CLINICAL PHARMACOLOGY & THERAPEUTICS
VOLUME 51 NUMBER 3 MARCH 1992

PHARMACOKINETICS AND DRUG DISPOSITION

The erythromycin breath test selectively measures P450IIIA in patients with severe liver disease

There are significant interpatient differences in the activity of a major drug metabolizing enzyme termed P450IIIA. Because P450IIIA uniquely catalyzes the *N*-demethylation of erythromycin, we have proposed that the P450IIIA activity of a patient may be determined from the rate of $^{14}CO_2$ production in the breath after an intravenous infusion of a test dose of [^{14}C-*N*-methyl]erythromycin. However, direct evidence that this erythromycin breath test selectively measures P450IIIA and not other major human liver P450s in patients has been lacking. We therefore administered the erythromycin breath test to nine patients with severe liver disease who were awaiting liver transplantation. Microsomes were prepared from liver samples obtained during surgery and the concentrations of P450IA2, P450IIC8, P450IIC9, P450IIE1, and P450IIIA were determined immunochemically. We found a significant correlation between patients' erythromycin breath test results and their liver P450IIIA levels ($r^2 = 0.56, p = 0.02$). In contrast, there was no correlation at all between the erythromycin breath test result and the microsomal levels of any of the other four P450s assayed. The correlation of the erythromycin breath test and P450IIIA did not appear related to the extent of liver disease because neither correlated with prothrombin time or albumin or bilirubin levels. These data provide the best evidence to date that the erythromycin breath test is a specific assay of in vivo P450IIIA activity in patients. (CLIN PHARMACOL THER 1992;51:229-38.)

Kenneth Lown, MD, Joseph Kolars, MD,[a] Kim Turgeon, MD, Robert Merion, MD, Steven A. Wrighton, MD, and Paul B. Watkins, MD[a,b]
Ann Arbor, Mich., and Indianapolis, Ind.

From the Departments of Internal Medicine and Surgery, University of Michigan, and the Department of Internal Medicine, Veterans Affairs Medical Center, Ann Arbor, and the Department of Drug Metabolism, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis.
Received for publication Aug. 8, 1991; accepted Oct. 1, 1991.
Reprint requests: Paul B. Watkins, MD, Clinical Research Center, University of Michigan Medical Center, 1500 East Medical Center Dr., Room A7119 Box 0108, Ann Arbor, MI 48109.
[a]Recipients of Veterans Administration Career Development Awards.
[b]Supported in part by the National Institutes of Health, Bethesda, Md. (GM 38149).
13/1/34055

The cytochromes P450 are hemoproteins that play a central role in the metabolism of many endogenous and exogenous (xenobiotic) compounds. The cytochromes P450 are most abundant in the liver, where they are the principal components of the mixed function oxidase system. The broad substrate specificity of the mixed function oxidase system is in part related to the existence of multiple P450 proteins, each differing in substrate binding affinities.[1,2] The cytochromes P450 have been divided into "families" and "subfamilies" of enzymes that share amino acid homology greater than 40% and 67%, respectively, as derived from complementary deoxyribonucleic acid sequences.[3]

P450IIIA is one of the major P450 subfamilies in humans and accounts for up to 20% of the total P450 content in the liver. [1,2] P450IIIA has been studied extensively and plays a central role in the metabolism of a number of commonly used drugs such as cyclosporine,[4,5] erythromycin,[6] nifedipine,[7] midazolam,[8] and lidocaine.[9] P450IIIA is also capable of bioactivating some xenobiotics into potential carcinogens or toxins. For example, P450IIIA has been implicated in the bioactivation of aflatoxin B1[10,11] and in the conversion of hexachlorobenzene into its porphyrinogenic metabolite.[12]

It has been shown recently that the catalytic activity of P450IIIA varies tenfold to twentyfold among patients.[7,13] It seems likely that this heterogeneity may account in part for interpatient differences in both dosing requirements of P450IIIA drug substrates and response to some environmental toxins. However, to test this hypothesis it will be necessary to develop a noninvasive means of measuring P450IIIA activity in patients. We have proposed the erythromycin breath test as such an assay on the basis of the fact that P450IIIA uniquely catalyzes the *N*-demethylation of erythromycin.[14] Because the majority of the carbon in the cleaved methyl group should appear in the breath as carbon dioxide, we postulated that after an intravenous injection of [^{14}C-*N*-methyl]erythromycin, the rate of production of $^{14}CO_2$ in the breath would reflect the P450IIIA activity in the liver.[6]

