0026-895X/92/020280-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:280-285

Anti-liver Microsomes Autoantibodies and Dihydralazine-Induced Hepatitis: Specificity of Autoantibodies and Inductive Capacity of the Drug

MOHAMMED BOURDI, JEAN-CHARLES GAUTIER, JASMINA MIRCHEVA, DOMINIQUE LARREY, ANDRE GUILLOUZO, CHANTAL ANDRE, CLAIRE BELLOC, and PHILIPPE H. BEAUNE

Institut National de la Santé et de la Recherche Médicale Unité 75, Centre Hospitalo-Universitaire Necker-Enfants-Malades, F-75730 Paris, Cedex 15, France (M.B., J.-C.G., J.M., C.B., P.H.B.), Institut National de la Santé et de la Recherche Médicale Unité 24, Hôpital Beaujon, F-92118 Clichy, France (D.L.), Centre Hospitalo-Universitaire Henri Mondor, F-94010 Créteil, France (C.A.), and Institut National de la Santé et de la Recherche Médicale Unité 49, Hôpital Pontchaillou, F-35033 Rennes, France (A.G.)

Received January 28, 1992; Accepted May 11, 1992

SUMMARY

Anti-liver microsomes (anti-LM) autoantibodies in patients with dihydralazine-induced hepatitis were found to react specifically with cytochrome P4501A2 (P4501A2) but not with P4501A1 expressed in yeast and bacteria. These results were confirmed by immunoinhibition of methoxyresorufin-*O*-demethylase activity (supported by the P4501A subfamily); anti-LM antibodies more strongly inhibited this activity in yeast expressing P4501A2 than in yeast expressing P4501A1. Anti-LM were shown to be specific to the disease; in three cases, these autoantibodies were present at high titers during disease, whereas the titers decreased upon recovery and became undetectable a few months after recovery. Thus, there exists a time-dependent relationship between the disease and the autoantibodies, which does not prove that the autoantibodies are causative of the hepatitis; they might only be a marker. The inductive capacity of dihydralazine toward P450 was also studied. In rats treated *in vivo* and in human hepatocytes treated *in vitro* with dihydralazine, a 2-fold increase in P4501A2- and P4501A-supported monooxygenase activities was found. The levels of the other P450 isoforms tested were unchanged during treatment, both *in vivo* in rats and *in vitro* in cultures of human hepatocytes. In human hepatocytes, dihydralazine produced a dose-dependent increase in the level of P4501A up to 0.1 mM; induction of P4501A was less strong at 0.2 mM and disappeared at 0.5 mM. The same treatment did not change the level of P4503A4, taken as control. The strong heterogeneity in the expression of P4501A enzymes in human liver and the capacity of these enzymes for induction by dihydralazine and by other compounds might be predisposing factors in this autoimmune disease.

Circulating autoantibodies reacting with liver/kidney microsomes (anti-LKM2) or with liver microsomes (anti-LM) have been found in sera from patients with tienilic acid- (1, 2) and with dihydralazine-induced hepatitis (3–6). Similar autoantibodies (anti-LKM1) were also found in chronic active hepatitis, a disease that is apparently not caused by a toxin. However, in one case of this disease, atypical anti-LKM were found (7). Target antigens recognized by anti-LKM2, anti-LM, and classical anti-LKM1 include P4502C8–10 (8, 9), P4501A2 (6), and P4502D6 (10), respectively. In the case cited above (7), the autoantibodies were found to be directed against P4501A2. The specificity of some autoantibodies has been further determined; anti-LKM1 recognized basically a small peptide within P4502D6 (10), whereas atypical anti-LKM recognized P4501A2 and not P4501A1 (7). Dihydralazine-induced hepatitis is the second type of drug-induced liver disease to be associated with anti-P450 autoantibodies. A simple scheme has been proposed to explain the triggering of these diseases and the appearance of autoantibodies (9); for dihydralazine, P4501A2 might metabolize the parent drug to produce a reactive metabolite able to bind to P4501A2 and subsequently to behave as an hapten, leading to the reaction of the immune system and the production of the autoantibodies. These antibodies might contribute to the pathogenesis of the liver disease caused by dihydralazine but might also be a marker of the disease.

In this paper, we have tried to determine (a) the specificity of the anti-LM autoantibodies, (b) the relationship between the disease and the autoantibody titer, and (c) the interaction between dihydralazine and the P4501A subfamily. We clearly

This work was supported by Grant MRT 91C 0542 from the Ministère de la Recherche et de la Technologie. M.B. was supported by a grant "Bourse Aguirre-Basualdo" from la Chancellerie des Universités de Paris.

