INTERPATIENT HETEROGENEITY IN EXPRESSION OF CYP3A4 AND CYP3A5 IN SMALL BOWEL

Lack of Prediction by the Erythromycin Breath Test

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ABSTRACT:

The CYP3A subfamily of cytochromes P450 metabolize many medications and environmental contaminants. *CYP3A4* and, in 25% of patients, *CYP3A5* seem to be the major CYP3A genes expressed in adult liver. Hepatic levels of CYP3A4 can be estimated by the erythromycin breath test and vary at least 10-fold among patients. CYP3A4 has also been shown to be present in small bowel where it is responsible for significant "first-pass" metabolism of orally administered substrates. However, it is not known whether there is significant interindividual variability in the intestinal expression of CYP3A4, or whether the liver and intestinal catalytic activities of CYP3A4 correlate within an individual. It is also not known whether *CYP3A5* is expressed in the small intestine. To address these questions, we administered the erythromycin breath test to 20 patients and obtained biopsies from their small bowel. There was a 6-fold variation in CYP3A catalytic activity (midazolam hydroxylation), an 11-fold variation in CYP3A4 protein content, and an 8-fold variation in CYP3A4 mRNA content in intestinal biopsies. There was an excellent correlation between intestinal CYP3A4 protein level and catalytic activity ($r = 0.86$; $p = 0.0001$); however, neither parameter significantly correlated with hepatic CYP3A4 activity as measured by the erythromycin breath test result ($r = 0.27$; $p = 0.24$ and $r = 0.33$; $p = 0.15$, respectively). We also found that CYP3A5 protein was readily detectable in biopsies from 14 (70%) of the patients, indicating that *CYP3A5* is commonly expressed in human small intestine. We conclude that there is marked interpatient heterogeneity in intestinal expression of *CYP3A4* and *CYP3A5*, which is likely to contribute to the heterogeneous kinetics of orally administered CYP3A substrates. We also conclude that hepatic and intestinal CYP3A4 activities do not correlate well within individuals. Therefore, assays of liver CYP3A4 catalytic activity, such as the erythromycin breath test, do not provide an accurate means of assessing intestinal CYP3A catalytic activity.

Members of the cytochrome P450 subfamily termed *CYP3A*[1] have been shown to metabolize a growing list of commonly used medications, including erythromycin, cyclosporin A, 17 α-ethinyl estradiol, midazolam, lidocaine, lovastatin, and some calcium channel blockers (reviewed in ref. 2). CYP3A enzymes have also been implicated in the metabolism of many potentially toxic environmental chemicals, such as mycotoxins (3), pyrrolizidine alkaloids (4), and aryl hydrocarbons (5).

Four different CYP3A cDNAs have been cloned from human liver libraries and have been termed *CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7* (1). CYP3A4 seems to be the major CYP3A enzyme present in adult liver (6) and has been detected in essentially all adult livers examined to date. The hepatic concentration of CYP3A4 protein and catalytic activity have been found to vary at least 10-fold among patients (2). The other major adult liver CYP3A enzyme seems to be CYP3A5; however, this enzyme is present in only 25% of adult livers (7, 8). Although CYP3A4 and CYP3A5 have over 85% amino acid identity, their catalytic properties seem to be different. For example, CYP3A4 *N*-demethylates erythromycin roughly 10-fold faster than does CYP3A5 (9). In addition, both CYP3A4 and CYP3A5 readily metabolize cyclosporin A, but the pattern of metabolites generated by each enzyme differs (7).

The activity of liver CYP3A4 can be estimated from the rate at which a patient exhales $^{14}CO_2$ after receiving a trace intravenous dose of [^{14}C-*N*-methyl]erythromycin (10, 11). This test is based on the observation that CYP3A4 exclusively catalyzes the *N*-demethylation of erythromycin in liver microsomes (10) and that the carbon atom in the resulting formaldehyde should largely appear in the breath as CO_2 (12). Several lines of evidence strongly support the idea that this ERMBT[2] specifically measures CYP3A4. First, ERMBT results significantly rise or fall in patients after they are given either inducers or inhibitors, respectively, of CYP3A4 (10). In addition, the ERMBT results predict the kinetics of the orally administered specific substrates for CYP3A4, cyclosporin A (13, 14) and OG37-325 (15). Finally, in patients with severe liver disease who underwent liver biopsy after receiving the ERMBT, the results of the breath test correlated significantly with the liver microsomal concentration of

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[1] The cytochromes P450 are a gene superfamily that has been divided into families that share >40% amino acid sequence identity and subfamilies that share >70% amino acid identity. In this study, we refer to specific P450s according to the recently recommended "CYP" nomenclature (1). P450s within the CYP3A subfamily have been termed "P450IIIA" in our former publications.

CYP3A immunoreactive protein (11). In this same study, the ERMBT results did not correlate at all with the microsomal concentration of four other major liver P450s.

It has recently been reported that CYP3A4 is also present in the epithelial cells (enterocytes) lining the small bowel (16) and that intestinal CYP3A4 seems to account for significant "first-pass" metabolism of orally administered cyclosporin A (17). It is possible that variations in intestinal expression of CYP3A enzymes may account for a significant portion of the variability in oral cyclosporin A pharmacokinetics (17). However, it is not known whether there is heterogeneity in the expression of intestinal *CYP3A4* in patient populations or whether *CYP3A5* is expressed in the intestine.

It is also not known whether the ERMBT is able to predict intestinal activity of CYP3A enzymes. For several reasons, it seems likely that the ERMBT primarily measures hepatic CYP3A4 activity. First, the intravenous administration of the test dose of erythromycin should in essence "bypass" the intestinal CYP3A4. Second, although the kidney expresses CYP3A enzymes, the liver contains a much larger mass of CYP3A enzymes than the kidney [the only other nongastrointestinal tract organ conclusively shown to express CYP3A enzymes (18)]. Finally, the liver specificity of the ERMBT is supported by the fact that the test results are dramatically lower than normal in patients with severe liver disease (11) and during the anhepatic phase of liver transplant surgery (19). However, if the *CYP3A4* gene were coordinately regulated in liver and intestine, the liver CYP3A4 activity measured by the ERMBT result should also correlate with intestinal CYP3A4 activity.