A variety of evidence suggests that our hypothesis is correct. First, we have shown that in rats pretreated with inducers of various cytochromes P450, the rate of production of $^{14}CO_2$ from [^{14}C-*N*-methyl]erythromycin correlates well with immunoreactive P450IIIA and erythromycin *N*-demethylase activity determined directly in hepatic microsomes prepared from these rats. In patients, we have indirectly validated the erythromycin breath test as a measure of P450IIIA activity by showing an increase or decrease in the rate of $^{14}CO_2$ released in the breath when patients are treated with known inducers or inhibitors of P450IIIA, respectively.[14] Further supportive evidence in humans has been obtained by showing that in patients receiving cyclosporine, which is primarily metabolized by cytochrome P450IIIA,[4,5] the erythromycin breath test predicts the mean trough blood levels of the drug.[15] Although these data, in aggregate, suggest that the erythromycin breath test selectively assays P450IIIA, direct proof of this in humans has been lacking. One way to directly test our hypothesis would be to administer the erythromycin breath test to patients and correlate the findings with P450IIIA levels determined in liver biopsy specimens simultaneously obtained from these patients. However, the risk of performing liver biopsies could not be justified in healthy individuals. We have therefore performed this study in patients with end-stage liver disease scheduled to undergo liver transplantation.

METHODS

Patient population. Nine patients (four men and five women) with severe end-stage liver disease were enrolled in this study. Each was an inpatient awaiting liver transplantation at the University of Michigan Medical Center (Ann Arbor, Mich.). Seven patients had chronic liver disease (four, alcohol related; one, non-A, non-B hepatitis; one, hemochromatosis; one, cryptogenic). One patient had acute heptic decompensation superimposed on chronic liver disease (Wilson's disease), and one patient had acute fulminant liver failure (non-A, non-B hepatitis). All patients were Child's class B or C. Biopsy specimens of all diseased livers were obtained at transplantation surgery and were submitted for pathologic evaluation. Histologic study of the liver specimens revealed that all patients had liver cirrhosis with the exception of the patient with acute fulminant non-A, non-B hepatitis.

Our reference control group for the erythromycin breath test results consisted of 86 patients (48 men and 38 women) who had no evidence of liver disease and who were not receiving any medications known or suspected to induce or inhibit P450IIIA. These patients received the erythromycin breath test as part of other studies and some of their results have been previously reported.[14,15]

As a control for P450 microsomal levels, microsomes were prepared from 14 patients without liver disease who were not taking any drugs known or suspected to induce cytochromes P450. The majority of these patients had undergone hepatic lobectomy to re-

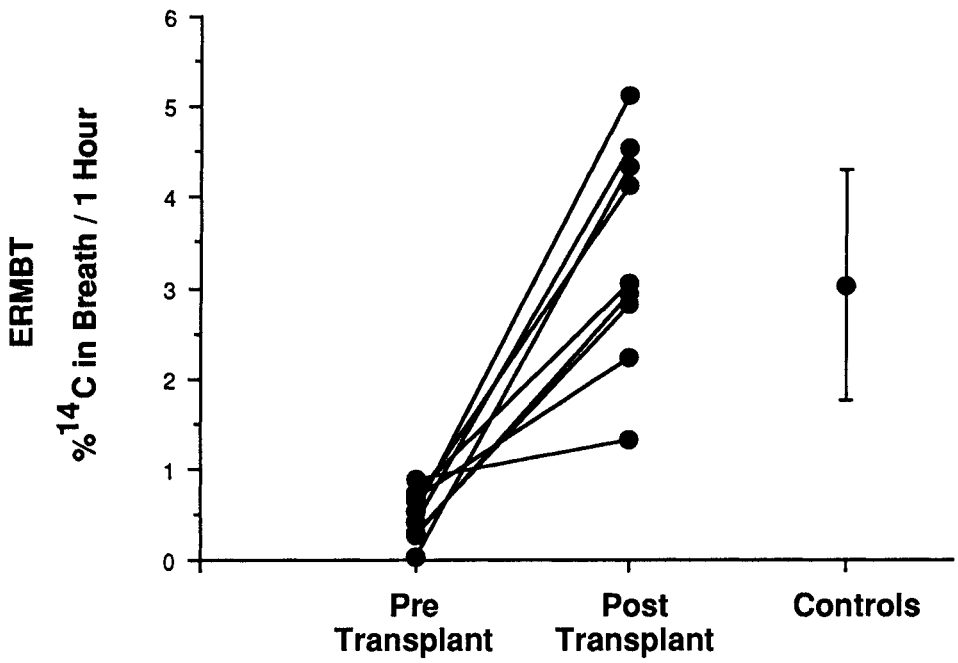

Fig. 1. The erythromycin breath test (ERMBT) results obtained before and after liver transplantation. The ERMBT is expressed as a percentage of ^{14}C dose administered that was exhaled in the breath during 1 hour. *Lines* connect each of the nine patients' respective test results. For comparison we have shown the results obtained from 86 control subjects who had no evidence of liver disease and who were not taking any medications thought to influence P450IIIA activity. The *vertical bars* indicate the SD of the mean. The mean of the pretransplant ERMBT results is significantly different from control subjects ($p = 0.0001$).

