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IMMUNOCHEMICAL STUDY ON THE PARTICIPATION OF CYTOCHROME b_5 IN DRUG OXIDATION REACTIONS OF MOUSE LIVER MICROSOMES

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Summary: Highly purified divalent and monovalent antibodies against cytochrome b_5, anti-b_5 immunoglobulin G (IG) and anti-b_5 Fab', were used in elucidating the role of this cytochrome in the drug-oxidizing enzyme system of mouse liver microsomes. Anti-b_5 IG strongly inhibited not only NADH-supported but also NADPH-supported oxidation of 7-ethoxycoumarin and benzo-(a)pyrene, but had no inhibitory action on the oxidation of aniline. Anti-b_5 Fab' also inhibited NADH-supported and NADPH-supported benzo(a)pyrene hydroxylation. These observations indicate an essential role of cytochrome b_5 in the transfer of electrons not only from NADH but also from NADPH to cytochrome P-450 in the microsomal oxidation of some drugs, but not of aniline.

It is generally accepted that cytochrome b_5 is involved in NADH-supported monooxygenase reactions catalyzed by liver microsomes (1-4). Cytochrome b_5 also plays an essential role even when NADPH is used as the electron source in stearyl CoA desaturation (5,6) and laurate hydroxylation (4) reactions of liver microsomes. However, general agreement has not yet been made as to the involvement of cytochrome b_5 in NADPH-supported drug oxidation reactions of liver microsomes. Hildebrandt and Estabrook (1) first proposed that the second of the two reducing equivalents required for one cycle of the oxidation process is supplied to cytochrome P-450 via cytochrome b_5 even in the NADPH-supported oxidation of ethylmorphine. On the contrary, several investigators (7,8) reported that the incorporation of detergent-purified cytochrome b_5 into the microsomal membrane inhibited NADPH-supported oxidation of aminopyrine and benzphetamine. Reconstitution experiments by Lu and co-workers (9) indicated that cytochrome b_5 was required for the maximal rate of NADPH-supported oxidation of chlorobenzene by purified cytochrome P-450, whereas it was not required for the oxidation of the same

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substrate by cytochrome P-448. Recently Imai and Sato (10) confirmed the stimulation by cytochrome b_5 of NADPH-dependent benzphetamine N-demethylation catalyzed by a reconstituted system, which supported the participation of cytochrome b_5 in NADPH-dependent drug oxidation reactions.

We studied the role of cytochrome b_5 in both NADH-supported and NADPH-supported oxidation of several drugs by mouse liver microsomes using highly purified divalent and monovalent antibodies against cytochrome b_5, and we confirmed the participation of the cytochrome in the oxidation of 7-ethoxycoumarin and benzo(a)pyrene. On the other hand, our immunochemical study did not support the participation of cytochrome b_5 in microsomal aniline hydroxylation. The role of cytochorme b_5 in the NADPH-dependent microsomal monooxygenase reactions varies significantly according to the substrates used.

MATERIALS AND METHODS

Male albino mice (DDD strain, 15-25 g body weight) were kept on a commercial rat chow (Nippon Clea Co.). They were starved for 20 h before use, and liver microsomes were prepared as described previously (11).

Cytochrome b_5 was purified from rat liver microsomes by the method of Omura and Takesue (12) as modified by Ito (13). Antibody against cytochrome b_5 was prepared as described previously (11). The immunoglobulin fraction was purified by ammonium sulfate fractionation and specific antibody was obtained by the affinity chromatography using an antigen-conjugated Sepharose 4B as described previously (11). Monovalent fragment of the purified antibody (Fab') was prepared by a modified procedure of Nisonoff et al. (14), the detail of which will be published elsewhere (15).

NADPH-cytochrome c reductase and NADH-cytochrome c reductase activities were determined as described previously (12). 7-Ethoxycoumarin O-deethylation, benzo(a)pyrene hydroxylation, and aniline hydroxylation were assayed as described by Ullrich and Weber (17), Nebert and Gelboin (18), and Imai et al. (19), respectively. When antibody was added to assay mixtures, microsomes were preincubated with the antibody for 10 min. The concentrations of immunoglobulin in each set of assay mixtures were made up to the same by the addition of control immunoglobulin obtained from non-immunized rabbits. Protein concentrations were determined by the method of Lowry at al. (16) using bovine serum albumin as the standard.