The primary aims of this study were, therefore, to determine whether there is significant interpatient heterogeneity in intestinal expression of CYP3A enzymes and, if so, whether the ERMBT predicts CYP3A catalytic activity in the intestine. To address these questions, we administered the ERMBT to 20 patients and analyzed biopsies from their small bowel for CYP3A4 and CYP3A5 proteins, CYP3A4 mRNA, and CYP3A catalytic activity.

Materials and Methods

Population. Subjects were recruited from among outpatients who had been previously scheduled by their attending physicians to undergo upper intestinal endoscopy for various clinical indications. Written informed consent was obtained from 20 subjects who agreed to participate in the study. Patients under the age of 18 or women of childbearing potential were excluded to minimize the theoretical risks associated with the use of carbon-14. Patients were also excluded if they were taking any medications known to induce or inhibit CYP3A activity, including glucocorticoids, macrolide antibiotics, antiseizure medications, or imidazole antimycotic medications (20). Finally, patients were excluded if they had any known small bowel disease or had any contraindications to having small bowel biopsies taken (*i.e.* abnormalities in blood clotting). This study was approved by the Institutional Review Board at the University of Michigan.

ERMBT. Patients were given the ERMBT, as previously described, before receiving any medications or sedation for the endoscopy. Briefly, patients were given an intravenous dose of 0.074 mmol (3 μCi) of [^{14}C-*N*-methyl]erythromycin. At timed intervals after the injection, patients were asked to exhale through a tube creating bubbles in a hyamine solution formulated to trap exactly 2 mmol of carbon dioxide. The vials were capped and the specific activity of ^{14}C determined by scintillation counting. Breath test results were expressed as the percentage of administered ^{14}C that was exhaled during the first hour after the injection of erythromycin (10). Because CYP3A5 does not readily metabolize erythromycin (9), the results of the ERMBT should not be influenced by the expression of CYP3A5 in liver.

Protein and RNA Isolation. Ten small bowel biopsies distal to the duodenal bulb were obtained from each patient at the time of endoscopy. Five of the biopsies were immediately placed in 400 μl of denaturing solution (4 M guanidine isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, and 0.7% 2-mercaptoethanol; pH 7.0) and frozen at −70°C. At a later time, the samples were quickly thawed and the RNA extracted by the method of Chomczynski and Sacchi (21). The other five samples were placed in solution D (0.05 M Tris-HCl, 20% glycerol, and 2 mM EDTA) (22). An S9 fraction was then prepared as previously described (23, 24).

RNA Analysis. Thirty μg of total RNA from each patient was subjected to electrophoresis on a formaldehyde agarose gel containing ethidium bromide. After visualizing the ribosomal bands with UV light, the RNA was transferred to nylon membranes by positive pressure using a Posiblotter (Stratagene, La Jolla, CA). The cDNA probes used were synthesized as follows. Thirty base pair synthetic oligonucleotide primers specific for *CYP3A4* (16) were used in the PCR. The 382 base pair DNA fragment amplified from cDNA made from human intestinal RNA was then cloned into pBluescript KS (Stratagene) using a modified TA cloning procedure (25) and sequenced at least 3 times to confirm its identity with the published sequence of *CYP3A4*. The same PCR cloning technique was used to generate and confirm the identity of a human villin cDNA probe. The cDNA probes were labeled by random priming with ^{32}P (Amersham Corporation, Arlington Heights, IL) to a specific activity of $\sim 1 \times 10^9$ cpm/μg. Hybridizations with cDNA probes were conducted at 65°C for 18 hr as previously described (26). The blots were washed twice at 65°C and placed with X-ray film for 3 and 12 hr for detection of CYP3A4 and villin mRNAs, respectively. The relative hybridization intensities with the CYP3A4 and villin cDNAs were quantitated by computer-assisted densitometry and expressed as arbitrary optical density units.

Protein Analysis. Protein concentrations were determined by a modification of the Lowry assay (27). Fifty μg of the S9 fraction were mixed in sample buffer containing SDS and 2-mercaptoethanol and heated at 100°C for 5 min. The samples were then subjected to electrophoresis in 10% polyacrylamide/0.1% SDS gels. After 2 hr of electrophoresis, serial dilutions of either purified CYP3A4 protein or intestinal S9 protein from a reference patient (to serve as standards for villin determinations) were second-loaded on the gel (28), and the electrophoresis was continued for an additional 2 hr. The samples were then transferred to nitrocellulose by the method of Towbin *et al.* (29). The blots were blocked for 1 hr in Tris-buffered saline containing 0.3% Tween 20 (Sigma Chemical Company, St. Louis, MO) and 5% nonfat dry milk (Carnation). The filters were then incubated with either a rabbit polyclonal antibody specific for CYP3A5 (9), a mouse monoclonal antibody raised against chick villin that has been shown to cross-react with human villin [(30) a generous gift from A. Brian West, Yale University], or a mouse monoclonal antibody specific for human CYP3A proteins (9, 31, 32). The latter antibody reacts with all CYP3A proteins. However, because CYP3A4 is the major CYP3A protein expressed in the intestine (16), the amount of antibody bound on the immunoblots should primarily reflect CYP3A4 protein levels. The protein identified by this antibody is, therefore, referred to as CYP3A4 in these studies.

After extensive washing with Tris-buffered saline containing 0.3% Tween 20 and 0.25% nonfat dry milk, the blots were incubated with a secondary antibody (either rabbit antimouse or goat antirabbit antibody) conjugated with peroxidase. Further washings were done with Tris-buffered saline containing 0.3% Tween 20 and 0.25% nonfat dry milk, with a final washing of Tris-buffered saline containing only 0.3% Tween 20. The filters were then developed with a chemiluminescence kit (Amersham) and exposed to Hyperfilm ECL (Amersham) for 1 sec to 15 min, depending on the intensity of the bands.

