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INHIBITION OF AZOREDUCTASE ACTIVITY BY ANTIBODIES AGAINST CYTOCHROMES P-450 AND P-448

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SUMMARY: Hepatic microsomal azoreductase activity in mice was induced with phenobarbital (PB) and 3-methylcholanthrene (3-MC). Antibodies against cytochrome P-450 inhibited azoreductase activity of PB-treated animals while antibodies against cytochrome P-448 inhibited liver azoreductase activity of 3-MC-treated animals, each by about 90%. These antibodies also inhibited microsomal 7-ethoxycoumarin-O-deethylase activity to the same extent. It is concluded that hepatic microsomal azoreductase activity is almost totally dependent on cytochromes P-450 and P-448 and the contribution, if any, of other microsomal components is negligible.

The study of the enzymatic reduction of the azo linkage is of clinical importance for a variety of reasons. Many azo compounds are carcinogenic and the reduction of these materials leads to their inactivation (1). In contrast, the activation of a recently proposed antineoplastic drug (2-4) depends on the cleavage of the azo linkage by a reductase so as to produce a short-lived, potent, alkylating agent, which in turn interacts with cancer cell chromatin.

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Hernandez et al. (5,6) suggested that three microsomal components are responsible for hepatic microsomal azoreductase activity. These are: I) NADPH-cytochrome c reductase; II) a CO sensitive component, presumably cytochrome P-450; and III) a CO insensitive component induced by 3-MC, which is different from I and II. For the sake of convenience, we tentatively term these components as "azoreductase I, II, and III" respectively, in the following discussion.

In 1978 Fujita and Peisach (7) demonstrated that azoreductase activity in liver microsomes, using amaranth as substrate, is mediated solely by cytochromes P-450 and P-448. This conclusion was drawn from experiments in which normal and DBA/2J mutant mice were treated with PB and 3-MC. In the mutant mice, there is a genetic defect in the Ah locus which is known to regulate cytochrome P-448 induction. Cytochrome P-450 induction by PB, however, is not impaired. It was found that in the mutant mice, 3-MC treatment did not cause the induction of cytochrome P-448, as expected, nor did it cause the induction of azoreductase activity. In addition, the levels of liver microsomal azoreductase activity in control, 3-MC- and PB-treated mutant and normal animals was directly proportional to the levels of P-450 type cytochromes. In all cases ~90% inhibition by CO was observed. These results appear to rule out azoreductase III and lend support to the view that azoreductase II is the sole enzymic catalyst for azo reduction in liver microsomes. It might be argued, though, that there is a specific azoreductase III gene that is somehow linked to the Ah locus in

mutant mice and therefore both the azoreductase gene and the P-448 gene are not expressed in the DBA/2J strain. Such co-segregation of two drug metabolizing enzymes had been observed previously with cytochrome P-448 and either UDP-glucuronyl-transferase (8) or epoxide hydratase (9). Also, one might argue that the use of CO inhibition to demonstrate the dependence of azoreductase activity on P-450 type cytochromes is fallacious, because of possible O_2 contaminants in the CO used in the experiments. O_2 has been shown to markedly inhibit liver microsomal azo reduction.

In order to clarify whether P-450 type cytochromes are totally responsible for hepatic microsomal azoreductase activity as proposed by Fujita and Peisach (7) or whether other components play a role, as suggested by Hernandez et al. (5,6), we have prepared antibodies against cytochromes P-450 and P-448. The effect of these antibodies on liver microsomal azoreductase activity in PB- and 3-MC-treated mice was studied. A comparison was also made with the effect of these antibodies on 7-ethoxycoumarin-O-deethylase activity, which had been previously shown to be dependent on P-450 type cytochromes (10).

MATERIALS AND METHODS

Preparation of Microsomes - Two groups of C57B/6J mice, each consisting of 10 animals were pretreated with a single i.p. injection of PB (100 mg/kg) or 3-MC (80 mg/kg). Animals were killed by decapitation 24 hr after the injection and the microsomes were prepared by differential contrifugation. Cytochrome P-450 content was determined according to the method of Omura and Sato (11,12). Protein concentrations were determined by the Lowry procedure (13).

Preparation of Antibodies - Cytochrome P-450 was purified from PB-treated rat liver microsomes by the method of Negishi et al.

(14) while cytochrome P-448 from β-naphthoflavone-treated animals by the method of Hashimoto and Imai (15). The specific contents of heme were 14.1 and 12.1 nmol/mg protein for cytochrome P-450 and P-448, respectively. 700 μg of purified cytochrome was mixed with an equal volume of Freund's complete adjuvant (DIFCO) and was injected subcutaneously into a white rabbit. Three weeks later, the rabbit's antibody titer was boosted with the same cytochrome preparation by i.v. injections through the ear vein. One week later, the serum was separated from the blood of the immunized animal and was fractionated with ammonium sulfate (0-45% saturation). Immunoglobulin fractions were dissolved in 10 mM sodium phosphate buffer, pH 7.5, containing 0.9% NaCl to a concentration of approximately 40 mg protein/ml.

Azoreductase Assay - Azoreductase activity at 25° was assayed by a method described earlier (7). In addition to an NADPH generating system and the substrate amaranth, antibodies against cytochromes P-450 and P-448 in concentrations 10 to 80 times those of microsomal protein were added to the reaction mixture. For controls, immunoglobulin fractions from non-immunized rabbit serum was added in corresponding concentrations.