move liver tumors and have been described in a previous report.[16] All patient study protocols were approved by the University of Michigan Committee for the Conduct of Human Research.

Breath test. The nine patients with liver disease received the erythromycin breath test before liver transplantation, usually within 24 hours of the operation. Each patient was retested after transplantation at a time when they were medically stable (range, 13 days to 6 months after transplant; mean, 54 days). The erythromycin breath test was performed essentially as previously described.[14] Patients were given 3 μCi (0.074 mmol) of [^{14}C-*N*-methyl]erythromycin intravenously while at rest, and breath samples were collected at 0, 3, 10, 20, 30, 40, 50, and 60 minutes after injection. The specific activity of ^{14}C was determined by scintillation counting. The erythromycin breath test results were expressed as the calculated percentage of administered ^{14}C that was exhaled during the first hour after injection as previously described.[14]

Microsomal preparation. Samples of diseased native liver were obtained from patients at the time of liver transplantation. The location of the biopsy specimen from the liver varied, but the specimen was always obtained from the right lobe. We have shown previously that the specific content of P450IIIA protein and of several other P450 proteins varies little among biopsy specimens obtained from diverse locations within a single liver.[17] Wedge sections of liver were excised within minutes of when the native liver was removed from the patient, and the tissue was immediately flash frozen in liquid nitrogen and stored at −80° C. The samples were later thawed and microsomes were simultaneously prepared by differential centrifugation.[18] Protein concentrations were determined by the method of Lowry with modifications.[19]

Immunoquantitation of P450 protein. Microsomal proteins were electrophoretically separated as described by Laemmli[20] in 10% polyacrylamide and 0.1% sodium dodecyl sulfate gels, and the separated proteins were transferred to nitrocellulose sheets.[21] The nitrocellulose sheets were then incubated with antibodies as described previously.[22] Specific monoclonal antibodies to P450IA2, P450IIC8, P450IIC9,

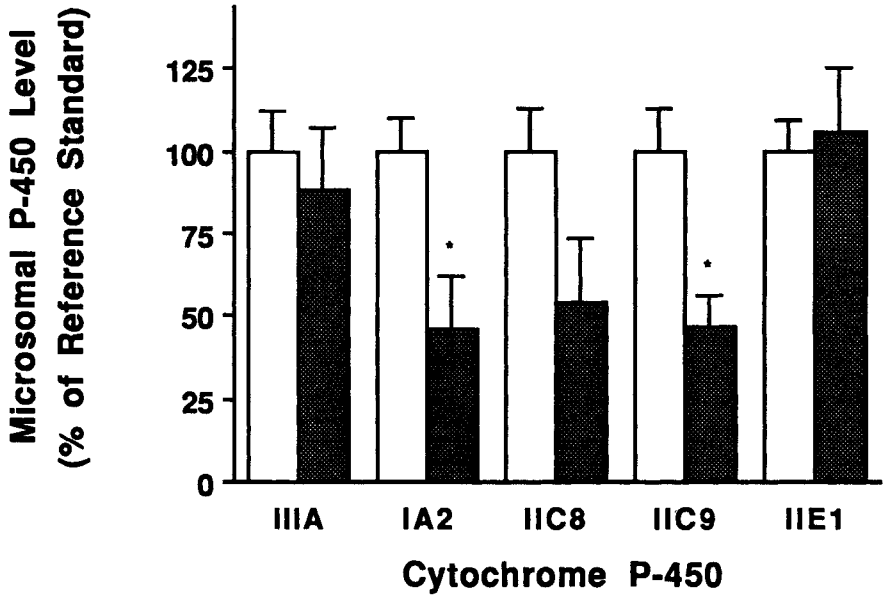

Fig. 2. The protein levels of five cytochromes P450 in hepatic microsomes prepared from normal and severe liver disease patients. Results are expressed as the percentage of immunoreactivity observed in the 14 normal liver microsomes (see Methods section). *Open bars* represent normal subjects. *Hatched bars* represent patients with liver disease. *Vertical lines* indicate the SEM. *Significantly different from the mean of the normal group ($p < 0.008$).