ABBREVIATIONS: anti-LKM, antibodies to liver and kidney microsomes; anti-LM, antibodies to liver microsomes; P450, cytochrome P450; MROD, methoxyresorufin-*O*-demethylase; EROD, ethoxyresorufin-*O*-deethylase; PROD, pentoxyresorufin-*O*-dealkylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide; PCR, polymerase chain reaction.

show that anti-LM autoantibodies recognized P4501A2 but not P4501A1, that their titer decreased upon recovery, and that dihydralazine was a P4501A subfamily inducer.

Materials and Methods

Chemicals. Electrophoresis reagents were from Serva Fine Biochemicals (Heidelberg, Germany), nitrocellulose sheets were from Bio-Rad Laboratories (Richmond, CA), peroxidase- and peroxidase anti-peroxidase-conjugated immunoglobulins were from Dako-patts (Copenhagen, Denmark), the luminol staining kit was obtained from Amersham (Buckinghamshire, UK), resorufin and dihydralazine were from Aldrich-chimie (Strasbourg, France), NADPH, 7-ethoxyresorufin, and 7-pentoxyresorufin were obtained from Boehringer (Mannheim, Germany), and 7-methoxyresorufin was a generous gift of Dr. R. Peter (Department of Pharmacology, Biocenter, Basel, Switzerland). Other reagents were of the highest quality available and were purchased either from Prolabo (Paris, France) or from Sigma (St. Louis, MO).

Animals. Male Sprague-Dawley rats (150–200 g) (Iffa Credo, Les Oncins, France) were treated with β-naphthoflavone (40 mg/kg) or dihydralazine (150 mg/kg) daily for 3 days (intraperitoneally); the products were dissolved in corn oil, which has no inductive effects. Rats had free access to water and to a standard diet (Usines d'Alimentation Rationnelle, Villemoisson, France). The dose of dihydralazine was the highest tolerated.

Human sera. Six serum samples containing anti-LM antibodies were obtained from patients suffering from dihydralazine-induced hepatitis. Two control sera were obtained from patients who had received dihydralazine for 1–6 months without hepatic disease.

Human liver. Human livers were obtained from donors for kidney transplantation. Livers were removed within 30 min after circulatory arrest and were frozen at −80°. Collection of human samples was done in compliance with French regulations. Microsomes were prepared as previously described (11). For hepatocytes in culture, examination indicated normal liver histology in all donors.

Assays. Immunoblotting analysis was performed as previously described (12–14) and developed (according to the manufacturer's recommendation) with 4-chloronaphthol or luminol as substrate. The protein concentrations were estimated by the method of Lowry *et al.* (15), using bovine serum albumin as standard. EROD, PROD, and MROD were measured as described (16, 17). In immunoinhibition studies, 0.05–2 μl of sera/pmol of P450 were preincubated with microsomes, and the monooxygenase activity was then measured and compared with the activity observed in the absence of serum (100%).

Yeast preparation. P4501A1 and P4501A2 were cloned by PCR and inserted in the yeast expression vector (18, 19).[1] The PCR product was checked by restriction mapping and sequencing. Yeast microsomes prepared according to the methods in Refs. 18–20 were checked, in immunoblots probed with anti-rat P450 1A1/2, for the presence of human P4501A1 and P4501A2. Both P4501A1 and P4501A2 possessed EROD and MROD activity.

Bacteria preparation. Human P4501A1 and P4501A2 were cloned by PCR, as described above, and were inserted into a bacteria expression vector, pGex 2T. The 42 carboxyl-terminal amino acids were missing in the P4501A1 construction. Fusion proteins with glutathione-*S*-transferase were produced in *Escherishia coli* JM101 and were isolated according to the methods in Refs. 21 and 22. These fusion proteins were then checked in immunoblots with anti-rat P4501A1/1A2 and were used to characterize the anti-LM autoantibodies.

Cell isolation and culture. Hepatocytes were isolated and cultures were performed as previously described (23, 24). The inducers dihydralazine and 3-methylcholanthrene were added to cultures 24 or 36 hr after cell seeding. Thereafter, inducers were added every day, with each renewal of the medium. Dihydralazine (0.05–0.5 mM) and 3-methylcholanthrene (5 μM) were dissolved in 0.2% DMSO (final concentration). Control cultures received the same concentration of vehicle (0.2% DMSO). The cultures were terminated 24 hr after the third treatment. Cells were washed with 33 mM HEPES, pH 7.6, at 4° and were collected in the same buffer, by scraping of the cells. After centrifugation, the pellets were stored at −80° until analysis; after thawing and sonication, they were homogenized in a Potter-Elvehjem device in 0.25 mM sucrose, 10 mM Tris, 1 mM EDTA buffer, pH 7.5.