CYP3A Catalytic Activity Analysis. We found that measurements of erythromycin or cyclosporin A metabolism that were previously used to quantitate CYP3A catalytic activity (16, 33) were not sufficiently sensitive for the purposes of this study (data not shown). This reflects the fact

that these substrates are not rapidly metabolized by CYP3A enzymes and that the enzymes are present in relatively low concentrations in patients who have not been receiving inducers of *CYP3A*. We therefore analyzed the ability of the biopsy S9 protein fractions to metabolize midazolam to its 1′- and 4-OH metabolites.

S9 supernatant from each intestinal biopsy sample was thawed and suspended in cold potassium phosphate buffer (0.1 M, pH 7.4) to reach a final protein concentration of 100 μg in a 1 ml volume. Midazolam was added to a final concentration of 4 μM, and the resulting mixtures were gently agitated within a Dubnoff metabolic shaking incubator at 37°C for 3 min. The reaction was started with the addition of NADPH (1 mM final concentration) and terminated after 5 min by the addition of 1 ml of 100 mM Na_2CO_3 (final pH 11). The samples were spiked with 10 ng of D_2-1′-OH midazolam and extracted twice with 2 volumes of ethyl acetate. After extraction, solvent was removed under nitrogen and the concentrated extracts were dissolved in 80 μl of derivatizing reagent [20% *N*-(tert butyldimethylsilyl)-*N*-methyltrifluoroacetamide in acetonitrile]. The samples were transferred to autoinjector vials and sealed before being heated to 80°C for 2 hr. Quantification of 1′-OH midazolam and 4-OH midazolam was performed by GC-negative chemical ionization-mass spectrometry operated in the selective ion monitoring mode. The mass spectrometer was a VG model Trio 1000 interfaced to a Hewlett-Packard 5890A gas chromatograph equipped with a Hewlett-Packard 7376A autoinjector and a DB-17 fused capillary column (30 m × 0.32 mm i.d., 0.25-μm film thickness; J & W Scientific, Ventura, CA). Helium carrier gas was used at a head pressure of 7 psi, and injections were made in the splitless mode. The injector and transfer line temperatures were held at 250°C and 290°C, respectively. The injection volume was 2 μl. The oven temperature was held at 110°C for 1 min, increased at 25°C/min to 290°C, held for 5 min, increased at 40°C/min to 300°C and then held for a final 3 min. Under these conditions, the derivatives of 4-OH midazolam and 1′-OH midazolam eluted with retention times of 9.4 and 10.0 min, respectively. The base peak fragment ions [M-tBu$(CH_3)_2$SiOH]$^-$ at m/z 323 were monitored for both metabolites. The D_2-1′-OH midazolam internal standard also eluted at a retention time of 10.0 min and was monitored at m/z 327, the base peak fragment ion of the ^{37}Cl isotopic species. Methane was used as the reagent gas and the source temperature held at 200°C. Concentrations of 1′-OH midazolam and 4-OH midazolam were quantified by comparing peak area ratios from unknown samples to their respective standard curves (area ratio *vs.* concentration) prepared from the addition of known amounts of 1′-OH midazolam, 4-OH midazolam, and D_2-1′-OH midazolam to human liver microsomes suspended in phosphate buffer.

TABLE 1

Patient characteristics and medication profiles

Patient	Age	Sex	Medications
1	65	F	Furosemide, levothyroxine, spironolactone
2	54	F	Cisapride, furosemide, insulin, metoclopramide, omeprazole, ranitidine
3	45	F	Atenolol, estrogen, famotidine, hydrocrotisone cream, tolmetin
4	34	M	Amitriptyline, hydroxychloroquine, ibuprofen, oxycodone/acetaminophen, ranitidine
5	61	M	Captopril, insulin, isosorbide dinitrate, thiethylperazine maleate
6	53	F	Alprazolam, omeprazole
7	54	M	Aspirin
8	66	F	Aspirin, diphenoxylate/atropine, estradiol patch
9	57	M	Aspirin, famotidine, ibuprofen, nifedipine
10	74	F	Diltiazem
11	40	M	Metronidazole
12	62	M	Aspirin, piroxicam, ranitidine, trazodone, terfenadine
13	51	M	Aspirin, vitamin C
14	34	M	Ranitidine
15	60	F	Levothyroxine, lorazepam, ranitidine
16	44	F	Dicyclomine, hydroxyzine pamoate, imipramine, oxycodone/acetaminophen, pancreatin, propranolol, omeprazole, triazolam, trimethobenzamide
17	76	F	Calcium, estrogen, ferrous sulfate, hydrocodone, misoprostol, sulfasalazine, sulindac, triamterene/hydrochlorothiazide
18	32	M	Ranitidine
19	26	M	Doxepin, ranitidine
20	49	M	Atenolol, omeprazole

Results

ERMBT Results. Twenty patients (11 men and 9 women) were enrolled in the study. A list of their age, sex, and medication profiles is shown in table 1. Each patient was given the ERMBT before receiving any medications for the endoscopy. Intestinal biopsies were obtained during the endoscopic procedure from the second portion of the duodenum and processed for either protein or RNA analysis as described in *Materials and Methods*. There was >7-fold interpatient heterogeneity in the ERMBT results (mean of 3.3 ± 1.3 SD % administered ^{14}C exhaled over 1 hr; see table 2). Women had a higher mean ERMBT value than did men, but this was not significant (3.7% *vs.* 3.0%, respectively, $p = 0.25$).

Protein and RNA Correction for Enterocyte Content of Biopsies. Because CYP3A enzymes are expressed exclusively in mature enterocytes in the intestine (16, 32), differences in the percentage of enterocyte content by weight in individual intestinal biopsies might alter our results. For example, a deep mucosal biopsy would contain a relatively low proportion of enterocytes, whereas a more superficial biopsy would have a relatively high proportion of enterocytes. A shallow biopsy should therefore have a higher concentration of CYP3A protein and mRNA/mg of tissue than a deep biopsy obtained from the same individual. Another potential source of variability in the CYP3A content of biopsies is proteolysis due to digestive enzymes. It is logical to assume that enterocytes, due to their location adjacent to the gut lumen, might be especially susceptible to proteolysis during tissue procurement and processing. To correct for these factors, it seemed essential to identify an enterocyte specific mRNA and protein that would have little intrapatient or interpatient variability to serve as an internal standard for our studies.