P450IIE1, and P450IIIA* were used. Purification of these proteins and the preparation of specific antibodies have been described.[6,13,17,23] After subsequent incubation with horseradish peroxidase conjugated secondary antibody, the nitrocellulose sheets were developed with diaminobenzidine tetrachloride and H_2O_2, and the densities of the resulting bands were analyzed by computer-integrated optical densitometry. Immunoblot analysis of the microsome samples was performed in triplicate by an investigator (S.A.W.) who was blinded to the erythromycin breath test results of each patient. The levels of each cytochrome P450 were then expressed as a percentage of the respective mean value obtained for that cytochrome P450 in the 14 control liver microsomes.

In vitro cyclosporine metabolism. The production of the M-1 and M-17 metabolites from cyclosporine was assayed in microsomes as described previously.[24]

*Ten families and 18 subfamilies of P450 genes have been identified in humans, and a universal gene nomenclature has been recently proposed.[3] We have used the term "P450IIIA" in this article to denote the protein products of the *CYP3A* gene subfamily, which contains at least four separate genes that may be expressed in human liver.[1,2] P450IA2, P450IIC8, P450IIC9, and P450IIE1 refer to the protein products of the *CYP1A2, CYP2C8, CYP2C9,* and *CYP2E1* genes, respectively.

Microsomal protein (100 μg) was incubated at 35° C in a reaction mixture consisting of 0.1 mol/L sodium phosphate buffer pH 7.4 and a NADPH-regenerating system. The reaction was started with the addition of ^{3}H-cyclosporine (Amersham Co., Arlington Heights, Ill.) to give a final concentration of 13 mmol/L (specific activity, 0.64 Ci/mmol). Preliminary experiments determined that the rate of cyclosporine metabolite production in liver microsomes remained constant for up to 30 minutes under these conditions. The incubations were therefore terminated at 15 to 30 minutes by the addition of methanol (−20° C). After centrifugation at 2000 rpm for 10 minutes, the supernatant fraction was filtered and assayed without further purification. Reversed-phase HPLC was performed by the addition of 5 to 25 ml of the supernatant to a microbore C_8 Hypersil column 20 cm × 2.1 mm (Hewlett-Packard Company, Andover, Mass.). Peaks were quantified by use of an on-line scintillation counter (B-Ram, IN/US Systems Inc., Fairfield, N.J.). The metabolites that corresponded to each peak were identified as described previously.[24]

RESULTS

Erythromycin breath test results. The erythromycin breath test was administered to the nine subjects be-

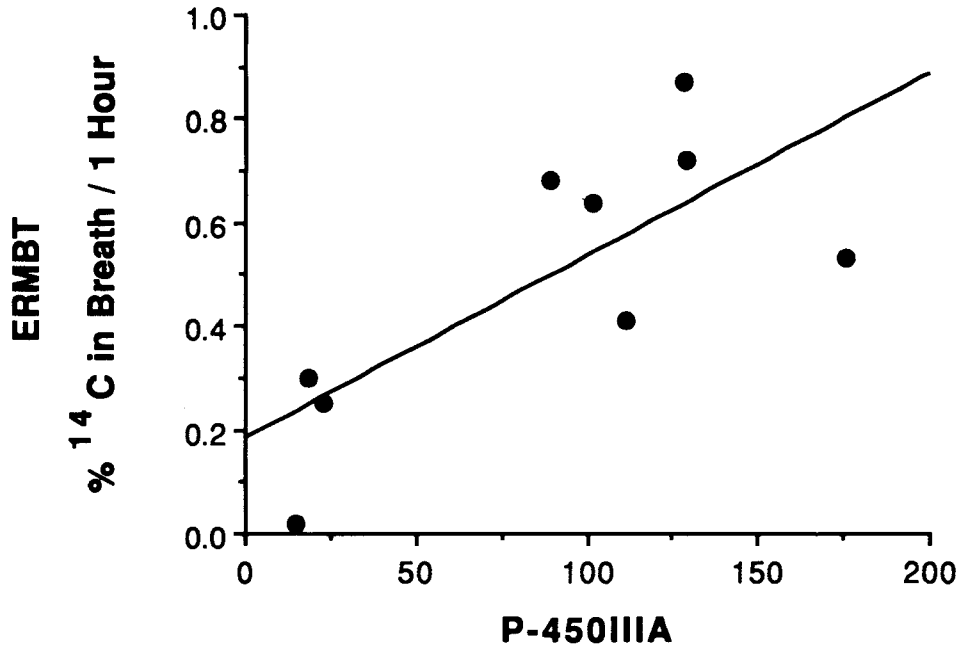

Fig. 3. Comparison of the ERMBT result with P450IIIA immunoreactive protein level determined in the microsomes of nine patients expressed as the percentage of immunoreactivity observed in the 14 normal liver microsomes. The linear correlation has an r^2 value of 0.56 ($p = 0.02$).