Results

Specificity of the autoantibody target. In order to determine whether sera from patients with dihydralazine-induced hepatitis reacted with P4501A1 or P4501A2, immunoblots were performed using cloned human P4501A1 and P4501A2 expressed in yeast. These immunoblots were first probed with the serum (LER) of a patient suffering from dihydralazine-induced hepatitis. A strong reaction with P4501A2 expressed in yeast was observed (Fig. 1), confirming our earlier results (6). In contrast, P4501A1 was not recognized by the serum of the same patient. This P4501A1 was recognized by antibodies directed against rat P4501A1/2, used as a control. Five sera from other patients with dihydralazine-induced hepatitis (anti-LM) were then tested. All of them recognized P4501A2 expressed in yeast and bacteria, but none of them recognized P4501A1 (data not shown). To confirm these results, we tested the ability of one patient serum to inhibit a monooxygenase activity (MROD) supported by both P4501A1 and P4501A2 expressed in yeast. We found that an anti-LM serum (NAL) inhibited MROD more strongly than did rabbit anti-human P4501A2 serum (Fig. 2A) and was a 2-fold better inhibitor of P4501A1 than was rabbit anti-rat P4501A1/2 serum (Fig. 2B). The patient serum more strongly inhibited the P4501A2 activity than P4501A1 (Fig. 2C). Only 0.04 μl of patient serum/pmol of P4501A2 was required for 50% inhibition of P4501A2 activity in yeast microsomes, whereas 0.6 μl (a concentration 1 order of magnitude higher) was required to obtain the same effect on P4501A1 as on P4501A2.

Evolution of autoantibodies during the disease. The autoantibody titer decreased after termination of the drug intake and disappearance of the disease. In three patients, autoantibodies were present during the disease and disappeared or drastically decreased after several months (Fig. 3). These immunoblot results were confirmed by immunoinhibition with two sera from the same patient (LER), one obtained at the time of the disease and the other 2 months after the drug was stopped. Serum at 0.03 μl/pmol of P450 inhibited 64% of the EROD activity (a P4501A subfamily-supported monooxygenase activity) at the time of the disease and only 25% 2 months later.

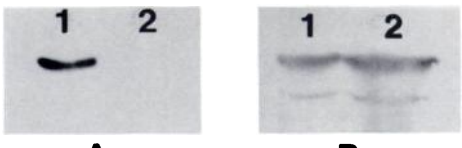

Fig. 1. Immunoblot of microsomes from yeast expressing human P4501A1 and P4501A2. *Lane 1*, 10 pmol of P4501A2; *lane 2*, 10 pmol of P451A1. The immunoblots were developed with anti-LM LER serum (diluted 1/1000) (A) and anti-rat P4501A1/2 (diluted 1/1000) (B). The immunoblot was developed with luminol.

[1] J.-C. Gautier. Yeast co-expression of human cytochrome P4501A, and microsomal expoxide hydrolase as a tool for the analysis of the first steps of benzo(*a*)pyrene activation. Manuscript in preparation.

