Erythromycin Breath Test as an Assay of Glucocorticoid-inducible Liver Cytochromes P-450

Studies in Rats and Patients

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Abstract

The major P-450IIIA gene family member present in human liver is HLp which, like its rat liver orthologue P-450p, is inducible by glucocorticoids and catalyzes erythromycin *N*-demethylation. To develop a practical method to estimate the amounts of HLp in patients [^{14}C]*N*-methyl erythromycin was injected into rats that had been pretreated with dexamethasone or with inducers of other forms of cytochrome P-450. The rate of demethylation of this substrate, measured simply as $^{14}CO_2$ in the breath, correlated well with the concentrations of immunoreactive P-450p protein ($r = 0.70$), holocytochrome P-450p ($r = 0.70$), or with erythromycin *N*-demethylase activity ($r = 0.90$) determined in the liver microsomes prepared from each rat. Next, [^{14}C]*N*-methyl erythromycin was administered to 30 patients and there was a sixfold interindividual variation in breath $^{14}CO_2$ production seemingly unrelated to medications, smoking status or age. However, the average breath test values were twofold greater in female as compared to male patients ($P < 0.01$). Breath $^{14}CO_2$ production rose in patients retested after treatment with the P-450IIIA inducers dexamethasone ($P < 0.05$) or rifampicin ($P < 0.05$) and was decreased after treatment with the HLp inhibitor triacetyloleandomycin ($P < 0.05$). We conclude that the erythromycin breath test provides a convenient assay of P-450IIIA cytochromes in rats and in some patients.

Introduction

The cytochromes P-450 are membrane bound hemoproteins abundant in the liver where they play a critical role in the metabolism of many endogenous and exogenous compounds including steroid hormones and drugs. Reactions catalyzed by cytochromes P-450 often produce metabolites less bioactive and more readily eliminated than are the parent compounds. However, reactions catalyzed by these enzymes have also been implicated in pathological processes including carcinogenesis, mutagenesis, cytotoxicity, and teratogenesis (1). To date, there have been at least 21 cytochromes P-450 purified from rat liver, each with characteristic structure, substrate binding affinities, and in some cases, differential regulatory responses to drugs or other xenobiotics (2). Because each cytochrome appears to reflect expression of a unique gene, a standard nomenclature has been proposed based on dividing the P-450 genes into families and subfamilies according to nucleotide sequence homology (3). Many of the genes in this "superfamily" are highly conserved in all mammalian species that have been examined, including man (3).

An important new development is the demonstration of significant interpatient differences in the liver concentrations and activities of some of the cytochromes P-450. For example, as many as 10% of caucasians are deficient in the ability to metabolize debrisoquine, an antihypertensive medication used in Europe (4). This deficiency is inherited as an autosomal recessive trait and appears to result from one of several mutations in the gene coding for a single liver cytochrome P-450 (P-450IID gene family) (4, 5). Patients with this defective cytochrome are at risk for adverse reactions to many medications (6), and may have altered risks for developing illnesses such as Parkinson's disease (7) and some cancers (1). At least eight other human liver cytochromes P-450 have now been identified (8, 9). It follows that selective defects in the structure or regulation of these cytochromes could also predispose individuals to adverse health outcomes. Investigating this possibility will require safe and noninvasive means to phenotype individuals in vivo and then to link this information to appropriate biochemical and molecular genetic studies of tissue samples (10, 11). One approach is illustrated by the aminopyrine breath test (12) in which [^{14}C]*N*-dimethyl aminopyrine is administered to patients and, because the radiolabeled carbon atom in the cleaved methyl group is largely converted to bicarbonate in vivo, the rate of demethylation can be conveniently monitored as the rate of production of $^{14}CO_2$ in the breath (13). Unfortunately, aminopyrine is demethylated twice (13) by a form(s) of cytochrome P-450 that has not yet been identified. In theory, however, by choosing an appropriate substrate, it should be possible to estimate the amounts of known forms of cytochrome P-450 in the liver in vivo by a breath test.

In the present study, we illustrate the utility of this approach in our studies of the major glucocorticoid-inducible cytochromes P-450 (P-450IIIA gene family [3]) present in the livers of rats (P-450p) and man (HLp). P-450p and HLp cDNA share 71% nucleotide and 73% derived amino acid sequence homology (11). Since HLp and P-450p are enzymes that catalyze and, in most instances, limit the rate of erythromycin *N*-demethylation in liver microsomes prepared from their respective species (14), we reasoned that the rate of production of breath $^{14}CO_2$ after injection of a test dose of [^{14}C]*N*-methyl

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erythromycin should provide a convenient and selective measure of the catalytic activity of these cytochromes in vivo. Erythromycin may be an ideal substrate for a breath test because demethylation appears to be its major metabolic pathway (15). Furthermore, *N*-desmethyl erythromycin is excreted in bile and does not appear to undergo a second demethylation (16). We also reasoned that because HLp is known to be induced in man by glucocorticoids (11, 14), and is inhibited by triacetyloleandomycin (TAO)[1] (14), these treatments would provide a way to help validate the breath test as an HLp-specific assay in man.

Methods

Materials. [^{14}C]*N*-methyl erythromycin (54.3 mCi/mmol) was purchased from New England Nuclear (Boston, MA); dexamethasone, phenobarbital, pyrazole, clofibrate and 3-methylcholanthrene were purchased from Sigma Chemical Co. (St. Louis, MO); nitrocellulose paper from Bio-Rad Laboratories (Richmond, CA); 3,3'-diaminobenzidine tetrahydrochloride from Pfalz and Bauer Inc. (Stanford, CT); erythromycin lactobionate and TAO were generous gifts from Pfizer Inc. (New York). All other reagents were of the highest purity commercially available.

Animals and treatments. Sprague-Dawley rats (175–200 g, Charles River Breeding Laboratories) housed in groups of two to four in wire-bottom cages, were given unlimited access to standard lab chow and tap water until the erythromycin breath test was administered. The various indicated amounts of dexamethasone and TAO were suspended in corn oil and administered by gavage. Clofibrate (500 mg/kg per d × 1), 3-methylcholanthrene (25 mg/kg per d × 3 d), phenobarbital (100 mg/kg per d × 4 d), and pyrazole (200 mg/kg per d × 3 d) were administered intraperitoneally.

Human subjects. All patients enrolled in these studies were inpatients at the University of Michigan Medical Center. Administration of the erythromycin breath tests and the treatment with TAO and rifampicin were performed according to a protocol approved by the Committee for the Conduct of Human Research. The patients were not acutely ill and all had normal serum tests of liver and kidney function (alkaline phosphatase, AST, ALT, bilirubin, creatinine, and BUN). These patients have not been used in prior reports.