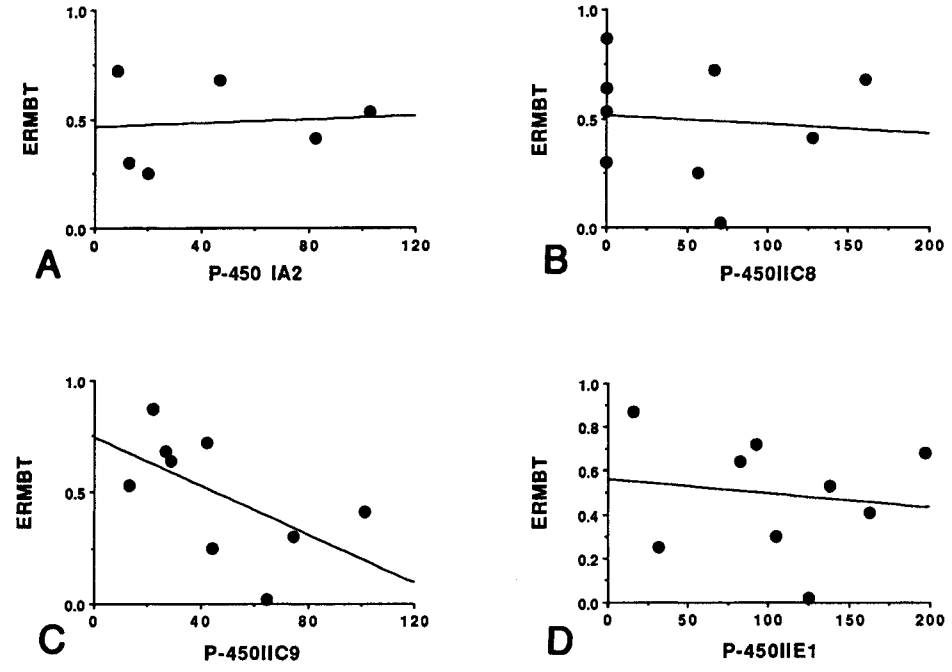

Fig. 4. Comparison of the ERMBT result expressed as a percentage of ^{14}C dose administered that was exhaled during 1 hour, with levels of four different P450 proteins in severe liver disease expressed as a percentage of control levels. **A,** P450IA2 ($r^2 = 0.008$, $p = 0.87$). **B,** P450IIC8 ($r^2 = 0.009$, $p = 0.81$). **C,** P450IIC9 ($r^2 = 0.334$, $p = 0.1$). **D,** P450IIE1 ($r^2 = 0.02$, $p = 0.72$).