RESULTS

As shown in Table I, purified anti-b_5 IG and anti-b_5 Fab' inhibited NADH-cytochrome c reductase activity of mouse liver microsomes without affecting NADPH-cytochrome c reductase activity, although the inhibition by the Fab' was significantly weaker than that by the IG. The specificity of

Table I

Effects of anti-b_5 IG and anti-b_5 Fab' on NADH- and NADPH-supported cytochrome c reductase activities of mouse liver microsomes.

	control IG (5 mg/mg Ms protein)	anti-b_5 IG	control Fab' (20 mg/mg Ms protein)	anti-b_5 Fab'
	(%)		(%)	
NADH-cyt. c reductase	100	21.6	100	69.6
NADPH-cyt. c reductase	100	94.9	100	97.7

The reaction mixture contained microsomes, antibody, 20 μM cytochrome c, 0.5 mM KCN, and 0.1 mM NADH or 0.1 mM NADPH in 2 ml of 0.1 M potassium phosphate buffer (pH 7.5). The amounts of microsomes were 0.05 and 0.1 mg for NADH-supported and NADPH-supported reactions, respectively. Control activities were 0.763 and 0.213 μmoles of cytochrome c reduced per min per mg of microsomal protein for NADH-supported and NADPH-supported reactions, respectively. Neither control IG nor control Fab' affected the activities. Ms stands for microsomes.

the antibody and the antigenic similarity between rat and mouse cytochrome b_5's were also confirmed by the Ouchterlony double diffusion analysis as described previously (11). The monovalent antibody did not form precipitates when incubated with the antigen.

In accordance with our previous observations (11), purified anti-b_5 IG strongly inhibited the NADH-supported O-deethylation of 7-ethoxycoumarin as shown in Fig. 1. The corresponding NADPH-supported reaction was also inhibited by the antibody, although the extent of inhibition was significantly lower than that of the NADH-supported reaction. The addition of NADH enhanced the rate of the NADPH-supported O-deethylation by about 20 %. Anti-b_5 IG not only exerted an inhibitory action on this enhanced portion of the reaction as reported by Mannering et al. for the N-demethylation of ethylmorphine by rat liver microsomes (20) but also further inhibited the reaction to the same level as when NADPH was the sole electron donor (Fig. 1).

The aniline hydroxylation was not sensitive to the inhibition by anti-b_5 IG (Fig. 2). Neither NADPH-supported nor NADH-supported reaction was inhibited by the antibody even at a concentration of 10 mg antibody per mg of microsomal protein where the oxidation of 7-ethoxycoumarin (Fig. 1) and benzo(a)pyrene (Fig. 3) were both strongly inhibited. Therefore, the

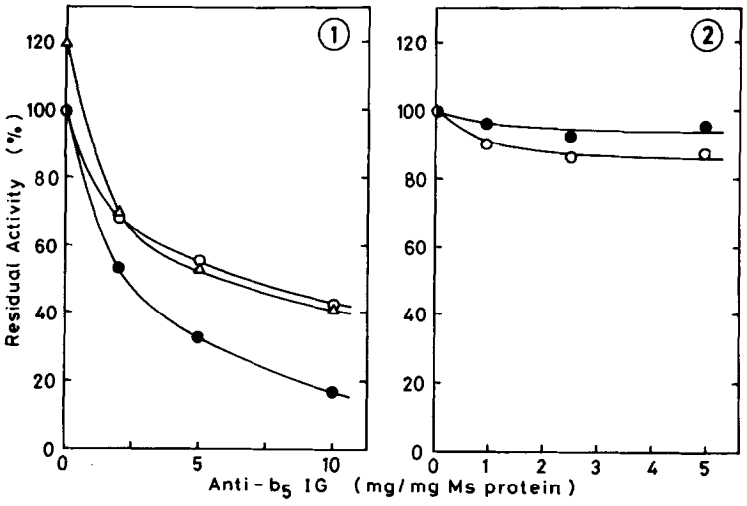

Fig. 1 Effects of anti-b_5 IG on 7-ethoxycoumarin O-deethylation. The reaction mixture contained microsomes, antibody, and 1 mM 7-ethoxycoumarin in 2 ml of 0.1 M Tris-HCl (pH 7.6). Microsomal protein concentrations were 0.1 and 0.2 mg for NADPH-supported and NADH-supported reactions, respectively. The assay was started by the addition of 0.1 mM NADPH and/or 0.1 mM NADH at 30 °C. Control activities were 4.50, 5.34, and 0.259 nmoles of product formed per min per mg of microsomal protein for NADPH-supported (o——o), NADPH plus NADH-supported (△——△), and NADH-supported (●——●) reactions, respectively. In order to indicate the synergistic effect of NADH, the control activity in the presence of NADPH plus NADH is shown in the Figure at 120 %.

