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RAPID COMMUNICATION

MONOCLONAL ANTIBODIES TO ETHANOL INDUCED CYTOCHROME P-450 THAT INHIBIT ANILINE AND NITROSAMINE METABOLISM

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Multiple forms of cytochrome P-450 are central to the metabolism of xenobiotics such as drugs, carcinogens, and environmental chemicals as well as endobiotics such as fatty acids, prostaglandins and steroids. Ethanol induced cytochrome P-450 (1-3) is of special importance and potential relevance to human biochemical epidemiology. First, its induction by ethanol makes its study important for the alcohol consuming population in respect to its genetics, possible polymorphisms and its role in xenobiotic and endobiotic metabolism. Second, the ethanol induced P-450 exhibits high activity towards nitrosamines (2,3), a class of carcinogens that are common in the environment and are believed to cause cancer in humans. Monoclonal antibodies (MAbs) (4-6) are epitope specific for individual and classes of cytochromes P-450 and are powerful tools for probing cytochrome P-450 multiplicity and function (7-9). We have constructed a library of MAbs (4-6) that can be used for simple immunopurification of cytochrome P-450 (7) and for quantitative tissue phenotyping by radioimmunoassay (8) for epitope specific individual and classes of cytochromes P-450. MAbs that inhibit the enzyme activity of the cytochromes P-450 to which they bind are also extremely useful for "reaction phenotyping", i.e. the quantitative determination of the contribution of the epitope containing cytochromes P-450 to the total activity of a tissue for any P-450 substrate. This technique has been successfully applied for analyzing specific cytochrome P-450 function in both animal and human tissues (9). In the present study we describe the isolation and characterization of two MAbs to ethanol induced cytochrome P-450 (P-450et). One of these MAbs has potent inhibitory activity toward the aniline p-hydroxylase (APH) and nitroso-dimethylamine demethylase (NDMAD) activities of the P-450et and acetone induced cytochrome P-450 (P-450ac). This MAb may be useful for both basic studies of these cytochromes P-450 and studies in human biochemical epidemiology related to ethanol consumption and susceptibility to nitrosamine carcinogenesis.

Young male Sprague-Dawley rats were fed a commercial laboratory diet (Ralston-Purina Co., St. Louis, MO). The ethanol treatment and acetone treatment were as described (2,3). Liver microsomes were prepared by differential centrifugation (2). P450et and P-450ac were isolated by a modified procedure of Tu and Yang (3). Microsomes were suspended in buffer A (50 mM sodium phosphate, pH 7.25, containing 25% glycerol) to a protein concentration of 3 mg protein/ml, and were solubilized by the addition of Emulgen 911 to a final concentration of 0.2%. The solubilized mixture was applied to a laurate-AH-sepharose 4B column. The latter was washed with buffer A containing 0.4% cholate and then with buffer A containing 0.4% cholate and 0.25 M NaCl. Elution with buffer A containing 4% cholate and 0.8% Emulgen 911 yielded the major fraction of P-450 with a 25-30% yield. The preparation

was further purified by a hydroxylapatite column to remove Emulgen 911. This partially purified preparation was used in all the studies described. The media and hydridoma growth conditions were as in our previous papers describing the preparation of MAbs (4-6). The myeloma cell line used for hybridoma formation was SP2/0-Ag24, an azaguanine resistant line and a non-producer of immunoglobulins (6). The immunizations were essentially as previously described (4-6), using 10 μg of partially purified P-450et injected intraperitoneally for 4 weeks. A week later, 10 μg of antigen was injected intravenously. After sampling the sera for antibody production, antibody producers were kept for 1 month followed by a similar second series of immunizations. After the final intravenous booster injection, the mice were killed and spleen cells were prepared as described previously (4-6). APH activity was determined by measuring the amount of p-aminophenol formed (10). The reaction mixture contained 100 μl of 0.5 M potassium phosphate buffer (pH 7.65), 10 μl NADPH (30 mM), 25 μl $MgCl_2$ (0.1 M) and 50 μl aniline (0.05 M) in a total volume of 1.0 ml. The enzyme source was either 500 μg microsomal protein or a reconstituted P-450 system as described in Tables 2 and 3. After incubation for 20 min at 37° the reaction was stopped with 0.5 ml of 20% trichloroacetic acid and centrifugation for 1 min. To 1.0 ml of the supernatant fraction 0.5 ml of 1 M $NaCO_3$ and 1.0 ml of 2% phenol in 0.2 N NaOH were added. The resulting blue color was measured at 630 nm after standing at room temperature for 20 min. The effect of antibody was measured as described in the tables.