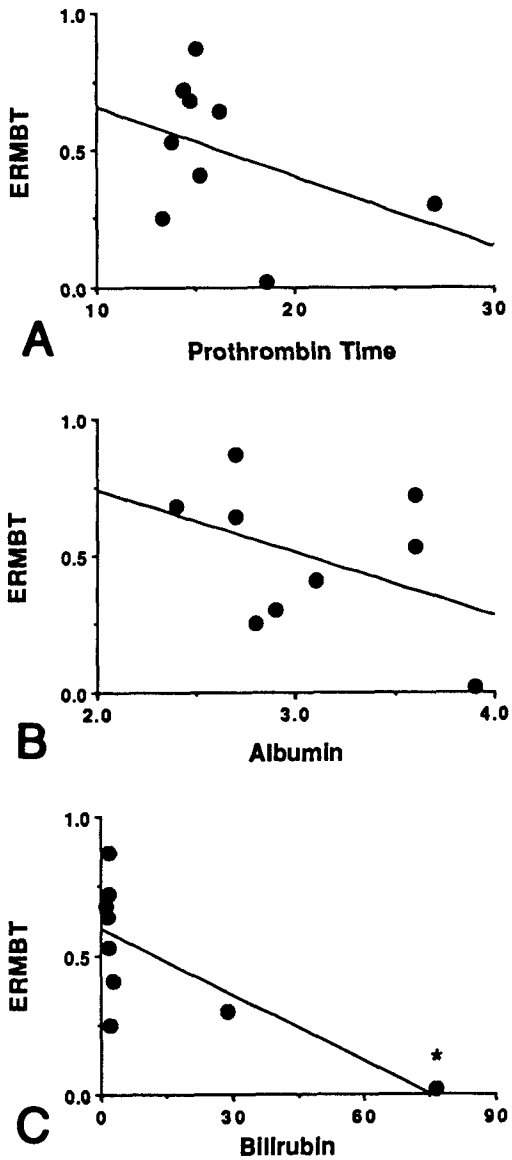

Fig. 5. Correlation of the ERMBT results (percentage of ^{14}C dose administered that was exhaled during 1 hour) and the traditional measures of liver function prothrombin time, serum albumin, and serum bilirubin. **A,** Prothrombin time in seconds ($r^2 = 0.016$). **B,** Albumin in grams per deciliter ($r^2 = 0.189$). **C,** Bilirubin in milligrams per deciliter ($r^2 = 0.556$, $p < 0.025$). The correlation with bilirubin was not significant when the point marked with the *asterisk* (*) was deleted (See text).

fore and after liver transplantation; the findings are shown in Fig. 1. The mean rate of production of $^{14}CO_2$ from [^{14}C-*N*-methyl]erythromycin determined in these patients before transplantation was just 16% of the mean value we observed in the 86 control patients without liver disease; this difference was highly significant ($p = 0.0001$). After transplantation, the rate of $^{14}CO_2$ production from erythromycin increased dramatically, and the mean value was not significantly different from that observed in the 86 control patients (Fig. 1).

Immunoquantitation of P450 protein. At the time of transplantation surgery, a biopsy specimen weighing at least 10 gm was obtained from the diseased liver and immediately frozen. At a later date, microsomes were prepared and the relative level of P450IIIA protein was determined as described in the Methods section. For comparison, the levels of four other major P450 proteins were also assayed. The findings of these analyses are shown in Fig. 2. The mean level of P450IIIA immunoreactive protein in these microsomes was not significantly different from levels observed in the microsomes prepared from 14 patients without liver disease. Likewise, the mean level of P450IIE1 immunoreactive protein in the diseased microsomes was essentially unchanged from the protein concentrations observed in the normal liver microsomes. However, in this small sample of patients there did appear to be selective suppression of some cytochromes P450 in diseased livers. P450IA2, P450IIC8, and P450IIC9 levels were all decreased in diseased liver microsomes, compared with the normal microsomes. This was statistically significantly for P450IA2 and P450IIC9. Of note, even in severe liver disease the level of P450IIIA protein varied twelvefold, which is comparable to the variability in protein levels seen in normal liver microsomes.

For each of the nine patients with liver disease, we next compared the erythromycin breath test results and the P450IIIA immunoreactive protein concentrations directly determined in their liver microsomes. As shown in Fig. 3, there was a linear correlation between these two parameters that was statistically significant ($p = 0.02$).

To confirm that the correlation between the erythromycin breath test result and P450IIIA was specific, we also compared the erythromycin breath test results with the respective microsomal concentrations of P450IA2, P450IIC8, P450IIC9, and P450IIE1. As shown in Fig. 4, there was no significant correlation between the erythromycin breath test result and any of the other four cytochromes P450 assayed in these patients. In fact, the P450IIC9 level appeared to have an inverse but not a significant correlation with the erythromycin breath test result.

One explanation for the association between the erythromycin breath test result and P450IIIA protein

