. 89, 237-248.

11. Dean, W.L. and Coon, M.J. (1977) J. Biol. Chem. 252, 3255-2361.

12. Guenthner, T.M. (1983) Trends Pharmacol. Sc. 4, 6.

13. Park, S.S., Cha, S.J., Miller, H. Persson, A.V., Coon, M.J. and Gelboin, H.V. (1982) Molec. Pharm. 21, 248-258.

14. Park, S.S., Persson, A.V., Coon, M.J. and Gelboin, H.V. (1980) FEBS Letters 116, 231-235.

15. Fujino, T., Park, S.S., West, D. and Gelboin, H.V. (1982) Proc. Nat. Acad. Sci. U.S.A. (New York) 79, 3682-3686.

16. Warner, M., Villa La Merca, M. and Neims, A.H. (1978) Drug Metab. Disp. 6, 353-362

17. Fazekas, D.S., Groth, S. and Scheidegger, D. (1980) J. Immunol. Meth. 35 1-21.

18. Bruck, C., Portelle, D., Glineur, C. and Bollen, A. (1982) J. Immunol. Meth. 53, 313-319.

19. Paye, M., Beaune, P. Kremers, P., Guengerich, F.P. and Gielen, J.E. (1983), submitted to Analytical Biochemistry.

20. Van Cantfort, J., De Graeve, J. and Gielen, J.E. (1977) Biochem. Biophys. Res. Commun. 79, 505-512.

21. Kremers, P., De Graeve, J., Azhir, A. and Gielen, J.E. (1977) Eur. J. Biochem. 82, 529-533.

22. Wolff, T., Deml, E. and Wanders, H. (1979) Drug Metab. Disp. 7, 301-305.

23. Greenlee, W.F. and Poland, A. (1978) J. Pharmacol. Exp. Thera. 205, 596-605.

24. Reik, L.M., Levin, W., Ryan, D.G. and Thomas, P.E. (1982) J. Biol. Chem. 257, 3950-3957.

25. Park, S.S., Fujino, T., West, D., Guengerich, F.P. and Gelboin, H.V. (1982) Cancer Res. 42, 1708-1808.

26. Boobis, A.R., Slade, M.B., Stern, C., Lewis, K.M. and Davies, D.S. (1981) Life Sciences 29, 1443-1448.