All patients were resting in bed or sitting in a chair for the duration of the breath test and all tests were performed within two hours of breakfast, lunch, or dinner. Repeat tests were performed at the same time of day as the initial test.

Erythromycin breath test. In rat studies, [^{14}C]*N*-methyl erythromycin (1.0 μCi/100 g body wt) was added to a syringe containing 2.5% dextrose in water, along with the desired amount of unlabeled erythromycin lactobionate (30 μmol/100 g body wt in most studies). Rats were lightly anesthetized with diethyl ether because the tail vein injection of erythromycin, a known venous sclerosant, appeared to cause transient pain. Preliminary experiments (data not shown) indicated that light diethyl ether anesthesia did not inhibit the rate of in vivo demethylation of erythromycin as measured by the breath test in control or treated animals. All breath tests were administered between 9 a.m. and 4 p.m. and a diurnal variation in results was not observed (data not shown). The rats were then placed in a water sealed polyurethane breath chamber and the chamber air was continously drawn through a vapor trap (acetone and dry ice) and then through two gas washing bottles connected in series (17). Solutions to trap CO_2 were prepared by mixing 270 ml methanol, 410 ml toluene, and 50 ml Liquifluor (New England Nuclear) for 20 min under nitrogen. Phenethylamine (270 ml) (Aldrich Chemical Co., Milwaukee, WI) was then added with stirring (under nitrogen) for 15 additional minutes (17). The initial gas washing bottle contained 45 ml of the resulting mixture to which 4 ml of toluene was added. The second bottle in series contained 30 ml of the mixture with 2 ml toluene added. A preliminary experiment confirmed that a third gas washing bottle in series trapped < 2% of the total counts during any collection interval and therefore only two bottles were used in these studies. After each collection interval, the total volume of mixture was determined and two 10-ml samples from each bottle were analyzed for ^{14}C content by scintillation counting. The mean of the two values was used to calculate the total $^{14}CO_2$ exhaled during the collection interval. Counting efficiency was > 85% in all samples, determined by internal addition of [^{14}C]toluene. The average rate of label elimination during the collection interval was then determined (expressed as percentage of administered radiolabel appearing in breath per minute) and plotted as a function of time. The best fit polyexponential curve was then determined by computer assistance (RSTRIP; Micromath Scientific Software, Salt Lake City, UT). Total label elimination in 135 min was determined as the area under the best-fit curve 0-135 min. The best-fit polyexponential equation was used to determine the terminal exponential decay constant, referred to as K_{breath} in the text.

In the clinical studies, 4 μCi of [^{14}C]*N*-methyl erythromycin (0.074 μmol) were dissolved in 2 cm^3 of 5% dextrose in water immediately before intravenous administration to the patients. At timed intervals thereafter, the patients were asked to exhale through a tube (which contained a valve to prevent accidental aspiration), creating bubbles in 4 cm^3 of hyamine hydroxide and ethanol (1:1) to which a trace amount of phenolphthalein had been added. When the blue color vanished (indicating trapping of 2 mmol of carbon dioxide) the vials were capped and transported to the laboratory. 11 ml of Aquasol (New England Nuclear) was added and the specific activity of carbon-14 determined by scintillation counting. The percentage of administered carbon-14 exhaled per minute was calculated assuming an endogenous carbon dioxide production equal to 5 mmol CO_2/M^2 body surface area/minute (18). This value remains relatively constant in sedentary individuals over a wide range in body weights (18).

Preparation of microsomes. Immediately after completing the breath collections in rats, each animal was killed and the liver was perfused through the portal vein with PBS and then frozen in liquid nitrogen. The tissue was stored at −70°C until used for preparation of microsomes by differential centrifugation (19).

Quantitation of immunoreactive P-450p in liver microsomes. Immunoreactive P-450p was determined by a quantitative immunoblot analysis as previously described (20). In brief, microsomes (up to 40 μg of protein) were subjected to electrophoresis on 10% polyacrylamide gels. Electrophoresis was interrupted after ~ 40% of the total running time had elapsed, and the sample wells were reloaded with known amounts of purified P-450p (21). The electrophoresis was then completed and the separated proteins were electrophoretically transferred to nitrocellulose sheets (600 mA current for 1 h and then 200 mA current for 1 h in buffer containing 50 mM Tris base, 385 mM glycine, and 40% methanol). The nitrocellulose sheets were incubated over night (room temperature) in 10 mM potassium phosphate buffer (pH 7.4) containing 10% (wt/vol) dialyzed calf serum, 3% (wt/vol) BSA and 0.4 M sodium chloride. The sheets were then placed sequentially in fresh 25-ml aliquots of this buffer containing: (*a*) a P-450p specific monoclonal antibody (1G8 [22]) at a concentration of 0.1 mg/ml (1 h); (*b*) peroxidase conjugated anti-mouse IgG (30 min); (*c*) 100 ml of 0.05 M Tris base (pH 7.5) containing 30 mg of 3,3'-diaminobenzidine tetrahydrochloride and 16.6 μl of 30% H_2O_2. The integrated density of the resulting brown bands was determined by computer-assisted microdensitometry. Because amounts of purified P-450p between 0.5 and 2.0 pmol give linear densitometric values, it was possible to quantitate

1. *Abbreviations used in this paper:* AUC, area under the curve; CER_{max}, maximal rate of $^{14}CO_2$ elimination in the breath; $ERMD_{max}$, estimation of the maximum rate of in vivo erythromycin demethylation; K_{breath}, terminal exponential decay constant derived from the $^{14}CO_2$ exhalation rate vs. time from injection curve; TAO, triacetyloleandomycin.

P-450p present in the microsomal samples, with the lower limit of quantitation being 0.013 nmol/mg microsomal protein.

Quantitation of holocytochrome P-450p in microsomes. P-450p and its closely related forms may be unique among rat liver cytochromes in converting TAO to a metabolite that then forms a spectral complex with P-450p thus providing a convenient assay for holocytochrome P-450p in liver microsomes (23, 24). Microsomes were diluted to 1 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.4) and 10 μl 2.0 mM K_3FeCN_6 per mg microsomal protein was added to dissociate any existing heme ligands (23). The microsomal suspension was then divided between sample and reference cuvettes of a dual beam spectrophotometer. TAO disolved in dimethylsulfoxide (3 μl of 100 mM stock) was added to the sample cuvette and 3 μl of dimethylsulfoxide was added to the reference cuvette. NADPH (final concentration of 1 mM) was added to both cuvettes to initiate the reaction and the absorption at 456 nm was monitored. The concentration of complex formed was calculated from the difference spectra using the extinction coefficient of 68 mM^{-1} cm^{-1} (24). In liver microsomes prepared from rats pretreated with TAO in vivo, the concentration of inactive (complexed) P-450p was determined by the difference spectra (456 nm) obtained after K_3FeCN_6 was added to the reference cuvette only. The concentration of active (uncomplexed) P-450p was determined as the additional spectral complex that could be formed in the sample cuvette (no preaddition of K_3FeCN_6 to the microsomes).