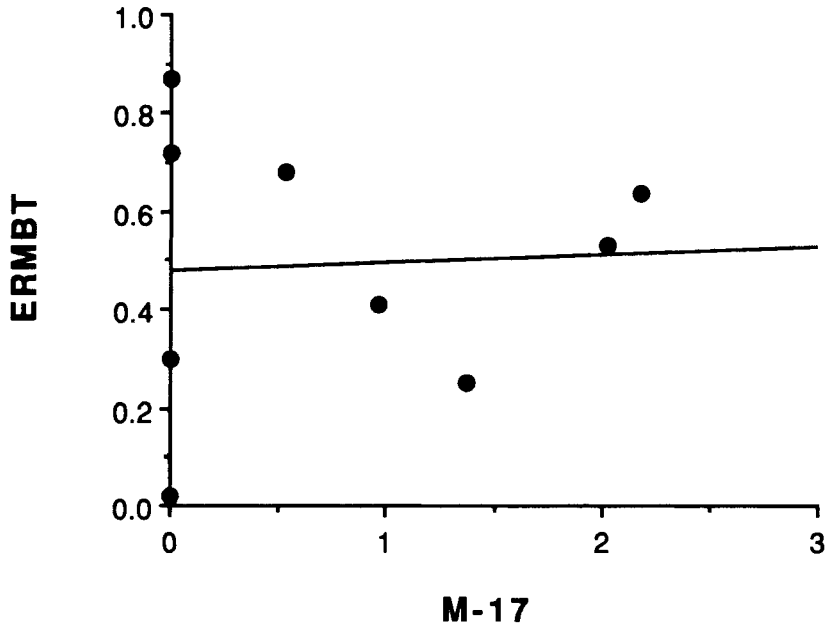

Fig. 6. Patients' ERMBT results (percentage of ^{14}C dose administered that was exhaled during 1 hour) compared with the rates of formation of the M-17 metabolite from cyclosporine in their liver microsomes. M-17 production is expressed in picomoles per minute per milligram of microsomal protein. No correlation between the two parameters was evident ($r^2 = 0.002$).

could be that both independently reflect the severity of the underlying liver disease. We therefore examined the traditional measures of liver function, such as prothrombin time, serum albumin, and serum bilirubin levels, that had been determined in the patients before liver transplantation. There was no correlation at all between the erythromycin breath test result and the prothrombin time and albumin level in the nine patients with liver disease, as seen in Fig. 5, *A* and *B*. There was a significant association seen between the erythromycin breath test results and bilirubin levels (Fig. 5, *C*). However, this correlation was determined largely by one patient whose bilirubin was 76 mg/dl. This patient had Wilson's disease and presented with a severe hemolytic anemia that required multiple blood transfusions. This level of bilirubin therefore largely reflected the acute hemolysis and not liver "function" per se. When this individual was excluded, the correlation between bilirubin and the erythromycin breath test result was no longer significant. In addition, comparisons of P450IIIA immunoreactive protein determinations with prothrombin time, albumin, and bilirubin levels did not reveal any significant association (data not shown).

In vitro cyclosporine metabolism. In human liver, P450IIIA catalyzes the metabolism of cyclosporine to its major metabolites.[4,5] We therefore examined cyclosporine metabolism in hepatic microsomes prepared from the diseased livers to determine the level of cytochrome P450IIIA activity. Microsomal protein was incubated with ^{3}H-cyclosporine and the rate of production of the M-1 and M-17 metabolites was determined by HPLC as outlined in the Methods section. We found no correlation between the erythromycin breath test results and the respective rates of production of M-17 (Fig. 6) and M-1 (data not shown) from cyclosporine. There was in fact no correlation between the microsomal content of P450IIIA protein and the rate of M-1 or M-17 formation in the microsome samples (data not shown). This lack of correlation was attributable in part to the fact that four of the nine liver microsome samples had no detectable cyclosporine metabolizing activity (Fig. 6), in spite of the presence of readily detectable concentrations of P450IIIA protein.

DISCUSSION

We have shown that, in patients with severe liver disease, the rate of production of $^{14}CO_2$ in the breath after intravenous injection of a trace dose of [^{14}C-*N*-methyl]erythromycin correlates significantly with the level of P450IIIA protein determined in their liver mi-

crosomes (Fig. 3). This correlation did not appear to be attributable to interpatient differences in liver functional ability that might have affected P450IIIA levels and the production of CO_2 from erythromycin in a coordinate but independent fashion, because no correlation was detected between either the liver specific content of P450IIIA protein or the erythromycin breath test results and the traditional measures of liver function in these patients (Fig. 5 and data not shown). Moreover, the correlation between the erythromycin breath test result and the level of P450IIIA protein was specific because the erythromycin breath test result did not at all correlate with the levels of any of the other four P450 proteins assayed (Fig. 4).

The correlation between the erythromycin breath test and P450IIIA levels existed in spite of the fact that the absolute rate of $^{14}CO_2$ production from erythromycin in these patients was significantly less ($p = 0.0001$) than the mean rate observed in patients without liver disease (Fig. 1). This marked reduction in in vivo erythromycin metabolism is consistent with our previous observations in rats with chronic liver disease induced by prolonged choline deficiency.[25] Furthermore, in both the patients with liver disease in this study (Fig. 2) and the choline deficient rats we found normal liver microsomal concentrations of P450IIIA protein in spite of the marked reduction in in vivo erythromycin metabolism.[25]

