Cytochrome P-450–Dependent Monooxygenases in Olfactory Epithelium of Dogs: Possible Role in Tumorigenicity

Abstract. *The respiratory tract epithelium of dogs, from the nose to the lungs, was examined for cytochrome P-450 and associated biotransformation activities. In the ethmoturbinates, where olfactory epithelium is located, the amount of cytochrome P-450 was comparable to that in the liver, when measured on the basis of activity per milligram of microsomal protein. The rest of the nasal region also contained large quantities of cytochrome P-450. The presence of these enzymes in the nose may be important in chemical-induced tumorigenesis. The nasal carcinogen hexamethylphosphoramide was shown to be metabolized by nasal microsomal enzymes to another nasal carcinogen, formaldehyde.*

The respiratory tract is continuously exposed to environmental chemicals and contains an array of mechanisms that respond to these chemicals (*1*). One such mechanism is biotransformation brought about by the xenobiotic metabolizing enzymes known as the cytochrome P-450–dependent monooxygenases (MO's). These enzymes facilitate the removal of lipid-soluble compounds by converting them to water-soluble metabolites that may be excreted. However, metabolites of some compounds are highly toxic. Among these compounds are benzo[*a*]pyrene and the solvent hexamethylphosphoramide (HMPA) which are metabolized, wholly or partly, to a dihydro arene oxide (*2*) and formaldehyde (*3*), respectively. The gasoline additive 1,2-dibromoethane (DBE), HMPA, and formaldehyde cause tumors of the nose when inhaled (*4–6*). Both DBE (*7*) and HMPA (*3*) are metabolized by MO's. Formaldehyde, a product of HMPA metabolism, is also a frequent product of other MO catalyzed oxidations. In this report we show, by using HMPA as a substrate, that the nasal enzymes rapidly produce formaldehyde.

Cytochrome P-450–dependent MO's are well known in the liver and in the lung where they are reported to be concentrated in the Clara cells of the conducting airways (*8*). Because the toxicity and fate of inhaled pollutants might be significantly affected by the presence of MO's in the nasal region and other parts of the respiratory tract as well as the lung, we examined these regions for MO's. The respiratory tract of the dog was chosen for these studies because this species shows deposition patterns for inhaled material similar to those in humans (*9*) and because the relatively large size of the animals would allow dissection of samples of sufficient size for enzyme analysis.

Table 1. Cytochrome P-450 content of dog nose, lung, and liver. For dog No. 1, microsomes were suspended in 20 percent sucrose, frozen in liquid nitrogen, and stored at −70°C for 12 hours prior to analysis. For dogs 2 and 3, freshly prepared microsomes were used. Microsomes from dog No. 2 were washed by resuspending the 100,000*g* pellet and recentrifuging; those from dog No. 3 were not washed.

Tissue	Cytochrome P-450 (pmole)					
	Dog No. 1		Dog No. 2		Dog No. 3	
	Per milligram of microsomal protein	Per gram of tissue	Per milligram of microsomal protein	Per gram of tissue	Per milligram of microsomal protein	Per gram of tissue
Nose						
Maxilloturbinate	21	187	42	174	63	239
Ethmoturbinate	166	917	262	896	278	1330
Nasal epithelial membrane	19	130	52	216	44	303
Lung						
Central parenchyma (including terminal airways)	< 10	35	76	298	44*	259*
Subpleural parenchyma	< 5	< 5	98	390		
Liver	264	5580	362	1750	490	6400

*For dog No. 3 a single sample of lung containing both central and subpleural parenchyma was used.

Table 2. Rates of metabolism of *p*-nitroanisole, aniline, and benzo[*a*]pyrene by dog nose, lung, and liver microsomes. All rates are expressed as picomoles per milligram of microsomal protein per minute. For dog No. 1, microsomes were suspended in 20 percent sucrose, frozen in liquid nitrogen, and stored at −70°C for 12 hours prior to analysis. For dog No. 2, freshly prepared microsomes were used.