The large panel of hybridoma clones was isolated from the fusion of the spleen cells of the cytochrome P-450et immunized mice and myeloma cells. Of 63 original clones, we found 31 clones that bound to either P-450et or P-450ac or to both. Previous studies have indicated that both ethanol and acetone induce the same form of P-450 (11). Of the 31 positive clones we isolated, only one was highly inhibitory and we report here detailed studies on that clone, MAb 1-91-3, as well as on MAb 1-98-1.

Table 1. Characteristics of Two Monoclonal Antibodies to Ethanol Induced Cytochrome P-450*

Monoclonal Antibodies (MAb)	Ig subclass	Radioimmunoassay binding to P-450 (RIA;cpm)		Double Immunodiffusion to P-450et
		P-450et	P-450ac	
NBS 1-48-5 (Control)	IgG_{2a}	281	523	--
1-91-3	IgG_1	1,348	7,648	+
1-98-1	IgG_1	8,480	10,218	++

*Culture fluids were used as the source of MAbs for the RIA determined binding and for the double immunodiffusion analyses (4-6). The P-450et and P-450ac were different preparations but these two inducers induce the same form of P-450 (11).

Table 1 shows the characteristics of these two MAbs. These clones were also subcloned three times to ensure their monoclonality. Both MAbs are of the IgG_1 subclass and both exhibit highly positive binding activity to the two preparations, P-450et and P-450ac, relative to the binding observed with the non-specific control MAb NBS-1-48-5. The MAb 1-91-3 showed considerably higher binding to the P-450ac than the P-450et. Since these preparations were only partially purified, differences in binding affinity would be affected by the nature of a contaminant in the preparations or by their degree of heterogeneity. Both of the MAbs affected precipitation of the P-450et as measured by double immunodiffusion analyses (Ouchterlony).

Table 2. Effect of Monoclonal Antibodies on Aniline p-hydroxylation by Reconstituted Purified P-450et and P-450ac

Monoclonal Antibodies (200 μg)	3-OH Aniline formed (nmoles/nmole P-450/min)			
	P-450et	% Control	P-450ac	% Control
A. Serum-free culture fluid				
HyHel-9 (Control)	3.99	100	2.88	100
1-91-3	0.28	7	0.05	2
1-98-1	1.64	41	0.62	27
B. Ascites fluid				
HyHel-9 (Control)	2.50	100	1.50	100
1-91-3	0.25	10	0.05	3
1-98-1	4.30	172	3.40	226

Purified cytochrome P-450 (5 μg) and MAbs (200 μg) of serum-free culture fluid concentrates were preincubated in PBS for 15 min at room temperature. The APH activity was measured in the reconstituted system containing NADPH cytochrome P-450 reductase (10 μg) and dilauroyl glycerol-3-phosphocholine (30 μg) at 37° for 20 min. Aniline hydroxylation was measured as described.