Fig. 2 Effects of anti-b_5 IG on aniline hydroxylation. The reaction mixture contained 0.4 mg microsomes, antibody, 10 mM aniline, 0.1 mM EDTA, and NADPH-generating system or 5 mM NADH in 2 ml of 50 mM Tris-HCl (pH 8.0). Incubation was started by the addition of aniline and continued for 20 min. Control activities were 3.16 and 1.16 nmoles of p-aminophenol formed per min per mg of microsomal protein for NADPH-supported (o——o) and NADH-supported (●——●) reactions, respectively.

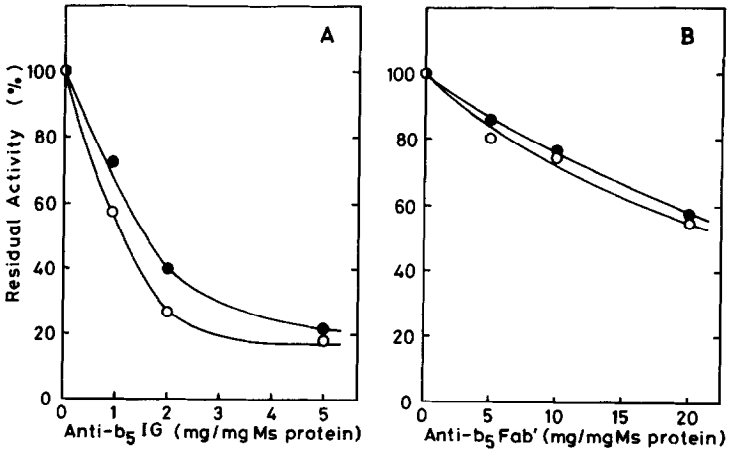

Fig. 3 Effects of anti-b_5 IG (A) and anti-b_5 Fab' (B) on benzo(a)pyrene hydroxylation. The reaction mixture contained 0.1 mg of microsomes, antibody, 0.08 mM benzo(a)pyrene, 0.1 mM EDTA, and NADPH-generating system

antibody against cytochrome b_5 exerts variable inhibitory action on microsomal drug oxidation reactions according to substrates used, suggesting different extent of participation of cytochrome b_5 in the reactions.

Although anti-b_5 IG used in this study was specific to cytochrome b_5 according to all immunochemical criteria examined, the addition of the divalent antibody to microsomes could have induced the aggregation of the antigen molecules in the membrane resulting in an alteration of the electron transport pathway from NAD(P)H to cytochrome P-450. We prepared a monovalent antibody preparation against cytochrome b_5, anti-b_5 Fab', and examined its inhibitory action on the oxidation of benzo(a)pyrene, which was most sensitive to the inhibition by anti-b_5 IG, as shown in Fig. 3. When we compare Fig.'s 3A and 3B, it is evident that the monovalent antibody was much less effective in inhibiting the hydroxylation of the substrate than the native antibody. However, both NADH-supported and NADPH-supported reactions were equally inhibited by anti-b_5 Fab' (Fig. 3B). The weaker inhibitory action of anti-b_5 Fab' on the drug oxidation reaction corresponds with its lower inhibition of the NADH-cytochrome c reductase activity (Table I) and could be explained by a weaker steric hindrance due to the decreased molecular size.

DISCUSSION

Antibodies are the only available site-specific inhibitors in studying the electron transport pathway of intact microsomes. In this study, highly purified antibody preparations against cytochrome b_5 were used in elucidating the participation of this cytochrome in microsomal drug oxidation reactions. The monovalent fragment (Fab') of the antibody was also prepared and used in order to eliminate the possibility of the antibody-induced rearrangement of the antigen molecules in the membrane.