Statistical analysis. In the rat studies, breath test results were subjected to a one-way analysis of variance followed by the Bonferroni multiple comparison procedure (25), which compares multiple treatment groups with a control group. In human studies, the relationships between breath test result and age, sex, and smoking status were analyzed by the Student *t* test method. The effects of drug treatment on an individual's breath test were analyzed by the paired *t* test method.

Other assays. Protein concentration was determined by colorimetric assay (26). Total microsomal concentration of cytochrome P-450 was determined as CO-binding protein (27). Polyclonal antibodies to purified P-450p were raised as previously described (24). Erythromycin demethylase activity was determined in liver microsomes by the Nash reaction as previously described (23). Immunoinhibition of microsomal erythromycin demethylase activity was performed as previously described (24).

Results

To see if demethylation of erythromycin in vivo resulted in detectable breath carbon dioxide, a trace amount of [^{14}C]*N*-methyl erythromycin (1.0 μCi/100 g body wt) was administered to untreated rats and breath CO_2 was trapped as described in Methods. Radioactivity was readily detected in the first breath collection (10 min). There was a biexponential decline in the subsequent rate of production of $^{14}CO_2$ over time (Fig. 1 *A*) from which three parameters can be derived from the best-fit equation: (*a*) the maximum $^{14}CO_2$ exhalation rate (CER_{max}), (*b*) the terminal exponential decay constant (K_{breath} [28]) and (*c*) the area under the $^{14}CO_2$ exhalation rate over time curve (shown in Fig. 1) during the first 135 min after injection (AUC_{0-135} min). These parameters have been frequently used in assessing $^{14}CO_2$ breath tests (28, 29).

If the liver concentration of P-450p were rate limiting in the conversion of the *N*-methyl carbon of erythromycin to breath carbon dioxide in vivo, then pretreatment of rats with dexamethasone, a potent inducer of P-450p protein (30), should increase the rate of production of $^{14}CO_2$ in the breath. Accordingly, female rats were pretreated with high doses of dexamethasone (300 mg/kg per d × 3) and then injected with trace amounts of [^{14}C]*N*-methyl erythromycin. The maximum rate of appearance of $^{14}CO_2$ in the breath (CER_{max}), again

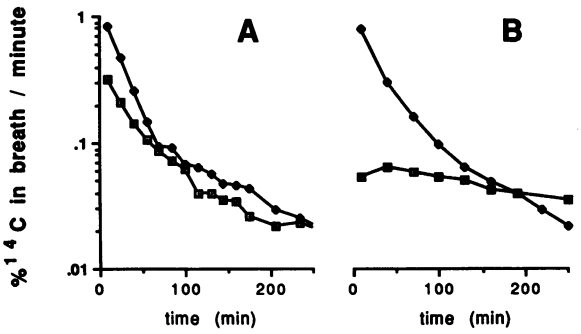

Figure 1. Elimination of $^{14}CO_2$ in control and dexamethasone pretreated rats given trace (*A*) and pharmacologic (*B*) doses of [^{14}C]*N*-methyl erythromycin. Adult female rats were gavaged with either dexamethasone (♦, 300 mg/kg per d) or vehicle alone (⊡) for 3 d. Each rat was then injected (tail vein) with [^{14}C]*N*-methyl erythromycin alone (A, 0.01 μmol/100 g b.w.) or mixed with cold erythromycin (B, 30 μmol/100 g b.w.). The rate of production of $^{14}CO_2$ in breath was determined at the indicated time points as described in Methods. Representative tracings from single rats in each of the treatment groups are shown.

noted at the initial collection, was threefold greater than that seen in the untreated animals (Fig. 1 *A* and Table I). Mean values for AUC_{0-135} and K_{breath} were also increased two- to threefold by dexamethasone pretreatment (Table I). The increase in K_{breath} was not significant, however, due to fluctuations in the production of breath radiolabel observed in each rat. When compared to the > 30-fold increase in hepatic P-450p protein we have previously observed in rats treated with dexamethasone (30), the two- to threefold increase in $^{14}CO_2$ production is modest. However, the true induction of P-450p was better approximated by the breath test when pharmacologic doses of unlabeled erythromycin (30 μmol/100 g body wt) were injected along with the radiolabeled compound (1.0 μCi/100 g body wt). In the induced rats, the pattern of

Table I. The Effect of Dexamethasone on the Production of Breath $^{14}CO_2$ after Injection of Trace and Pharmacologic Amounts of [^{14}C] N-Methyl Erythromycin

Treatment	Dose	CER_{max}	K_{breath}	$AUC_{0-135min}$
Control (*n* = 3)	Trace	0.27	0.056	11.5
		(0.14)	(0.026)	(2.5)
DEX (*n* = 3)	Trace	0.90	0.115	26.85
		(0.09)	(0.070)	(0.64)
Control (*n* = 5)	Pharmacological	0.054	0.047	5.1
	amounts	(0.017)	(0.024)	(2.1)
DEX (*n* = 5)	Pharmacological	0.78	0.16	27.6
	amounts	(0.11)	(0.04)	(4.7)

Rats were pretreated with dexamethasone (DEX, 300 mg/kg per d) or vehicle (control) for 3 d and then injected with trace amounts of [^{14}C]N-methyl erythromycin alone (trace) or combined with 30 mmol/100 g body wt of unlabeled erythromycin. The rate of label elimination in breath was calculated at the time points shown in Fig. 1 and the listed parameters were determined (see Methods). The values shown are the mean and standard deviations (in parentheses) of the results obtained in the indicated number of rats.

production of radiolabel in the breath (Fig. 1 *B*) and the derived kinetic parameters (Table I) were similar to those observed when trace doses of radiolabel were administered alone. In untreated rats, however, the coadministration of pharmacologic doses of unlabeled erythromycin resulted in marked inhibition in the production of $^{14}CO_2$ in the breath (Fig. 1 *B*). Therefore, there were marked differences between control and dexamethasone pretreated rats in mean CER_{max} (> 10-fold) and in mean $AUC_{0\text{-}135}$ (> 5-fold) (Table I).