Tissue	Dog No. 1			Dog No. 2		
	O-Demethylation (*p*-nitroanisole)*	Aromatic hydroxylation (aniline)*	Arylhydrocarbon hydroxylation (benzo[*a*]pyrene)*	*O*-Demethylation (*p*-nitroanisole)*	Aromatic hydroxylation (aniline)*	Arylhydrocarbon hydroxylation (benzo[*a*]pyrene)*
Nose						
Maxilloturbinate	0†	20	0†	80	30	0†
Ethmoturbinate	270	190	0	520	540	100
Nasal epithelial membrane	Trace‡	60	0	120	130	30
Lung						
Central parenchyma (including terminal airways)	Trace‡	Trace‡	0	Trace‡	60	70
Subpleural parenchyma (including pleura)	0	Trace‡	0	80	50	0
Liver	170	220	130	270	440	2000

*Test substrates. †Less than 5 pmole per milligram of microsomal protein per minute. ‡Detected activity, not double blank value.

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Samples for investigation of the MO's in the complete respiratory tract were obtained from two normal male beagle dogs aged 713 days (dog No. 1) and 791 days (dog No. 2). The animals were born and raised in the Lovelace Inhalation Toxicology Research Institute colony. Nasal tissues, liver, and lung parenchyma obtained from another dog, 815 days old (dog No. 3), were used to demonstrate formaldehyde production from HMPA by nasal microsomal enzymes. The care and management of the colony have been described (*10*). The dogs were anesthetized with nitrous oxide and exsanguinated by cardiac puncture. The pelt of the head was removed and the skull was cut longitudinally along the midline with a bone saw. The thoracic and peritoneal cavities were opened and the liver and lungs were purfused with ice-cold isotonic saline solution. The heart-lung block, skull, and liver, and all specimens derived from them were kept at 0° to 5°C throughout the procedure unless stated otherwise.

For studies of the complete respiratory tract (*11*) we dissected the system into 12 parts: maxilloturbinates (including the dorsal and ventral concha), ethmoturbinates, nasal cavity epithelial membrane, nasopharyngeal epithelium, frontal sinus epithelium, laryngeal epithelium, trachea, carina, major bronchi and second bifurcations, major airways of the lung lobes, central lung parenchyma and small airways, and the pleura and subpleural lung parenchyma. For the study of the production of formaldehyde from HMPA, dissection of the lung and postnasal portions of the skull was omitted.

The specimens were placed in ice-cold 0.1*M* tris buffer containing 1.15 percent KCl, *p*H 7.4 (buffer A). The tissues were blotted dry and weighed. Specimens were diced and homogenized in a Tekmar Tissumizer (Cincinnati) and then were centrifuged at 10,000*g* for 15 minutes (5°C). The supernatant was centrifuged at 100,000*g* for 60 minutes (5°C). For dog No. 1, the pellets were resuspended in 20 percent sucrose-tris buffer, frozen in liquid nitrogen, and stored at −70°C for 12 hours. The thawed microsomes were recentrifuged at 100,000*g* for 60 minutes. For dog No. 2, the pellets were not frozen but were resuspended in buffer A and recentrifuged at 100,000*g* for 60 minutes. Final pellets from dogs 1 and 2 were resuspended in 0.1*M* tris buffer at *p*H 7.4 (buffer B) for determination of protein content (*12*), *p*-nitroanisole *O*-demethylase (*13*), aniline hydroxylase (*14*), and arylhydrocarbon hydroxylase (AHH) (*15*). Microsomes from dog No. 3 were resuspended in buffer B after the initial 100,000*g* centrifugation and were used in the formaldehyde production assays (*16*) with aminopyrine and HMPA being used as substrates. For the enzyme assays the samples were incubated at 37°C with shaking (100 oscillations per minute) under oxygen (> 1000 ml/min) in a Dubnoff incubator (Precision Scientific, Chicago). Cytochrome P-450 content was determined with an Aminco DW-2A spectrophotometer by the method of Omura and Sato (*17*) or Miyake *et al.* (*18*).

Table 3. Rates of production of formaldehyde by dog nose, lung, and liver microsomes with HMPA and aminopyrine substrates. The rates are expressed as picomoles of formaldehyde formed per milligram of microsomal protein per minute. The microsomes, which were from dog No. 3, were not washed after centrifuging at 100,000*g*.