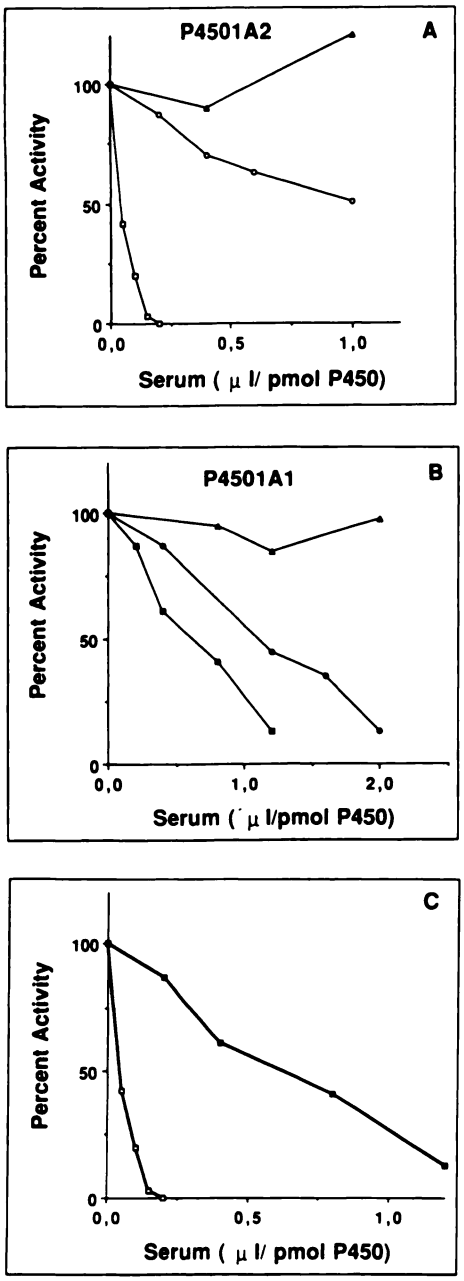

Fig. 2. Immunoinhibition of MROD activity. A, P4501A2 expressed in yeast (uninhibited activity, 0.1 mol/min/mol of P450), inhibited by anti-LM NAL serum (□), anti-human P4501A2 serum (○), and control serum (△). B, P4501A1 expressed in yeast (uninhibited activity, 0.9 mol/min/mol of P450), inhibited by anti-LM NAL serum (■), anti-rat P4501A1/2 serum (●), and control serum (▲). C, P4501A2 (□) and P4501A1 (■) expressed in yeast, inhibited by anti-LM NAL serum. Each *point* represent the mean of duplicate experiments.

***In vivo* effect of dihydralazine on P450s in rat liver microsomes.** EROD and MROD activities rose significantly in microsomes from dihydralazine-treated rats (Fig. 4). In contrast, PROD (P4502B-supported) was unchanged. These results were confirmed by immunoblots, which showed that dihydralazine, like β-naphthoflavone, induced P4501A2 in rat liver microsomes and that both compounds provoked the appearance of P4501A1, known to be undetectable in microsomes from untreated rats (Fig. 5). Dihydralazine treatment had no effect upon P4502B1/2, similar to PROD activity, but it decreased P4502C11 expression (Fig. 6). The P4501A subfamily was the only subfamily tested that was increased by dihydralazine treatment; P4502C11 was decreased and the other tested P450s remained unchanged. The overall P450 pattern was profoundly modified.

Fig. 3. Immunoblots of human liver microsomes (50 µg) probed with sera of patient suffering from dihydralazine-induced hepatitis. Patient ALM: *lane 1*, during disease; *lane 2*, 6 months after disease; patient ALE: *lane 1*, during disease; *lane 2*, 6 months after disease; patient LER: *lane 1*, during disease; *lane 2*, 2 months after cessation of dihydralazine. All sera were diluted 1/350.

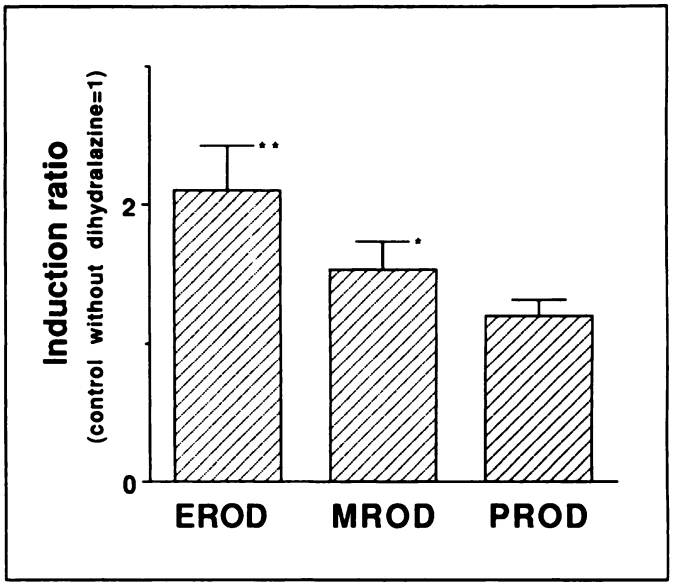

Fig. 4. Induction of rat hepatic microsomal EROD, MROD, and PROD with dihydralazine. Enzymatic determinations were performed in duplicate; results represent mean ± standard deviation for five treated animals. The experiment was repeated three times. *, $p < 0.05$; **, $p < 0.001$.

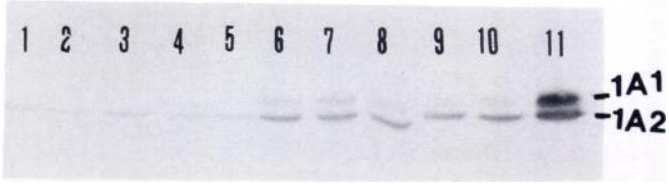

Fig. 5. Immunoblot of rat liver microsomes developed with anti-rat P4501A1/2 (diluted 1/500). *Lanes 1–5*, untreated rats (30 µg); *lanes 6–10*, dihydralazine-treated rats (30 µg); *lane 11*, β-naphthoflavone-treated rats (5 µg).