Next, we gave rats pretreated with high and low doses of dexamethasone varying amounts of unlabeled erythromycin combined with a fixed amount (1.0 μCi/100 g body wt) of [^{14}C]*N*-methyl erythromycin. For each rat, the maximum rate of demethylation ($ERMD_{max}$) was estimated as the highest rate of production of $^{14}CO_2$ attained during the breath test (expressed as percentage of injected radiolabel appearing in breath per minute) multiplied by the total administered dose of erythromycin. In control rats, $ERMD_{max}$ remained roughly constant over a wide range of erythromycin doses (5–35 μmol/100 g body wt) (Fig. 2). The maximum value (~ 0.02 μmol/min) should represent the V_{max} for the reaction in vivo. In rats pretreated with low dose dexamethasone, much larger doses of erythromycin (> 20 μmol/100 g body wt) were needed to approach V_{max}, and V_{max} was increased fivefold over that observed in control rats (Fig. 2). A V_{max} could not be achieved in animals pretreated with high doses of dexamethasone (Fig. 2) even when we gave the highest subtoxic dose of erythromycin (35 μmol/100 g body wt). Interanimal variation prevented our using the data shown in Fig. 2 to precisely calculate the K_m for erythromycin demethylation in vivo in untreated or induced rats (data not shown). In summary, these results (Fig. 2) indicate that the rate limiting step in the conversion of the *N*-methyl carbon of erythromycin to breath CO_2 in vivo is saturable in control rats, that the rate limiting step in this series of reactions can be induced at least 10-fold by dexamethasone, and that the magnitude of this induction is evident only when pharmacologic doses of erythromycin are used in the breath test (Fig. 1 and Table I). The total amount of radiolabel exhaled in 135 min ($AUC_{0\text{-}135\ min}$) was the most reproducible kinetic parameter (Table I and data not shown).

To directly examine the relationship between the amounts of P-450p protein and P-450 activity in the liver and the breath test results, groups of rats were treated with high or low doses of dexamethasone to induce P-450p. Induction of other major liver cytochromes P-450 was accomplished by treating additional groups of rats with phenobarbital (P-450b [31]), 3-methylcholanthrene (P-450c [31]), clofibrate (P-450 lauric acid ω-hydroxylase [32]), or pyrazole (P-450j [33]). The breath test values measured as $AUC_{0\text{-}135\ minutes}$ and the erythromycin demethylase activity assayed in vitro in liver microsomes subsequently prepared from each rat gave a linear correlation which extrapolated to the origin ($r = 0.91$) (Fig. 3). In making this calculation, we omitted the three values in the "plateau" region where in vitro activity exceeded 5 nmol/mg per min (see Discussion). Moreover, only in rats pretreated with P-450p inducers, dexamethasone ($P < 0.01$) or phenobarbital ($P < 0.05$), and not with 3-methylcholanthrene, pyrazole, or clofibrate (Fig. 3), were breath test values significantly increased above those obtained in control animals.

Next, in each rat the breath test results were compared to the specific content of immunoreactive P-450p (Fig. 4 *A*), determined by quantitative immunoblotting of the liver microsomes with a P-450p specific monoclonal antibody. The specific content of P-450p was below the limit of quantitation (< 0.013 nmol/mg) in control rats and in those receiving 3-methyl-cholanthrene, pyrazole, or clofibrate. In the rats pretreated with dexamethasone or phenobarbital, the $AUC_{0\text{-}135}$ values were directly proportional to the microsomal concentration of immunoreactive P-450p (Fig. 4 *A*), although, unlike the relationship between breath test results and microsomal erythromycin demethylase activity (Fig. 3), this linear correlation ($r = 0.71$) did not extrapolate through the origin. We also compared the breath test result in each animal to its respective

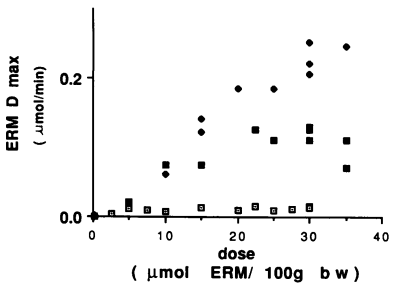

Figure 2. The maximal rate of in vivo demethylation as a function of dose of erythromycin administered in control and dexamethasone-pretreated rats. Adult female rats were gavaged with high dose dexamethasone (300 mg/kg per d × 3, ♦), low dose dexamethasone (20 mg/kg per d × 2, ■) or vehicle alone (2 d, ⊡). Each rat then received intravenous injections containing identical amounts of [^{14}C]*N*-methyl erythromycin to which various amounts of cold erythromycin was added as indicated on the *x* axis. The rate of production of $^{14}CO_2$ was determined (see Methods) and the maximal rate of demethylation of both labeled and unlabeled erythromycin (ERM D max) was then calculated as described in the text for each rat. Each point represents the values obtained from a single rat.

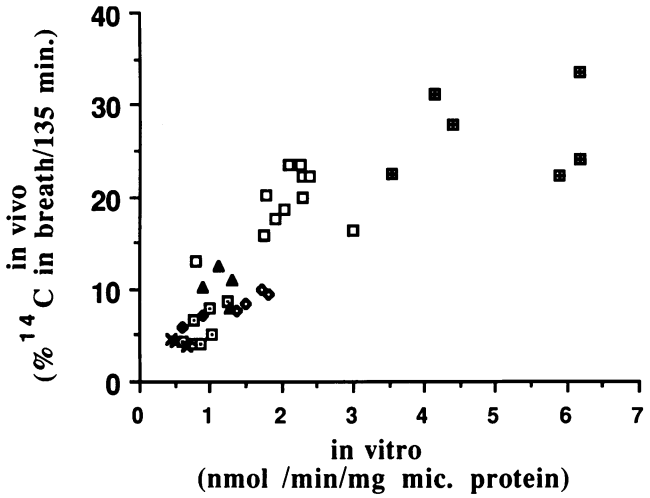

Figure 3. Erythromycin demethylase activity measured in rats (in vivo) and in the corresponding liver microsomes (in vitro) in response to various inducers. Adult female rats were treated with high dose (300 mg/kg per d × 3) or low dose (20 mg/kg per d) dexamethasone or with other inducers of cytochrome P-450. Each rat was then given the erythromycin breath test (in vivo) and immediately killed. The breath test result was then compared with the erythromycin demethylase activity determined in the liver microsomes prepared from each rat (see Methods). Each point represents the values obtained from a single rat (⊞, high DEX; □, low DEX; ▲, phenobarbital; ◇, pyrazole; ×, 3-methyl cholanthrene; ♦ clofibrate; ⊡, vehicle alone (control).