Tissue	Substrate	
	HMPA	Aminopyrine
Maxilloturbinate	841	0
Ethmoturbinate	4524	913
Nasal epithelial membrane	1158	264
Lung*	884	288
Liver	2576	1577

*A single sample of lung containing both central and subpleural parenchyma was used.

Only the tissues recorded in Tables 1 and 2 contained measurable amounts of cytochrome P-450 or associated activities. The microsomes prepared from the major airways of the lung lobes also showed some activity, but this probably resulted from parenchyma contamination which was not possible to eliminate completely. Of particular interest is the amount of cytochrome P-450 (Table 1) and associated enzyme activities in the ethmoturbinates (Table 2). Although the amount of AHH activity in the ethmoturbinates was small in comparison to that in the liver, the activities of both *O*-demethylase and aromatic hydroxylase were almost as high as or higher than in the liver when measured on the basis of activity per milligram of microsomal protein. The cytochrome P-450 content per milligram of microsomal protein in the ethmoturbinates was slightly less than that of liver (Table 1). Comparison of the MO's from dogs 1, 2, and 3 shows that the resistance of the nasal cytochrome P-450 and associated activity to denaturation by freezing is more like that of liver than that of lung. Measurement of the amount of cytochrome P-420, the denatured form of cytochrome P-450 (*19*), showed that the original amounts of cytochrome P-450 were about the same in all three dogs for the samples listed in Table 1.

The known nasal carcinogen HMPA was rapidly *N*-demethylated by dog nasal microsomes to produce formaldehyde (Table 3). The ethmoturbinate microsomes metabolized HMPA at a faster rate than did liver microsomes, whereas the reverse was true for aminopyrine, which is a classic substrate for *N*-demethylation. The observation that the nasal microsomal enzymes produce formaldehyde from HMPA strengthens the suspicion that this known carcinogen is also responsible for the carcinogenicity of HMPA.

Benzo[*a*]pyrene is metabolized mainly by a type of cytochrome P-450 that exhibits maximum absorption at 448 nm in the optical difference spectrum of its carbon monoxide adduct. Nasal cytochrome P-450 from all three regions examined exhibited maximum absorption at 452 to 453 nm in the optical difference spectrum. This shift may be related to the absence or very low concentration of cytochrome P-448 in nasal epithelial membrane.

The remarkably high activities toward some substrates for cytochrome P-450–mediated reactions observed in the nasal microsomes, particularly that of the ethmoturbinates, may have important consequences. The presence of these enzymes may not only help explain the carcinogenicity or tumorigenicity of such compounds as HMPA and DBE in the nasal cavity, but should also alert toxicologists to the potential nasal carcinogenicity of all compounds that are metabolized to produce formaldehyde or other carcinogenic substances and that might be absorbed in the nose during inhalation. The observation that the ethmoturbinates, where olfactory epithelium is highly concentrated, are also high in MO activity may indicate that the biotransformation enzymes are important in the removal of odorants from the olfactory tissue. If odorants remained on the olfactory epithelium for long periods, a high background of odor would exist which might decrease the acuity of the sense of smell.

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A Monoclonal Antibody That Reacts with Nonallelic Enzyme Glycoproteins

Abstract. *One of six monoclonal antibodies raised against purified human placental alkaline phosphatase cross-reacts with the adult and fetal forms of intestinal alkaline phosphatase. The placental and intestinal enzymes are nonallelic. A new electrophoretic titration procedure was used to assess the relative reactivities of the different enzymes with the antibody. The placental enzyme was the most reactive. However, the adult intestinal enzyme showed greater reactivity than the fetal enzyme. The determinants to which the antibody binds on these three forms of alkaline phosphatase presumably differ in their detailed molecular configurations.*

The human alkaline phosphatases (ALP's) are glycoproteins (*1–3*). At least three gene loci code for the protein moieties of the various ALP's that occur in different human tissues: one for the polymorphic placental ALP's, at least one for the intestinal ALP's, and at least one for the liver, bone, and kidney ALP's (*4*). Two forms of intestinal ALP, adult and fetal, have been identified (*2, 5, 6*). These two forms resemble each other closely in thermostability and in their sensitivity to inhibition with a series of different inhibitors, and they are clearly differentiated in these respects from placental and from liver, bone, and kidney ALP's. They differ from one another in that fetal intestinal ALP contains sialic acid residues that do not occur in adult intestinal ALP (*2, 5, 6*). Also it has been found that even after removal of these sialic acid residues with neuraminidase, fetal intestinal ALP still has a slightly greater anodal electrophoretic mobility than the adult form. It is not clear whether this small difference in electrophoretic mobility is due to a difference in the protein moieties of the ALP's which might imply that they are coded at separate loci, or whether they are due to differences in the carbohydrate moieties of the enzyme glycoproteins and reflect posttranslational differences.