Villin, a constitutively expressed protein that is enterocyte-specific (30), seemed to be an ideal candidate for our purposes. To verify the usefulness of villin as a standard, we endoscopically obtained biopsies from the second portion of the duodenum in two patients. In one patient, six biopsies were obtained within mm of each other, and, in the second patient, six biopsies were obtained at least 1 cm apart. Each biopsy was homogenized separately and run as an individual sample on an immunoblot. The blot was developed first with a monoclonal antibody specific for CYP3A and then redeveloped with a monoclonal antibody specific for human villin. There was significant interbiopsy variation in the content of CYP3A immunoreactive protein, both among biopsies obtained at a single site (fig. 1*A1*, mean 304.9 ± 43.2 SD optical density units/mg protein) and among biopsies

TABLE 2

Values for ERMBT and enterocyte CYP3A4 protein, CYP3A4 mRNA, and 1'-OH midazolam rate

CYP3A4 protein, CYP3A4 mRNA, and 1'-OH midazolam formation rates are expressed relative to villin expression to compensate for interbiopsy variations in enterocyte content and protein degradation.

Patient	ERMBT	CYP3A4 Protein	CYP3A4 mRNA	1'-OH Midazolam
	% ^{14}C exhaled/hr	*fmol/mg protein/ unit villin*	*arbitrary units/ unit villin*	*pg/min/unit villin*
1	2.7	2.63	92.8	48.5
2	2.9	2.34	118.9	32.4
3	1.8	5.19	93.9	165.9
4	3.5	2.33	44.6	76.4
5	0.9	1.84	19.1	29.0
6	4.9	7.16	150.9	277.6
7	3.5	4.81	61.9	109.5
8	3.2	3.02	110.6	60.8
9	4.3	1.93	40.9	41.1
10	1.8	3.71	32.5	57.8
11	3.0	1.46	33.6	71.1
12	3.5	3.09	136.9	124.2
13	2.0	2.44	121.2	78.5
14	4.3	2.15	112.8	40.5
15	4.7	5.41	52.4	86.8
16	4.4	1.59	76.2	65.8
17	6.8	3.49	164.7	114.9
18	2.4	3.01	—	83.8
19	2.3	0.63	35.0	5.8
20	3.1	2.27	88.7	95.6

obtained at multiple sites (fig. 1*B1*, mean 128.6 ± 47.6 SD optical density units/mg protein). However, there was an excellent correlation between the CYP3A4 and villin protein levels as determined by optical density ($r = 0.958$, $p = 0.003$ for same-site biopsies; $r = 0.948$, $p = 0.006$ for separate site biopsies; see fig. 1, *A1* and *B1*). When the ratios of the optical densities of CYP3A and villin immunoreactive proteins were calculated (see fig. 1, *A2* and *B2*), the interbiopsy variation was significantly reduced (mean 1.06 ± 0.04 SD, same-site biopsies, mean 0.468 ± 0.065 SD for separate site biopsies). These observations suggest that interbiopsy variation in enterocyte content does exist but that this could be controlled for by expressing CYP3A protein concentration relative to villin protein concentration. In addition, it seems that, with villin correction, the site of the biopsy does not appear to be an important variable, at least within the second portion of the duodenum.

We were unable to repeat the above study looking at CYP3A and villin mRNAs, because the amount of RNA obtained from a single biopsy was insufficient for hybridization analysis. However, because the regulation of villin is believed to be at the transcriptional level or by RNA stabilization (34), it seems reasonable to assume that villin mRNA could serve as an appropriate control for our RNA studies.

Expression of CYP3A4 mRNA in Human Intestine. Intestinal RNA obtained from each patient was analyzed on RNA blots. The RNA from patient 18 had degraded as judged by the absence of clear ribosomal bands on the ethidium-stained agarose gels and did not detectably hybridize with villin cDNA (fig. 2). We were therefore unable to normalize the CYP3A4 mRNA expression to villin, and this patient was omitted from the RNA analysis. As shown in fig. 2, the *CYP3A4* cDNA hybridized to two distinct RNA species of 2.2 and 3.0 kb characteristic for the *CYP3A4* gene (6). Because prior studies have shown that the ratio of the intensity of the two bands is constant (6), we quantitated only the more prominent 2.2 kb band by computer-aided densitometry. The blot was then stripped and rehybridized with a villin cDNA probe under stringent conditions (fig. 2). Two RNA species were identified (2.8 and 3.6 kb), which correspond to the two known villin mRNAs (35). As the two bands are always equal in intensity (35), the lower band was chosen for quantitation by computer densitometry.

There was a >7-fold variability in CYP3A4 mRNA concentration in the biopsies. This variability was not due to differences in biopsy content of intact enterocytes, because there was an 8-fold variability in CYP3A4 mRNA when corrected for villin mRNA expression (CYP3A4 mRNA/villin mRNA; see table 2). Mean levels of CYP3A4 mRNA were higher in women than in men in both absolute terms (446 *vs.* 333 optical density units) and when corrected for villin mRNA (99.2 *vs.* 69.5 optical density units). However, these differences did not achieve statistical significance ($p = 0.29$ and $p = 0.15$, respectively). When the ERMBT results were compared with the biopsy content of CYP3A4 mRNA (uncorrected), there was a positive trend toward a correlation that was not significant ($r = 0.27$, $p = 0.26$). However, when the ERMBT results were compared with the CYP3A4 mRNA corrected for villin mRNA, a modest, but significant, positive correlation was observed ($r = 0.49$, $p = 0.033$; fig. 3).