Our results demonstrate the involvement of cytochrome b_5 in the oxidation of some drugs, such as 7-ethoxycoumarin and benzo(a)pyrene, even

or 5 mM NADH in 1 ml of 0.1 M Tris-HCl (pH 7.5). The incubation was started by the addition of benzo(a)pyrene and carried out at 37 °C for 10 min. Control activities were 0.208 and 0.106 for NADPH-supported (o—o) and NADH-supported (●—●) reactions, respectively.

when the reducing equivalents for the reaction are donated by NADPH. However, cytochrome b_5 does not seem to be involved in the hydroxylation of aniline. Lu et al. (9) suggested the involvement of cytochrome b_5 in microsomal drug oxidation reactions based on their observations using reconstituted systems, but they concluded (3) that the cytochrome is not an obligatory component of the systems when NADPH was used as the source of reducing equivalents. Our observations using intact microsomes strongly indicate the essential role of cytochrome b_5 in NADPH-supported oxidation of benzo(a)pyrene, since the reaction was almost completely inhibited by anti-b_5 IG.

We recently presented (11) immunochemical evidence for the participation of NADPH-cytochrome c reductase in NADH-supported drug oxidation reaction as well as in NADPH-supported reactions. Our present study confirmed the participation of cytochrome b_5 in the microsomal oxidation of some, but not all, drugs even when NADPH was used as the electron source. In the case of aniline hydroxylation, in which cytochrome b_5 does not participate, both first and second electrons are possibly donated to cytochrome P-450 by NADPH-cytochrome c reductase. On the other hand, it is very likely that the second electron for the benzo(a)pyrene hydroxylation reaction, which requires cytochrome b_5 as an essential component, is solely supplied via cytochrome b_5. The different dependency of cytochrome P-450-catalyzed drug oxidation reactions on cytochrome b_5 is possibly due to different affinities of multiple forms of cytochrome P-450 to cytochrome b_5. The isolation of a species of microsomal cytochrome P-450 having a high affinity to cytochrome b_5 was recently reported (21).

REFERENCES

1. Hildebrandt, A. & Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 66-79.
2. Jansson, I. & Schenkman, J. B. (1977) Arch. Biochem. Biophys. 178, 89-107.
3. Lu, A. Y. H., West, S. B., Vore, M., Ryan, D. & Levin, W. (1974) J. Biol. Chem. 249, 6701-6709.

4. Sasame, H. A., Thorgeirsson, S. S., Mitchell, J. R. & Gillette, J. R. (1974) Life Sci. 14, 35-46.
5. Oshino, N., Imai, Y. & Sato, R. (1971) J. Biochem. 69, 155-167.
6. Oshino, N. & Omura, T. (1973) Arch. Biochem. Biophys. 157, 395-404.
7. Jansson, I. & Schenkman, J. B. (1973) Mol. Pharmacol. 9, 840-845.
8. Hrycay, E. G. & Estabrook, R. W. (1974) Biochem. Biophys. Res. Commun. 60, 771-778.
9. Lu, A. Y. H. & Levin, W. (1974) Biochem. Biophys. Res. Commun. 61, 1348-1355.
10. Imai, Y. & Sato, R. (1977) Biochem. Biophys. Res. Commun. 75, 420-426.
11. Noshiro, M. & Omura, T. (1978) J. Biochem. 83, 61-77.
12. Omura, T. & Takesue, S. (1970) J. Biochem. 67, 249-257.
13. Ito, A. (1979) J. Biochem. in press.
14. Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960) Arch. Biochem. Biophys. 89, 230-244.
15. Masters, B. S. S., Noshiro, M., Harada, N. & Omura, T. in preparation.
16. Lowry. O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
17. Ullrich, V. & Weber, P. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1171-1177.
18. Nebert, D. W. & Gelboin, H. V. (1968) J. Biol. Chem. 243, 6242-6249.
19. Imai, Y., Ito, A. & Sato, R. (1966) J. Biochem. 60, 417-428.
20. Mannering, G. J., Kuwahara, S. & Omura, T. (1974) Biochem. Biophys. Res. Commun. 57, 476-481.
21. Miki, S. & Yamano, T. (1978) Seikagaku (in Japanese) 50, 784.