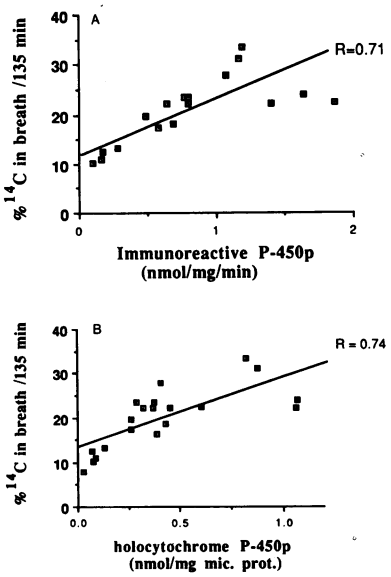

Figure 4. Correlation between expired $^{14}CO_2$ and the microsomal concentration of immunoreactive P-450p (*A*, $r = 0.71$) and holocytochrome P-450p (*B*, $r = 0.74$). The breath test results from each rat were compared to the concentrations of immunoreactive P-450p (*A*) and of holocytochrome P-450p determined in the liver microsomes as described in Methods. P-450p could be quantitated by either method only in microsomes prepared from rats pretreated with either phenobarbital or dexamethasone. Each point shown, therefore, represents a single rat receiving one of these treatments.

specific content of holocytochrome P-450p, measured in liver microsomes as TAO inactivatable cytochrome P-450. Once again (Fig. 4 *A*) the two parameters were linearly correlated (Fig. 4 *B*, $r = 0.74$) but the extrapolated curve did not pass through the origin. Of course, the holocytochrome measurements correlated with the respective values for immunoreactive P-450p ($r = 0.85$) (data not shown). Finally, we found a poor correlation ($r = 0.27$) between breath test results and the corresponding microsomal contents of total CO-binding protein in the rats not receiving phenobarbital or dexamethasone (data not shown). In aggregate, these results suggest that the erythromycin breath test results largely reflect the liver content of P-450p proteins rather than total cytochrome P-450, although in untreated rats, some other forms of cytochrome P-450 may contribute to erythromycin demethylation.

The liver content of P-450p and related proteins is higher in adult males as compared to females (22, 23). The mean $AUC_{0\text{-}135\ min}$ in our untreated male rats (not shown) was greater than it was in untreated female rats (6.80±1.54 SD, $n = 3$ vs. 5.10±2.1 SD, $n = 5$) but this difference was not statistically significant. Similarly, the mean liver microsomal erythromycin demethylase activity in males was greater than in females (1.19±0.13 SD, $n = 9$ vs. 0.88±0.20 SD, $n = 7$) and this difference was statistically significant ($P < 0.01$). Preincubation of untreated male and untreated female liver microsomes with anti-P-450p IgG (20 mg/nmol total P-450) resulted in 50 and 65% decreases, respectively, in erythromycin demethylase activity compared to parallel incubations with an identical amount of nonimmune IgG (data not shown). These results further support the idea that erythromycin demethylation in vivo is largely but not exclusively a function of P-450p in uninduced rats.

We carried out two final series of experiments with TAO to confirm that the erythromycin breath test mainly assays P-450p catalytic activity in induced rats. TAO is not only an inducer of P-450p but also a potent and selective inhibitor of this cytochrome (24). For example, liver microsomes prepared from rats given maximally inducing doses of TAO (20) contained high concentrations of holocytochrome P-450p (2.43 nmol/mg protein), measured as the sum of TAO-metabolite complex that had formed in vivo (inactive P-450p) plus the additional TAO-metabolite complex which could be formed in vitro (active) (Table II). The average content of active holocytochrome P-450p and the erythromycin demethylase activity determined in these microsomes (0.29 nmol/mg protein and 2.05 nmol/mg per min, respectively) were similar to those seen in rats pretreated with low dose dexamethasone (0.33 nmol/mg and 2.22 nmol/mg per min protein, respectively) (Table II). However, the breath test values obtained in TAO-treated rats were considerably lower than those obtained in the dexamethasone-pretreated rats (10.83 and 19.59%, respectively, of the administered label exhaled in 135 min).

The inhibition of P-450p function by TAO in vivo was further examined by pretreating rats with low doses of dexamethasone (20 mg/kg per d × 2), and then administering a single intraperitoneal injection of TAO followed 3 h later by the breath test. Acute administration of TAO appeared to result in significant induction of P-450p over that seen in rats given dexamethasone alone (1.06 nmol/mg microsomal protein and 0.33 nmol/mg microsomal protein respectively) (Table II). The majority of P-450p was inactive (0.78 nmol/mg microsomal protein) in these rats due to formation of the TAO-metabolite complex in vivo (Table II). The microsomal concentration of catalytically active P-450p (0.28 nmol/mg protein) and the microsomal erythromycin demethylase activity (1.83 nmol/mg per min) in these rats were similar to those seen in rats receiving dexamethasone alone (0.33 nmol/mg

Table II. The Effect of Dexamethasone and TAO on the Breath Test Results and on Liver P-450p

			P-450p		
Treatment	ERMBT	ERM D	Active	Inactive	Total
Control ($n = 4$)	5.35 (2.37)	0.88 (0.26)	<0.05	<0.05	<0.05
TAO ($n = 3$)	10.83 (0.83)	2.05 (0.18)	0.29 (*)	2.14 (0.29)	2.44
DEX ($n = 11$)	19.59 (3.4)	2.22 (0.47)	0.33 (0.10)	<0.05	0.33
DEX/TAO ($n = 3$)	6.79 (0.48)	1.83 (0.14)	0.28 (0.04)	0.78 (0.04)	1.06

Adult female rats were gavaged with vehicle (control), TAO (480 mg/kg per d × 6), dexamethasone (DEX, 20 mg/kg per d × 2) alone or DEX followed by a single 500-mg dose of TAO (DEX/TAO) and then administered the erythromycin breath test (ERMBT, results expressed as $AUC_{0\text{-}135\ min}$). Liver microsomes were then prepared and assayed for erythromycin demethylase activity (ERMD, units = nmol formaldehyde formed/mg microsomal protein/minute), the concentration of P-450p which was not complexed to the TAO metabolite (active P-450p, units = nmol/mg per microsomal protein) and P-450p which was complexed to the TAO-metabolite (inactive P-450p, units = nmol/mg per microsomal protein). All values shown are the mean and standard deviations (in parentheses) for rats receiving identical treatments.