Expression of CYP3A4 Protein in Human Intestine. The concentrations of CYP3A4 and villin immunoreactive protein were determined in the S9 fraction prepared from the biopsies of each patient using a quantitative immunoblot technique (described in *Materials and Methods*). There was an 11-fold variation in the biopsy concentration of either absolute CYP3A4 protein (130.4 ± 69.3 SD fmol/μg protein) or the villin adjusted CYP3A4 protein (3.03 ± 1.57 SD fmol/μg protein/villin optical density units; see table 2). Thus, the interpatient variation in intestinal CYP3A4 protein content greatly exceeded the relatively small degree of intrapatient variability we observed when multiple biopsies were obtained from the same patient (see fig. 1). Women were found to have significantly higher biopsy concentrations of CYP3A4 protein than did men (165 fmol/mg protein *vs.* 102 fmol/mg protein, $p = 0.039$) and also had higher CYP3A4 to villin ratios than did the men (3.84 fmol/unit villin *vs.* 2.36 fmol/unit villin, $p = 0.032$). There was no correlation between the ERMBT result and biopsy concentration of CYP3A4 protein corrected for villin ($r = 0.273$, $p = 0.24$). There was also no significant correlation between the CYP3A4 to villin protein ratios and CYP3A4 to villin mRNA ratios ($r = 0.367$, $p = 0.12$). Likewise, there was no significant correlation between biopsy concentrations of CYP3A4 mRNA and CYP3A4 protein when neither were corrected for villin expression ($r = 0.241$, $p = 0.31$).

Expression of CYP3A5 Protein in Human Intestine. CYP3A5 has been shown to migrate slightly more slowly than CYP3A4 on SDS-polyacrylamide gel electrophoresis. The presence of both CYP3A4 and CYP3A5 protein in liver microsomes therefore produces a "doublet" on an immunoblot (7, 8). We did not observe doublets in any of the intestinal samples, and the abundance of CYP3A4 protein prevented attempts to visualize CYP3A5 by prolonging the exposure of the immunoblots. To provide a more sensitive means of detecting CYP3A5 protein, duplicate immunoblots were probed with an antibody that has been shown to recognize selectively CYP3A5 and not any of the other CYP3A proteins (9). Protein from five of the patients (patients 1, 6, 7, 14, and 17) readily reacted with the antibody

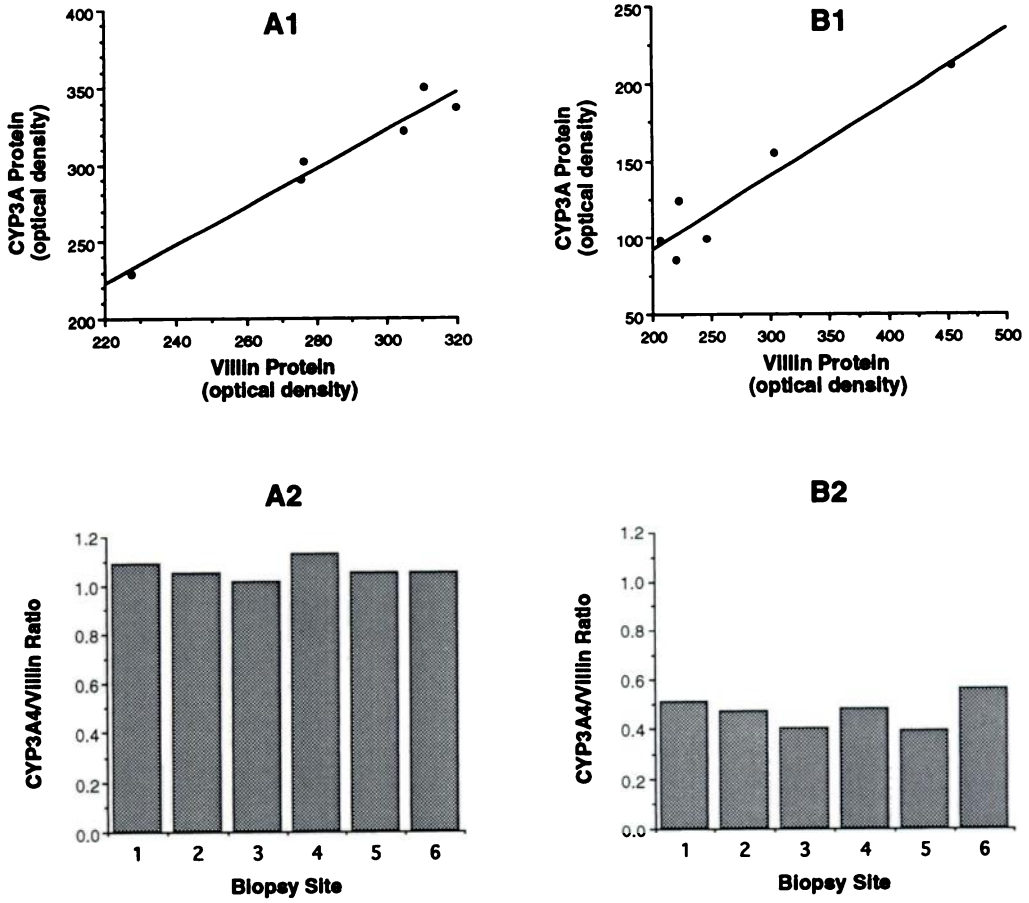


FIG. 1. *Comparison of immunoreactive CYP3A4 and villin protein in single duodenal biopsies.*

In the first patient (*A*), six biopsies were obtained within mm of each other, and in the second patient (*B*), six biopsies were obtained at least 1 cm apart. CYP3A4 and villin protein content were determined by immunoblot analysis of whole homogenate prepared from each biopsy. Biopsy concentrations of CYP3A4 and villin protein were found to vary over 2-fold; however, there was an excellent intrapatient correlation between the amount of CYP3A4 and villin protein in biopsies obtained at either the same site (*A1*; $r = 0.96$, $p = 0.003$) or different sites (*B1*; $r = 0.95$, $p = 0.006$). When CYP3A4 protein was expressed as a ratio with villin protein, the interbiopsy variation was significantly reduced with either same-site (*A2*, mean 1.06 ± 0.04 SD) or different site biopsies (*B2*, mean 0.468 ± 0.065 SD).