7-Ethoxycoumarin-O-Deethylase (ECD) Assay - ECD activity was assayed according to a method described by Ullrich (10). The assay mixture contained (final concentrations) 0.2 mM 7-ethoxycoumarin, 1 mM glucose-6-phosphate, 0.6 units of glucose-6-phosphate dehydrogenase and 0.02 mg/ml microsomal protein, in 0.1 M Tris HCl buffer, pH 7.5. Antibody was added to a total volume 2.5 ml. The reaction was started by the addition of (40 μM) NADP. The increase in fluorescence was followed using a Hitachi 204-A fluorimeter. The excitation wavelength was set at 380 nm with a slit width of 10 nm and the emission wavelength was set at 460 nm with a slit width of 20 nm.

RESULTS AND DISCUSSION

The azoreductase activity of liver microsomes from PB- (PB microsomes) and 3-MC- (3-MC microsomes) treated mice studied at 25° (Table I) is about one-third of the activity studied at 37° (7). As before (7), there is no significant difference between PB and 3-MC microsomes in the specific activity of azoreductase based on P-450 type cytochrome content. In contrast, 3-MC-microsomes show greater ECD activity than do PB-microsomes, clearly indicating that cytochrome P-448 is more effective in O-deethylation of 7-ethoxycoumarin than is cytochrome P-450.

TABLE I

Activities of Azoreductase and 7-Ethoxycoumarin-O-Deethylase in Liver Microsomes from PB- and 3-MC-Treated Mice Studied at Room Temperature

Treatment	Azoreductase		7-Ethoxycoumarin-O-deethylase	
	nmol/mg/min	mol/P-450/min	nmol/mg/min	mol/P-450/min
PB	1.9 ± 0.1	1.50	3.4 ± 0.1	2.7
3-MC	1.8 ± 0.1	1.46	4.2 ± 0.1	3.4

Assay methods are described in the text. Enzyme activities are expressed as nmol amaranth reduced or nmol 7-hydroxycoumarin formed/mg microsomal protein/min at 25°. The cytochrome P-450 content of microsomes from PB-treated animals was 1.26 ± 0.02 nmol/mg protein while those from 3-MC-treated animals was 1.23 ± 0.02 nmol/mg protein.

Fig. 1 demonstrates that azoreductase activity of PB-microsomes (A) is inhibited about 90% by anti-P-450 antibodies while azoreductase activity of 3-MC microsomes is inhibited about 90% by anti-P-448 antibodies. The extent of inhibition is about the same as that caused by CO (7). Anti-P-448 antibodies slightly inhibit azo reduction in PB-microsomes while anti-P-450 antibodies show some inhibitory effect on azo reduction in 3-MC microsomes. Certainly, the greater inhibitory effect is observed with the antibody specific to the appropriate cytochrome.

Fig. 1 also indicates the effect of these antibodies on ECD metabolism. ECD activity in PB microsomes (A) is inhibited by anti-P-450 antibodies by about 90% and by anti-P-448 antibodies about 20%. This same activity is never inhibited by anti-P-448 antibodies more than 70% in 3-MC microsomes (B). If however, P-450-antibodies

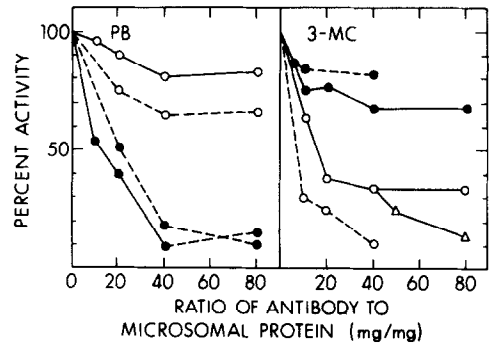

Fig. 1. Mouse hepatic microsomal azoreductase and 7-ethoxy-coumarin-O-deethylase (ECD) activities from animals treated with (left) phenobarbital and (right) 3-methylcholanthrene. Activities in the presence of antibodies to cytochrome P-450 and cytochrome P-448 were measured as described in the text. Antibody protein concentrations of up to 80 times that of microsomal protein were present in the assay mixture. For a control, the immunoglobulin fraction from non-treated rabbits was added in the same protein concentrations as anti-P-450 or anti-P-448 antibodies. ● : % activity in the presence of anti-P-450 antibodies; o : % activity in the presence of anti-P-448 antibodies; ---: azoreductase activity; —— : ECD activity; Δ : ECD activity in the presence of additional anti-P-450 antibodies.

now supplement the already 70% inhibited 3-MC microsomes, further inhibition is observed. These results demonstrate that the extent of inhibition of azoreductase activity by anti-P-450 and P-448 antibodies is comparable to the extent of inhibition of ECD activity, already known to be cytochrome P-450 dependent. This rules out azoreductase III since no 3-MC inducible azoreductase, other than cytochrome P-448, can be demonstrated. Although azoreductase I is active in purified systems (8), its contribution to microsomal azo reduction is negligible. Thus, azoreductase II, the P-450 type cytochromes, is almost completely responsible for azo reduction in isolated liver microsomes.

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