* Mean of two measurements only.

protein, and 2.22 nmol/mg per min, respectively) (Table II). However, the breath test results in these rats were approximately one-third of the values obtained in the dexamethasone-pretreated rats (6.79 and 19.59%, respectively) (Table II).

Human studies. We previously used immunoinhibition to show that HLp was the major erythromycin demethylase present in liver microsomes prepared from a patient receiving dexamethasone (14). To establish that this is true also in the livers of uninduced patients, we tested the effect of incubating anti P-450p IgG with liver microsomes prepared from a man and from a woman who had received neither steroids nor macrolides (these patients were designated as patient 19 and patient 21, respectively, in a prior report [34]). Confirming our expectations, an antibody concentration of 20 mg per nmol total spectral P-450 produced > 95% inhibition of erythromycin demethylase activity in both samples when compared to parallel incubations containing an identical amount of nonimmune IgG.

These biochemical tests of human liver suggested that the erythromycin breath test would reflect HLp activity in man. When trace amounts (4 μCi) of [^{14}C]*N*-methyl erythromycin were administered by intravenous injection to 30 hospitalized patients (Table III) and two mmoles of exhaled CO_2 were trapped 3 min after the injection (see protocol outlined in Methods), radiolabel was readily detected in the breath of all patients. Unlike the rat receiving trace doses of erythromycin (Fig. 1), the peak rate of radiolabel elimination was not attained until the 20-min time point in some patients (Fig. 5). The subsequent fall in the rate was biexponential for the majority of patients and, as was seen in the rat, fluctuations in the terminal rate of elimination in man resulted in large errors in the estimates for K_{breath} (data not shown). For all tests given to all patients, there was a linear correlation between the total amount of radiolabel eliminated in 1 h and the rate of radiolabel elimination measured in the single collection at 10 min (r = 0.98 for each) (data not shown). The range of breath test values was four- to sixfold among patients not taking medications known to influence the activities of either P-450p in rats or the related cytochromes in other species (Table III). However, the result for a given patient was consistent. On repeat testing of 10 patients (still not receiving medications likely to influence HLp activity) the breath test results were not

Table III. Patient Information

No.	ERMBT	Age/sex	Primary disease	Medications
1	2.93, 2.33	44/F	Sickle cell anemia	Meperidine, methadone, synthroid, HCTZ, estradiol
2	3.58	44/F	Endocarditis	Vancomycin, glipizide, warfarin, diazepam
3	1.38, 1.47	64/M	COPD	Cefazolin, tetracycline, ranitidine, sucralfate, piroxicam
4	3.34	61/F	Brain tumor	Sucralfate, antacids
5	3.41	75/M	Cardiac arrhythmia	Amiodarone, methazolamide, TMP/SMZ, diltiazem
6	2.43	66/M	Cervical laminectomy	None
7	1.00, 1.31	46/M	Endocarditis	Warfarin, gentamycin, synthroid, mezlocillin, furosemide, triazolam
8	2.34	66/M	Lung cancer	Ampicillin, triazolam, codeine, terpin hydrate
9	5.93	48/F	Brain tumor	Medroxyprogesterone, estrogen
10	4.52	79/F	Cellulitis	Vancomycin, TMP/SMZ, heparin
11	4.04, 4.70	39/F	Bile duct adenoma	Medroxyprogesterone, estrogen, HCTZ, triampterene
12	2.70	22/M	Spinal injury	Acetaminophen, heparin, triazolam
13	3.61	45/F	Spinal cord tumor	None
14	2.20	20/M	Endocarditis	Dicloxacillin, codeine
15	3.04	35/M	Brain tumor	None
16	3.70, 2.96	51/M	Cardiac arrhythmia	Amiodarone, nitrates, atenolol, codeine, nifedipine, insulin, dipyridamole
17	1.28, 1.98	32/M	Wrist infection	Vancomycin, naproxen, methocarbamol
18	0.99, 1.07	65/M	FUO	Ceftriaxone, TMP/SMZ
19	2.37	65/F	Rheumatoid arthritis	Prednisone, ferrous sulfate, trilisate, ciprofloxacin
20	0.58	57/M	Cardiac arrest	Digoxin, furosemide, captopril, amiodarone, nitrates
21	0.96, 1.41	44/M	Osteomyelitis	Ibuprofen, meperidine, vistaril
22	4.69	48/F	Cellulitis	Nafcillin, iron sulfate
23	1.86	23/M	Endocarditis	Gentamycin, penicillin, meperidine, acetaminophen
24	2.94, 2.41	67/M	Pyelonephritis	Ampicillin, phenazopyridine, enalapril
25	6.97	33/F	Bursitis/cellulitis	Baclofen, heparin
26	2.84	51/M	Colon cancer	Chlorpropamide, ranitidine
27	3.44, 2.92	40/F	Psoriasis	Estrogen, medroxyprogesterone acetate
28	1.31	69/M	Osteomyelitis	Diltiazem, insulin, ticarcillin, clavulanate, furosemide
29	2.36	58/M	Diabetes	Furosemide, insulin, nitrates, digoxin
30	1.22	63/M	Myocardial infarction	Atenolol, nitrates, aspirin, carafate, cefazolin

The erythromycin breath test (ERMBT) values are given as the percentage of administered radiolabel eliminated in breath in one hour. Patients with duplicate numbers were retested within 2 wk and did not receive glucocorticoids or macrolide antibiotics. Primary disease abbreviations: COPD, chronic obstructive pulmonary disease; FUO, fever of unknown origin; ca, carcinoma; HCTZ, hydrochlorothiazide; TMX/SMX, trimethoprim/sulfamethoxasole.

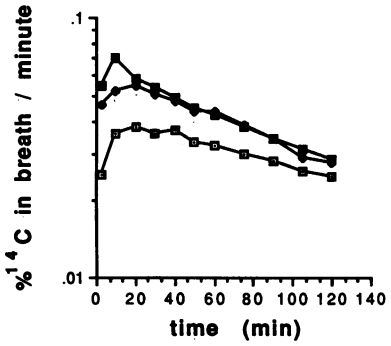

Figure 5. The rate of production of $^{14}CO_2$ in the breath of patient 23 injected with [^{14}C]*N*-methyl erythromycin on three occasions during treatment with rifampicin. The patient received the breath test before starting rifampicin (□) and at days 2 (♦) and 4 (■) of rifampicin treatment (600 mg oral dose each a.m.; the tests were done 24 h after the last dose).

changed by > 27% (Table III). This was true despite the fact that at least 2 d elapsed between tests and medical regimens had occasionally been altered.