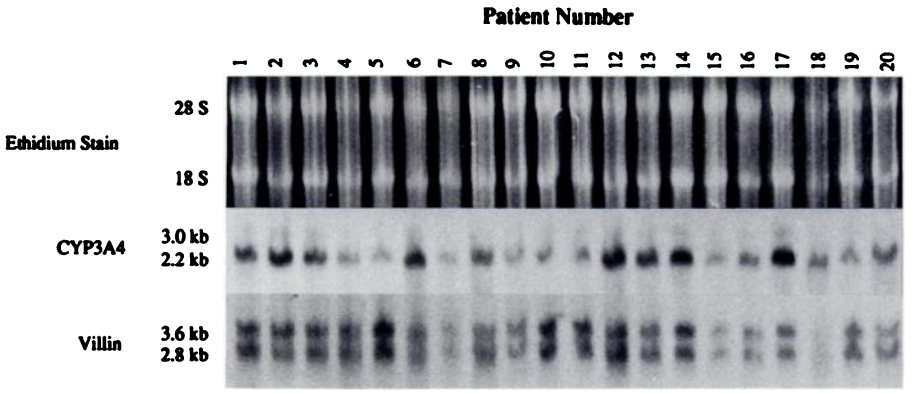


FIG. 2. *RNA blot analysis of intestinal RNA for expression of CYP3A4 and villin.*

Intestinal RNA (30 μg) from the 20 patients was subjected to electrophoresis in a formaldehyde containing agarose gel and transferred to nitrocellulose. Densitometry of the ethidium-stained gel revealed significant degradation of the RNA only from patient 18. The blot was sequentially hybridized with a CYP3A4 cDNA and a villin cDNA as described in *Materials and Methods*. The resulting bands are shown above with approximate molecular weights indicated at the left.

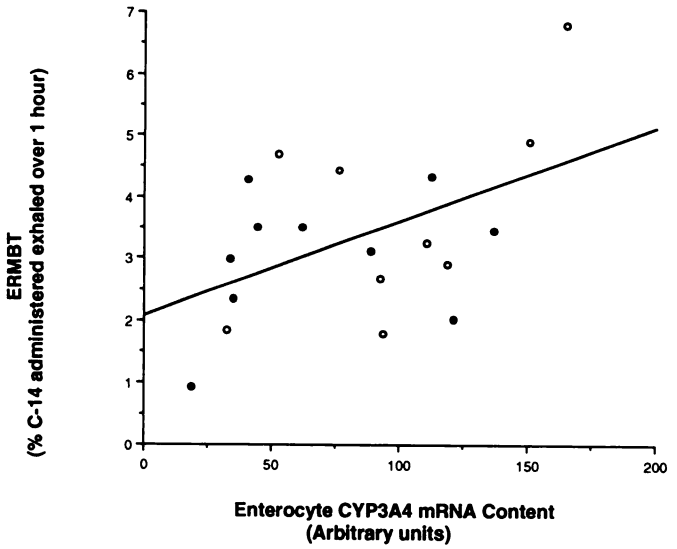


FIG. 3. *Correlation between the ERMBT result and enterocyte content of CYP3A4 mRNA.*

Enterocyte CYP3A4 mRNA expression was estimated in the 19 patients with evaluable RNA data using the ratio of CYP3A4 mRNA to villin mRNA content as determined by the integrated optical densities of the appropriate bands on the RNA blot shown in fig. 2. A significant correlation was observed between the ERMBT result and enterocyte CYP3A4 ($r = 0.49$, $p = 0.03$). ●, male; O, female.

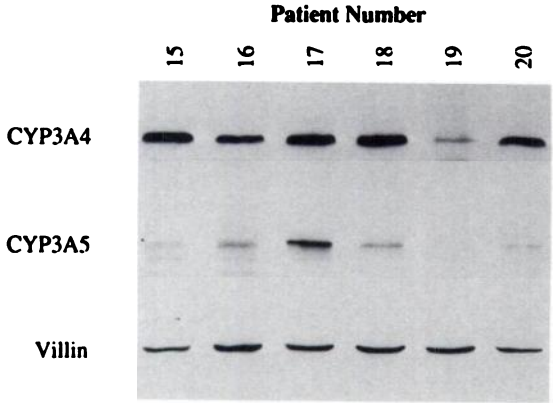


FIG. 4. *Comparison of representative immunoblot analyses of small bowel protein for expression of CYP3A4, CYP3A5, and villin.*

Triplicate immunoblots were developed with either an antibody that preferentially identifies CYP3A4, an antibody that specifically identifies CYP3A5, or an antibody that specifically identifies villin (see *Materials and Methods*).

producing distinct bands (see fig. 4 for representative samples). This reactive protein comigrated with purified CYP3A5 on polyacrylamide gels (not shown). With prolonged exposure of the film, we were able to detect CYP3A5 protein in all but six patients (patients 5, 6, 8, 12, 13, and 19; data not shown). The immunoreactive protein could not be quantitated, because of the absence of an appropriate standard on the blots. Nonetheless, there was clearly marked heterogeneity in expression of this protein, and there was no correlation between the biopsy content CYP3A5 and CYP3A4 immunoreactive protein (fig. 4 and data not shown).

CYP3A Catalytic Activity Analysis. The biopsy S9 protein fractions were analyzed for their ability to metabolize midazolam to its 1′- and 4-OH metabolites. These reactions have been shown to be rapidly catalyzed by CYP3A4 in liver microsomes (36). There was a >6-fold variability in the 1′-hydroxylation and the 4-hydroxylation of this substrate after correction for villin protein concentration (see table 2). The ratio of the 1′-OH to 4-OH metabolite formation rates was 5.26 ± 1.59 SD, but there was a nearly perfect correlation between these two measurements ($r = 0.98$, $p < 0.0001$). CYP3A5 has also been reported to hydroxylate midazolam (37), but the presence of detectable CYP3A5 in 14 patients did not seem to influence either the total production of the hydroxylated metabolites, or the ratio of the rates of production of the two metabolites measured (data not shown). Equivalent results were obtained using either the 1′-OH or the 4-OH metabolite formation rate in the following comparisons; therefore, only the correlations for the 1′-OH metabolite formation rate are given.