Antiserums raised in rabbits against purified human placental ALP do not cross-react with liver, bone, and kidney ALP but cross-react with both adult and fetal intestinal ALP's (*7*). When tested by Ouchterlony double diffusion against placental ALP, the adult and fetal intestinal ALP's give precipitin lines of partial identity. The cross-reacting antibodies can be absorbed with either adult or fetal intestinal ALP leaving an antiserum reacting only with placental ALP. Since such antiserums are polyclonal, the findings indicate that the unabsorbed antiserum to placental ALP contains a mixture of antibodies, some of which cross-react with determinants on the intestinal ALP's and others which do not. We have recently described a series of monoclonal antibodies prepared against purified human placental ALP by the mouse hybridoma technique (*8*). Quantitative binding studies by radioimmunoassay were carried out on six of these antibodies against an extensive panel of placental ALP's of different electrophoretic phenotypes and it was found that each monoclonal antibody gave a distinctive pattern of reactivities with the various phenotypes, suggesting that each is directed at a different antigenic determinant on the surface of the placental ALP molecule. It was of interest to find out whether any of these monoclonal antibodies raised against placental ALP also reacted with either adult or fetal intestinal ALP and if so whether or not differences in the degree of reactivity could be demonstrated. In the present report we describe experiments aimed at answering these questions.

We have recently described how placental ALP–monoclonal antibody complexes can be demonstrated by gel electrophoresis (*9*). In the present study we applied this method to find out whether any of the six monoclonal antibodies whose reactivities with placental ALP's had already been studied in detail also reacted with the different forms of human intestinal ALP. As a source of antibody we used ascites fluid from mice that had been injected intraperitoneally with the hybridoma cells, since these fluids contain much larger quantities of antibody than hybridoma culture fluids. After incubation of ALP containing tissue extracts with ascites fluid, the samples were examined by gel electrophoresis. Typical results are shown in Fig. 1. Only one of the six monoclonal antibodies, Sp2/5, gave evidence of cross-reac-

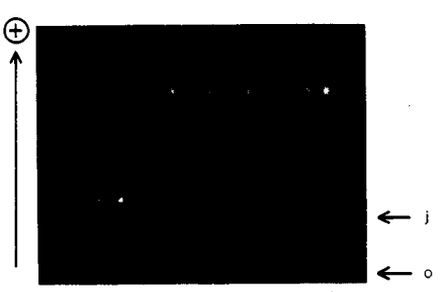

Fig. 1. Electrophoresis of human adult intestinal ALP complexed with six different monoclonal antibodies raised against purified human placental ALP (*8*). Vertical acrylamide slab gel electrophoresis was performed in 9.5 percent acrylamide layered with 5 percent acrylamide prepared as described (*9*) but with the addition of 0.01 percent Zwittergent (TM 314, Calbiochem). The ALP samples were prepared as described (*12*). Portions (50 μl) of the sample, diluted to 0.2 IU/ml, were incubated for 6 hours at 5°C with 10 μl of ascites fluid containing the particular monoclonal antibody (heated at 56°C for 1 hour to destroy any mouse ALP present). Electrophoresis was for 16 hours with a constant current (25 mA). Gels were stained with 4-methylumbelliferyl phosphate as described (*9*). Channels a and h contain uncomplexed adult intestinal ALP; channels b to g contain adult intestinal ALP after incubation with Sp2/5, Sp2/2, Sp2/11, Sp2/3, Sp2/4, and P3/1 ascites fluids, respectively. The letters o and j indicate, respectively, the origin and the junction between 9.5 and 5 percent acrylamide layers.

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