Table 2 shows the inhibitory effects of these two MAbs on the APH activity of purified P-450et and P-450ac in a reconstituted mixed-function oxidase system. The hybridoma culture fluid containing MAb 1-91-3 showed a marked and almost complete inhibition of the APH activity of both the P-450et and P-450ac, the respective inhibition being 93 and 98%. This indicates that this MAb is directed to an epitope on a single or epitope defined class of cytochromes P-450 in the P-450et preparation that contributes from 93 to 98% of the total APH activity. This experiment does not distinguish whether one or more than one species of P-450 in the partially purified preparation are responsible for APH. The results, however, definitively indicate that MAb 1-91-3 is directed at an epitope which when bound by the MAb essentially prevents the cytochrome P-450 from functioning as an APH. The MAb 1-98-1 culture fluid only partially inhibited the respective P-450 preparations (59-73%). When the MAbs were obtained by growing the hybridomas as ascites in mouse intraperitoneal cavities, the MAb 1-91-3 again exhibited potent, almost complete (90-97%), inhibition of APH. MAb 1-98-1 stimulated this activity. We do not understand this stimulatory activity but it may be due to allosteric or other modifying effects of the proteins (some undefined) in the ascites fluid.

Table 3 shows the effects of the two MAbs on NDMAD activity of the purified cytochrome P-450ac. Here, the MAb 1-91-3 inhibited NDMAD activity by 92% and MAb 1-98-1 exhibited essentially no effect. Thus, MAb 1-91-3 is directed at an epitope on a single or class of cytochrome P-450 in the partially purified preparation whose binding by the MAb prevented 92% of the NDMAD activity of the preparation.

Table 4 shows the effects of the MAbs on both APH and NDMAD of liver microsomes from acetone-treated rats. The MAb 1-91-3 inhibited these two activities by 54 and 77%. Thus, the "reaction phenotyping" of these two reactions with MAb 1-91-3 indicates that in the acetone induced preparation of rat liver microsomes at least 54% of APH and 77% of NDMAD are due to cytochromes P-450 which contain the MAb 1-91-3 directed epitope. The binding of this epitope by the MAb inhibits the NDMAD activity of this P-450 and quantitates its contribution to the total NDMAD activity of the microsomes. MAb 1-98-1 exhibited virtually no inhibitory effects on this microsomal preparation in respect to either of the enzyme activities measured.

Table 3. *Effect of Monoclonal Antibodies on Nitroso-dimethylamine* Demethylase (NDMAD) Activity of Reconstituted Purified P-450ac

Monoclonal Antibodies (Ascites)	NDMAD* (nmoles HCHO/nmole P-450ac/min)	% Control
HyHel-9 (Control)	4.5	100
1-91-3	0.4	8
1-98-1	4.2	93

*The NDMAD assay system consisted of 0.1 μmole P-450ac (specific content of 13 μmoles/mg protein), 1 unit of P-450 reductase (1.0 μmole cytochrome P-450 reduced/min), 22 μg dilauroylphosphatidyl-choline, 4 mM NDMA, an NADPH generating system and MAb at a final concentration of 100 μg. The incubation volume was 250 μl. The assay was as previously described (2).

Table 4. Effect of MAbs on Aniline p-hydroxylase (APH) and Nitroso-dimethylamine Demethylase (NDMAD) Activities of Liver Microsomes from Acetone Treated Rats*

Monoclonal Antibodies (culture fluids)	APH (nmoles 3OH-aniline/ mg protein/min)	% Control	NDMAD (nmoles HCHO/ mg protein/min)	% Control
NBS 1-48-5 (Control)	1.36	100	--	--
HyHel-9 (Control)	--	--	3.5	100
1-91-3	0.63	46	0.8	23
1-98-1	1.34	99	3.9	111

*Microsomes (500 μg) and MAbs (200 μg) of serum-free culture concentrates were pre-incubated for 15 min at 37° and enzyme activities were as described in Table 3. NBS and HyHel-9 control MAbs were non-specific for cytochromes P-450.

The MAb 1-91-3 has demonstrated binding activity towards both P-450et and P-450ac and exhibited inhibition of their catalytic activities towards both aniline and nitroso-dimethylamine, a highly carcinogenic nitrosamine. This MAb may be useful for tissue and reaction phenotyping required for studies identifying hypersusceptibility in human populations related to alcohol consumption and cancer induced by the nitrosamine class of carcinogens.

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