Women had higher midazolam hydroxylation rates than men in terms of both absolute (44.7 *vs.* 29.0 pg 1′-OH/min/mg protein) or villin-corrected 1′-OH metabolite formation (101.1 *vs.* 68.7 pg 1′-OH/min/arbitrary villin unit), although this was not significant ($p = 0.20$ and $p = 0.23$, respectively). There was no significant correlation between the rates of midazolam metabolism and the ERMBT results, whether the catalytic activities were expressed/mg intestinal protein ($r = 0.40$, $p = 0.08$) or corrected for biopsy content of villin ($r = 0.33$, $p = 0.15$; fig. 5). There was, however, an excellent correlation between the enterocyte CYP3A4 protein content and the midazolam hydroxylation activity ($r = 0.856$, $p < 0.0001$; fig. 6). This correlation was highly significant even when the patient with the highest CYP3A4 protein and catalytic activity (patient 6) was excluded ($r = 0.725$, $p = 0.0005$).

Discussion

There are well-established interpatient differences in the liver content and catalytic activity of CYP3A4 that are believed to

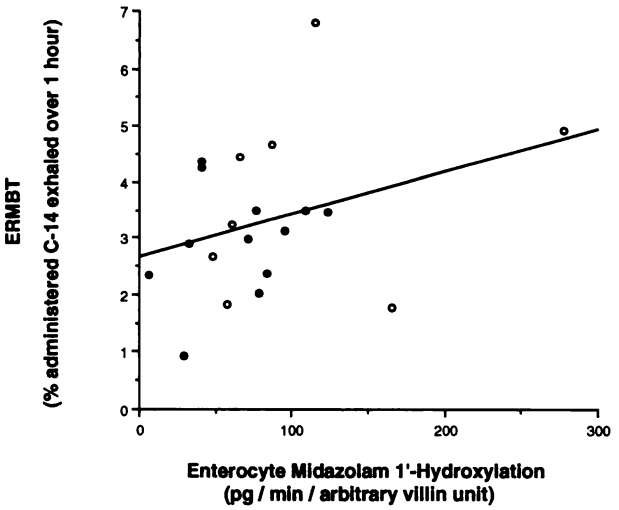


FIG. 5. *Comparison of the ERMBT result and enterocyte catalytic activity (midazolam 1′-hydroxylation).*

Midazolam 1′-hydroxylation activity was determined in the S9 fractions prepared from intestinal biopsies. To correct for interbiopsy variation in enterocyte number and variation in protein degradation, the catalytic rates were divided by the biopsy content of villin protein (expressed in arbitrary optical density units measured on immunoblots). The correlation observed was not significant ($r = 0.33$, $p = 0.15$). ●, male; O, female.

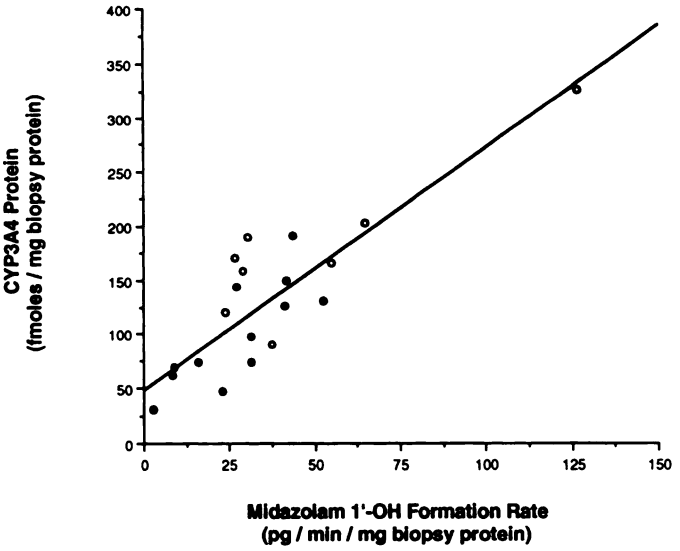


FIG. 6. *Correlation between the concentration of CYP3A4 protein and midazolam 1'-hydroxylase activity in intestinal biopsies.*

CYP3A4 protein and midazolam 1'-hydroxylase activity assays were performed on aliquots of the same S9 protein fractions; therefore, no correction by villin was necessary. The observed correlation is highly significant ($r = 0.86$, $p < 0.0001$). When the patient with the highest CYP3A4 protein and catalytic activity was excluded, the correlation remained highly significant ($r = 0.73$, $p = 0.0005$). ●, male; ○, female.

account, in part, for interpatient differences in the dosing requirements of some drugs. In this study, our first objective was to determine whether interindividual heterogeneity in the expression of CYP3A4 also exists in the small bowel. We found that there was indeed extensive variability in the expression of this enzyme. There was a >8-fold variability in CYP3A4 mRNA content, a >11-fold variation in CYP3A4 protein content, and a >6-fold variation in catalytic activity characteristic of CYP3A enzymes (1'-OH midazolam formation). This heterogeneity was unchanged when the results were expressed relative to the expression of villin, an enterocyte-specific protein (see table 2). Our data, therefore, indicate that there is significant heterogeneity in intestinal expression of *CYP3A4* and that this heterogeneity is comparable with that found in liver.

We also detected CYP3A5 protein in the intestinal samples of 70% (14 of 20) of the patients studied. This enzyme is present in roughly 25% of adult livers and, although we had no way to determine which of our patients had hepatic expression of CYP3A5, our results suggest that CYP3A5 may be more commonly expressed in the intestine than in the liver. The intestinal concentration of CYP3A5 protein, which seemed to vary greatly among patients, was not at all correlated with that of CYP3A4 protein (illustrated in fig. 4). This suggests that, in the intestine, as in the liver, these two enzymes are independently regulated. It is likely that CYP3A5 is expressed at lower levels than CYP3A4 in the intestine, because we could only detect the presence of CYP3A5 using an antibody specific for this protein. In addition, the presence of detectable CYP3A5 did not seem to influence the total midazolam metabolism measured (data not shown). Furthermore, we have previously been unable to detect CYP3A5 mRNA on RNA blots of human small intestinal RNA using a CYP3A5-specific oligonucleotide probe (16). We conclude that CYP3A5 is heterogeneously expressed in the small bowel, but, when compared with CYP3A4, it seems to be a relatively minor enzyme.