Female patients had significantly higher breath test results than did males (4.13±1.35 SD; n = 11 vs. 2.03±0.93 SD; n = 19; $P < 0.01$). Patient age, smoking status (n = 10), or the time of day that the test was administered did not appear to influence the breath test results (data not shown). Because short treatment with oral dexamethasone had been prescribed to patients 4 (24 mg daily for 9 d), 6 (18 mg daily for 2 d), 12 (32 mg over 2 d) and 13 (32 mg over 2 d) and because a short course of hydrocortisone had been prescribed to patient 15 (350 mg i.v. over 2 d), it was possible to determine the effects of glucocorticoids on the breath test in man. In each patient, treatment with glucocorticoids resulted in significant increases in breath test values (Fig. 6 *A*) (average increase of 55%; $P < 0.05$). In patients 21, 22, 23, 25, and 29, treatment with rifampicin (600 mg daily for 4 d) (Fig. 6 *B*) resulted in a 120% average increase in the breath test values, which was also statistically significant ($P < 0.05$). The time taken to attain the maximum rate of radiolabel elimination decreased after rifampicin treatment in each patient (Fig. 5 and data not shown). Moreover, the effects of rifampicin were reversible. In patient 21 breath test results had returned to within 20% of the baseline value when he was retested 7 d after discontinuing rifampicin (data not shown).

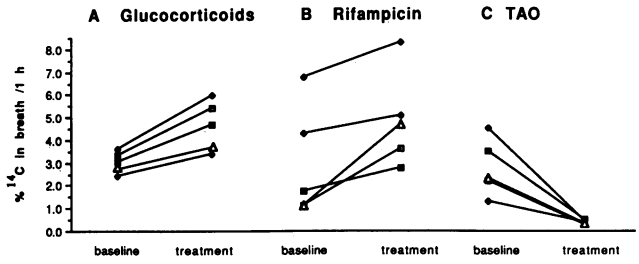

Figure 6. The effect of various medications on patients' breath test results. The erythromycin breath test was administered to patients receiving glucocorticoids (*A*), rifampicin (*B*), or TAO (*C*) (see Methods for details of this treatment). For each patient, these posttreatment results were compared with the (baseline) value obtained when the patient was not receiving the medication in question.

Patients 5, 10, 14, 27, and 28 were retested 2 h after they received a single oral dose of TAO (500 mg). Dramatic inhibition in radiolabel elimination was observed in each patient. The average decrease in the breath test results (Fig. 6 *C*) was 80%, which was statistically significant ($P < 0.05$).

Discussion

In this manuscript we present compelling evidence that the rate of in vivo metabolism of erythromycin, as measured by a simple breath test, can predict the catalytic activity of liver P-450p in rats. In rats given [^{14}C]*N*-methyl erythromycin, the ability to convert the radiolabeled carbon to breath carbon dioxide is saturable (Fig. 2), increases dramatically after treatment with dexamethasone (Fig. 2), and correlates well with the erythromycin demethylase activity of their liver microsomes (Fig. 3). Furthermore, in rats treated with P-450p inducers, the production of $^{14}CO_2$ correlates well with the specific content of both immunoreactive P-450p (Fig. 4 *A*) and holocychrome P450-p at all but very high levels of induction. Evidence for specificity includes the lack of increase in erythromycin breath test values in rats pretreated with inducers of cytochromes P-450 other than P-450p (Fig. 3) and the lack of correlation of breath test results with the liver content of total cytochrome P-450.

These results indicate that the erythromycin breath test is a specific assay for the catalytic activity of P-450p in animals with induced levels of P-450p. However, enzymes other than P-450p may also catalyze erythromycin *N*-demethylation. There were relatively small differences between the sexes in breath test results or in the erythromycin demethylase activity measured in the liver microsomes even though liver P-450p concentration is severalfold lower in females (23). Furthermore, a linear correlation between the breath test result and microsomal content of P-450p (measured immunochemically or as TAO-metabolite complex) did not extrapolate to the origin in female rats (Fig. 4, *A* and *B*). Indeed, others have recently shown that erythromycin demethylation is catalyzed by both high and low K_m enzymes in liver microsomes prepared from untreated rats (35). There appear to be multiple cytochromes structurally related to P-450p (22, 36) and the livers of female rats contain a dexamethasone-inducible protein that does not react with the monoclonal antibody utilized in our immunoquantitations, and yet is immunochemically related to P-450p (22). In as much as anti-P-450p IgG blocked 50% of the erythromycin demethylase activity in control female liver microsomes, it is likely that other P-450III gene family members are largely responsible for the non-P450p erythromycin demethylase activity.

The failure of breath test results to correlate with P-450p protein or catalytic activity at high levels of induction (Figs. 3 and 4) may simply reflect complete metabolism of the administered substrate. This is because about half of administered erythromycin undergoes demethylation before elimination in the bile (15) and, only one of the two radiolabeled methyl groups is removed during liver metabolism of this fraction (15, 16). Therefore only 25% of the administered radiolabel should be converted to formaldehyde in the liver. Furthermore, when [^{14}C]formaldehyde is administered intravenously to rats, only 50–60% of the radiolabel appears as breath $^{14}CO_2$ during the subsequent 2 h (unpublished observations). Based on these observations, the expected maximum exhalation of radiolabel

in untreated rats would be 12.5–15%, a range that approximates the $AUC_{0\text{-}135\ min}$ obtained when trace doses of erythromycin were administered to untreated rats (Table I). If all the erythromycin administered were to undergo demethylation (rather than 50%) the maximum recovery of radiolabel expected in breath would be 30%, a figure that closely approximates the "plateau" value seen in induced rats (Figs. 3 and 4).

We carried out these extensive experiments in rats because comparison of breath test results with biochemical analysis of nondiseased liver will not generally be possible in patients. Nevertheless, we have been able to assemble sufficient information to conclude that the erythromycin breath test largely reflects the amount of HLp present in the livers of at least some patients. First, in human liver microsomes prepared from both untreated and glucocorticoid-treated patients, erythromycin demethylase activity is blocked by anti-P-450p IgG. Moreover, the total elimination of radiolabel in breath during the 1 h after injection increased significantly in each of four patients after treatment with dexamethasone (Fig. 6 *A*). These results are in line with our prior observations that the concentration of HLp protein and HLp mRNA was highest in liver biopsies obtained from patients receiving dexamethasone at the time of surgery (11, 14) and that dexamethasone stimulates production of HLp mRNA in the human hepatoma cell line Hep G2 (11). Furthermore, the production of $^{14}CO_2$ increased in patient 15 after his treatment with hydrocortisone (cortisol). Likewise, corticosterone, the endogenous glucocorticoid in rats, induces P-450p in primary hepatocyte cultures (37). It is therefore possible that endogenous glucocorticoids are involved in the regulation of HLp in man. However, because each patient was retested at the exact time of day as the original test was given, diurnal variation in the level of circulating glucocorticoids could not have contributed to differences observed in our induction and inhibition studies.