The basis for this decreased metabolism in our patients could not be determined from this study. It is well known that there are multiple changes in severe liver disease that can contribute to poor metabolism of drugs, including the reduced delivery of drug to the hepatocyte because of changes in hepatic circulation or abnormal hepatic architecture. However, reduced delivery of erythromycin to the hepatocyte should have a relatively small effect on the absolute rate of metabolism of the drug because erythromycin has relatively low hepatic extraction and its hepatic clearance should therefore be independent of hepatic blood flow,[26] that is, drug delivery. The major factor accounting for the reduced in vivo erythromycin metabolism we observed is more likely to be the reduction in total hepatocyte mass available for drug metabolism. Total hepatocyte mass is likely to have been comparable in this group of patients because they all had hepatic failure to a degree sufficient to justify liver transplantation but not severe enough to cause immediate death. In any case, twofold or threefold variations in hepatic mass would be expected to have far less effect on erythromycin metabolism than the twelvefold variation in P450IIIA levels that we observed in these patients' microsomes (Figs. 2 and 3). The rate of erythromycin metabolism in vivo (the erythromycin breath test) would therefore mainly reflect the "intrinsic clearance" of the hepatocyte that our data indicate directly correlated with the hepatocyte content of P450IIIA.

We were unsuccessful in our attempt to correlate the erythromycin breath test results directly with P450IIIA catalytic activity as measured by cyclosporine metabolism in the microsomes. This was not surprising because we also found no correlation between the rates of cyclosporine metabolism and the level of P450IIIA in the microsome samples. These findings are at odds with previous studies of microsomes prepared from surgical liver biopsy specimens where cyclosporine metabolism correlated closely with the content of immunoreactive P450IIIA.[5] One likely explanation for this discrepancy is that there was inactivation of the enzyme in our liver samples. This may be the result of the loss of the prosthetic heme from the P450IIIA protein during the prolonged period of devascularization (up to 2 hours) of the native liver that occurred during the transplant surgery before the liver was removed from the body (and subsequently available to us for biopsy). This conclusion is supported by a recent study that showed very rapid decline in liver P450 activity after death.[27] Moreover, we were unable to detect cyclosporine metabolism at all in four liver microsome samples that contained readily detectable P450IIIA protein.

Finally, we found that the relative levels of P450IA2 and P450IIC9 were significantly reduced compared with levels in normal hepatic microsomes, whereas P450IIC8, P450IIE1, and P450IIIA levels were not significantly changed (Fig. 2). Although we cannot completely rule out the possibility that perioperative ischemia may affect the stability of some P450 proteins more than others, previous studies in rats have suggested that there is differential regulation of P450 proteins in liver disease.[25,28,29] This conclusion is further supported by the only other published report examining P450 isozymes in diseased human liver in which Guengerich and Turvy[30] analyzed microsome samples prepared from 36 normal and 42 cirrhotic livers for the content of P450IA2, P450IIC, P450IIE1, and P450IIIA. They found that the levels of P450IIIA were unchanged and that the levels of P450IA2 were significantly decreased in the diseased livers, supporting our findings. In addition, these investigators found no alterations in P450IIC levels, although we found a significant decrease in P450IIC9 but not in P450IIC8. It is possible that Guengerich and Turvy[30] measured a

summation of the changes in the levels of various members of the P450IIC subfamily. Also, in contrast to our study, these investigators found a significant reduction in P450IIE1 in diseased livers. Because the P450IIE1 subfamily is thought to contain only one gene,[3] it is unlikely that differences in antibodies used would account for these differences, which remain unexplained. Nonetheless, our data support the conclusions of Guengerich and Turvy[30] that liver disease may have selective effects on the regulation of different P450 isozymes.

In summary, our study of nine patients undergoing liver transplantation for severe liver disease provides the best evidence to date that the erythromycin breath test is a specific assay of in vivo P450IIIA activity in patients. Because these patients maintained more than a tenfold interpatient variability in erythromycin breath test results that appeared to be independent of the severity of liver dysfunction, the erythromycin breath test may not be useful as a general assessment of liver functional ability. However, the erythromycin breath test has some potential advantages over an analogous test, the aminopyrine breath test, which has been widely used in the research setting as a measure of liver function.[31] Unlike the P450 isozymes involved in aminopyrine demethylation, P450IIIA has been relatively well characterized in humans for medications that are likely to affect P450IIIA activity,[32] so that these could be avoided or compensation made for their effects. The erythromycin breath test may therefore have advantages over the aminopyrine breath test as a way to monitor the progressive loss of liver function in a given individual over time.

Finally, it is possible that by determining the relative P450IIIA activity in patients with liver disease, the erythromycin breath test may provide useful information to help physicians determine appropriate administration of medications metabolized by P450IIIA. We have already shown that in patients with healthy livers, the erythromycin breath test predicts the relationship between the daily dose of cyclosporine and its trough whole blood levels.[15] Similar studies should now be undertaken in patients with liver disease.

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