The second major aim of our study was to determine whether the ERMBT result predicted intestinal CYP3A4 catalytic activity. It did not (fig. 5). It is unlikely that the lack of a correlation between the ERMBT and intestinal midazolam metabolism was due to variable inactivation of the enzyme during processing of the tissue, because there was an excellent correlation between the catalytic activity and the biopsy content of CYP3A4 protein. In addition, when CYP3A4 protein and catalytic activity measurements were corrected for protein degradation and enterocyte content (by villin content), no correlation with the ERMBT results was observed. The discrepancy between the ERMBT result and intestinal expression of CYP3A enzymes is illustrated by patient 19, who had an ERMBT result of 2.33%. This result is normal for a man (10, 19) and comparable with the ERMBT values of patient 18 (2.35%) and patient 20 (3.11%). However, whereas both patients 18 and 20 had relatively high levels of CYP3A4 protein, patient 19 had almost no detectable CYP3A4 protein (fig. 4 and table 2), no detectable CYP3A5 (fig. 4), and the lowest midazolam 1'-hydroxylation rate of all of the patients studied (table 2). This could not be explained on the basis of protein degradation in patient 19's biopsies, because villin protein was present at roughly the same level as was observed in the intestinal biopsies from the other patients (fig. 4).

Because our study was performed in patients and not in healthy volunteers, it is possible that some of the heterogeneity in *CYP3A4* expression we observed was due to the effects of medications or disease. Five patients were receiving known substrates for CYP3A enzymes [patients 3 and 8 (estrogen), patient 9 (nifedipine), patient 12 (terfenadine), and patient 16 (triazolam); see table 1]. However, removal of these patients from the data base did not alter any conclusions (not shown). In addition, none of the patients were receiving any known inducers or noncompetitive inhibitors of CYP3A enzymes (table 1), and all biopsies were obtained from normal-appearing tissue.

Because liver *CYP3A4* seems to be measured by the ERMBT, our observations suggest that regulation of the *CYP3A4* gene in the liver and the intestine is not closely linked. Discordant regulation of liver and intestinal CYP3A proteins has also been reported in rats treated with certain diets (38). Our data may provide some insight into this issue. In a previous study, we showed that CYP3A4 mRNA and protein were induced in the intestine of volunteers taking rifampin (16). This indicates that pretranslational control of *CYP3A4* gene expression can occur in the intestine as has been shown in the liver (20). Although it is speculative, our finding that the ERMBT result correlated with the intestinal concentration of CYP3A4 mRNA (fig. 3) may indicate a degree of coordinate regulation between the liver and the intestine at the pretranslational level. We did not, however, find a correlation between the concentrations of CYP3A4 mRNA and CYP3A4 protein or catalytic activity in the biopsies. This may suggest that posttranslational factors are also involved in the regulation of CYP3A4 in the intestine. It is unlikely that incorporation of heme into the enzyme protein is an important step in the regulation of intestinal CYP3A4 catalytic activity, because there was an excellent correlation between intestinal protein and catalytic activity measurements in this study (fig. 6).

It seems reasonable to assume from our studies that heterogeneity in intestinal expression of CYP3A4 may be responsible for part of the variable first-pass metabolism of orally administered CYP3A4 substrates. This idea is supported by a recent study in

which the pharmacokinetics of oral and intravenously administered cyclosporin A were determined in normal volunteers before and after treatment with rifampin (39). Rifampin was found to reduce dramatically the oral clearance of cyclosporin A while having a relatively small effect on the systemic clearance of the drug. The authors concluded that this drug interaction was primarily due to induction by rifampin of intestinal metabolism of cyclosporin A. We have previously shown that treatment of patients with rifampin results in a 4- to 8-fold induction in CYP3A4 mRNA expression and in CYP3A4 catalytic activity in the enterocyte (16). Therefore, the 8- to 11-fold variation in CYP3A4 expression we observed in these 20 patients in the absence of known inducers should be of sufficient magnitude to influence significantly the oral bioavailability of drugs that are CYP3A4 substrates. Differences in intestinal CYP3A activity may therefore in part account for the variation in cyclosporin A kinetics that was not accounted for by differences in the ERMBT result in our previous studies (13, 14). In particular, this may account for the fact that the ERMBT results did not at all correlate with the peak cyclosporin A blood levels (14), a parameter that should largely reflect first-pass metabolism. The techniques developed for the current study, which allow accurate estimation of enterocyte CYP3A4 activity from a single pinch biopsy of small bowel mucosa, will make it possible to test these hypotheses directly in future studies.

Intestinal CYP3A enzymes have recently been shown to convert dietary aflatoxin B_1 to reactive metabolites that form aflatoxin B_1 macromolecular adducts within enterocytes (40). These adducts should pass harmlessly in stool as the enterocytes are shed. In perhaps a similar manner, intestinal CYP3A enzymes have also been shown to bioactivate heterocyclic amines to mutagenic metabolites (41). In addition, CYP3A4 has been shown to convert aflatoxin B_1 to Q_1, which may represent a detoxification pathway (42). For these reasons, intestinal CYP3A enzymes may provide a protective barrier to dietary xenobiotics. Interindividual variation in intestinal content of CYP3A enzymes may therefore represent a previously unrecognized risk factor for certain environmental diseases.

As a final point, it has been reported that liver microsomes obtained from women have a higher specific activity of CYP3A enzymes than do liver microsomes obtained from men (43). Gender differences in the expression of CYP3A have also been demonstrated in rats in both the liver and the intestine (44, 45). Each parameter of intestinal *CYP3A4* expression that we examined (1′-OH metabolite formation, 4-OH metabolite formation, CYP3A4 protein, and CYP3A4 mRNA) was higher in women than in men. Although our sample size was small, our observations suggest that there may be gender differences in intestinal CYP3A4 expression in humans.

In summary, we have shown that there is a marked interpatient heterogeneity in enterocyte expression of CYP3A4 and CYP3A5. It seems likely that this heterogeneity accounts, at least in part, for interpatient differences in the kinetics of orally administered CYP3A substrates. In addition, our results suggest that metabolic probe-based assays that exclusively measure liver CYP3A4, such as the ERMBT, are unlikely to predict accurately the first-pass metabolism of orally administered substrates. In the future, if the aim is to develop a test that will be useful in orally dosing medications that are CYP3A4 substrates, it would seem logical to administer the metabolic probe orally.

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