Further evidence that the breath test reflects HLp activity is that test results were also increased in each of five patients after treatment with rifampicin. Although this commonly used antibiotic does not at all induce P-450p in the rat (23) it is an effective inducer of the orthologous liver enzyme, LM3c (23), in the rabbit. Treatment of patients with rifampicin increases production of 6 β-hydroxysteroids in the urine (38). Because HLp appears to be the major steroid 6 β-hydroxylase in human liver (39) induction of HLp by rifampicin would provide an explanation for these earlier observations and the breath test results presented here.

Finally, the elimination of radiolabel was dramatically reduced in five patients after they received a single dose of TAO (Fig. 6 *C*). The acute administration of TAO to dexamethasone-pretreated rats also resulted in a marked decrease in $^{14}CO_2$ production that exceeded that predicted from analysis of the net functional P-450p in the liver microsomes (Table II). For example, the breath test values were greatly reduced in the dexamethasone-pretreated rats who had also received TAO versus rats receiving dexamethasone alone, even though the erythromycin demethylase activity in the liver microsomes was about the same (Table II). Inhibition of steps involved in the production of CO_2 after demethylation is unlikely because TAO has no effect on the rate of in vivo formation of CO_2 from formaldehyde (unpublished observations). Since TAO has a higher affinity for the P-450p substrate binding site than does erythromycin (40), it is likely that competitive inhibition by unmetabolized TAO, together with inactivation of P-450p by complex formation, best explains the decrease in $^{14}CO_2$ production occurring after the acute administration of TAO to rats. The basis for the TAO-mediated decrease in $^{14}CO_2$ production in patients is likely to be similar as HLp also binds unmetabolized TAO (14) and characteristically forms the TAO-metabolite complex (14).

In summary, when coupled with previous and present biochemical characterizations of human liver microsomes, we conclude that the changes in erythromycin breath test results produced by treating patients with glucocorticoids, rifampicin, or TAO reflect corresponding changes in the in vivo catalytic activity of HLp. It is highly unlikely that factors other than these drugs account for the observed differences because the patients' medical conditions were stable and their medication regimens were not altered in any other consistent fashion between tests. Because the breath test was an accurate predictor of liver P-450p activity in rats only at doses of erythromycin that appeared to saturate the enzyme in vivo, (Table I) the increases or decreases in the clinical studies that used trace doses of erythromycin may actually underestimate the changes in liver content and activity of HLp. Nevertheless, the test provides a way to investigate new drugs or other agents for their effects on humans.

The present clinical studies have already provided new insights into the expression of HLp because the breath test results were significantly higher in females than males. The whole body clearance of erythromycin has also been reported to be faster in women than in men (41). Prompted by these observations, we reanalyzed data derived from liver biopsies randomly obtained from patients not receiving steroids or macrolide antibiotics (reference 34 and unpublished observations). The mean concentration of immunoreactive HLp was $\sim$ 30% higher in 9 females than it was in 10 males although this difference was not statistically significant. The reverse sex differences occur in rats where liver P-450p content and erythromycin demethylase activity are significantly higher in males (35). While sexual dimorphism in liver gene expression is well established for rats, the present sex differences in P-450III gene expression would represent the first such example for the human cytochromes P-450.

Gender does not fully account for the inter-individual differences in breath test results, however, as a three- to fourfold range of values were observed in patients of the same sex (Table III). These differences were reproducible (Table III) and did not appear to correlate with patient age, smoking status or the time of day that the breath test was administered. We have also observed a three- to fourfold variation in the content of immunoreactive HLp measured in liver biopsies obtained from patients not receiving steroids or macrolide antibiotics (34; and unpublished observations) and it seems likely that the similar range of breath test values in uninduced patients reflects variation in HLp activity. However, variables other than HLp activity (such as hepatic uptake of erythromycin or one carbon pool kinetics) might be involved and therefore it will be necessary to make multiple direct comparisons between the breath test results and the liver content of HLp and of the many other cytochromes P-450 present in the liver to prove that the erythromycin breath test selectively measures HLp activity in all types of patients. Nevertheless, it is likely that regulation of HLp is influenced by genetic factors, disease, dietary differences, or medications in addition to steroids and macrolides that can be defined with the use of this breath test.

A potentially important observation was that virtually identical information was obtained whether breath test results were expressed as the total radiolabel eliminated in 1 h, as the maximal rate of radiolabel elimination, or as the rate of radiolabel elimination measured just once 10 min after the intravenous injection. Thus, it may be possible to perform HLp phenotyping in 10 min. Furthermore, the specific activity attained at this early time point should allow the test to be performed with as little as 1 μCi of [^{14}C]*N*-methyl erythromycin and at a cost of less than $10.00.

Just over 10 years ago, it was noted in Europe that a subpopulation of patients was hypersensitive to the pharmacologic effects of debrisoquine (4). This hypersensitivity results from a relative inability of the liver to form 4-hydroxy debrisoquine, the usual major metabolite of this drug. Therefore, after receiving a single dose of debrisoquine, metabolizer phenotype could be determined in patients by measuring the ratio of parent compound to 4-hydroxy metabolite in the urine. This convenient assay, the subsequent purification of the relevant cytochrome P-450 (P-450DB) from human liver (42, 43), and the cloning of P-450DB cDNA (5) made it possible to correlate the poor metabolizer phenotype with specific defects in the P-450 DB gene (5). In this case, characterization of the molecular structure of P-450 DB was the culmination of research initiated based on clinical observations. The studies presented here illustrate the opposite approach. We have previously characterized a prominent human liver cytochrome P-450 (HLp) (14), its cDNA (11), and its gene (unpublished observations). The erythromycin breath test should provide a means to determine genetic and environmental factors involved in HLp regulation. This research should have immediate clinical implications since recent experiments suggest that HLp or a closely related cytochrome P-450 (44) may play major roles in the metabolism of such commonly used medications as quinidine (45), nifedipine (46), and cyclosporine A (47). Perhaps more importantly, development of the erythromycin breath test exemplifies an approach to studying the control of other newly identified human liver cytochromes P-450.

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