***In vitro* effect of dihydralazine on induction of the P450s in primary cultures of human hepatocytes.** To confirm this inducing effect in humans, cultures of human hepatocytes were maintained for 72 hr in the absence or in the presence of dihydralazine (0.1 mM). 3-Methylcholanthrene (5 µM), a well known P4501A inducer, was used as a positive control. Dihydralazine was also an inducer of P4501A2 in humans (Fig. 7); the induction ratio (P4501A in treated versus untreated hepatocytes) was 2.28 ± 0.5 for 0.1 mM dihydralazine

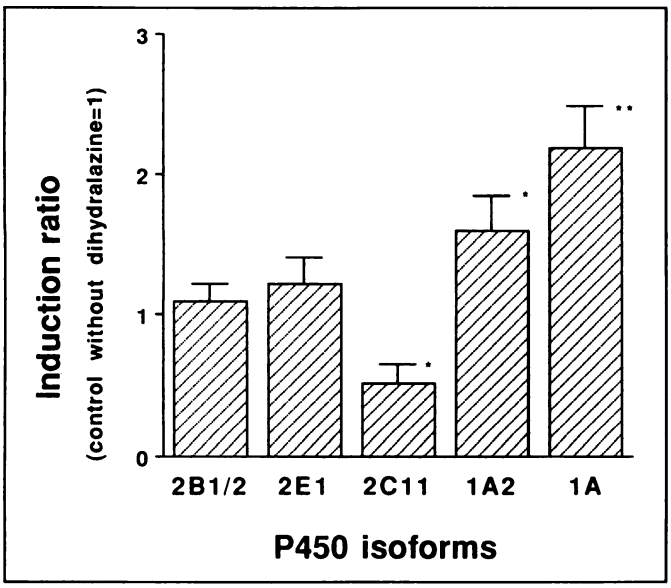

Fig. 6. Immunoquantification of P450s in liver microsomes from untreated or dihydralazine-treated rats. Results shown are mean ± standard deviation for five treated animals. Results significantly different from the control group are indicated; *, $p < 0.05$; **, $p < 0.001$. 1A is the sum of P4501A1 plus P4501A2.

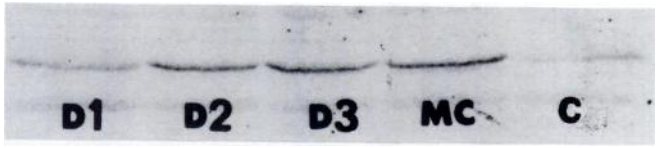

Fig. 7. Immunoblot of homogenates from human hepatocytes in primary culture, developed with anti-rat P4501A1/2 (diluted 1/500). *D1* to *D3*, hepatocytes cultured in the presence of 0.2, 0.1, and 0.05 mM dihydralazine, respectively (100 μg); *MC*, hepatocytes cultured in the presence of 5 μM 3-methylcholanthrene (100 μg); *C*, hepatocytes cultured in the absence of inducer (100 μg). These results are representative of experiments from two or three different cultures; the peroxidase-antiperoxidase system was used for immunoblot development, as described previously.

and 2.5 ± 0.3 for 5 μM 3-methylcholanthrene (three experiments). Conditions of electrophoresis for immunoblots did not enable a separation of P4501A1 and P4501A2, and the antibodies used were unable to distinguish between the two enzymes. Thus, the observed increase could not be specifically attributed to P4501A1 or P4501A2. These primary cultures of human hepatocytes were tested in the presence of increasing concentrations of dihydralazine (0.05, 0.1, 0.2, and 0.5 mM). The increase in the P4501A subfamily was dose dependent up to 0.1 mM; induction was lower at 0.2 mM and disappeared at 0.5 mM (Fig. 8). At this concentration toxicity was morphologically observed, and homogenates at 0.2 and 0.5 mM were blue-green, indicating heme degradation. Dihydralazine (0.1 mM) did not modify significantly the level of the other forms of P450 tested in this work, i.e., P4502D6, P4502C subfamily, and P4503A4 (Fig. 9). We tested the effect of the dose (0.1, 0.2, and 0.5 mM) of dihydralazine on the quantity of two other P450 isoenzymes, i.e., P4502C subfamily and P4503A4, and found that the treatment did not influence any of the isoenzymes tested (Fig. 8). Again, as in rats, the P450 pattern was drastically modified by dihydralazine treatment.

Discussion

In immunoblots, all tested sera of patients with dihydralazine-induced hepatitis clearly recognized P4501A2 but not

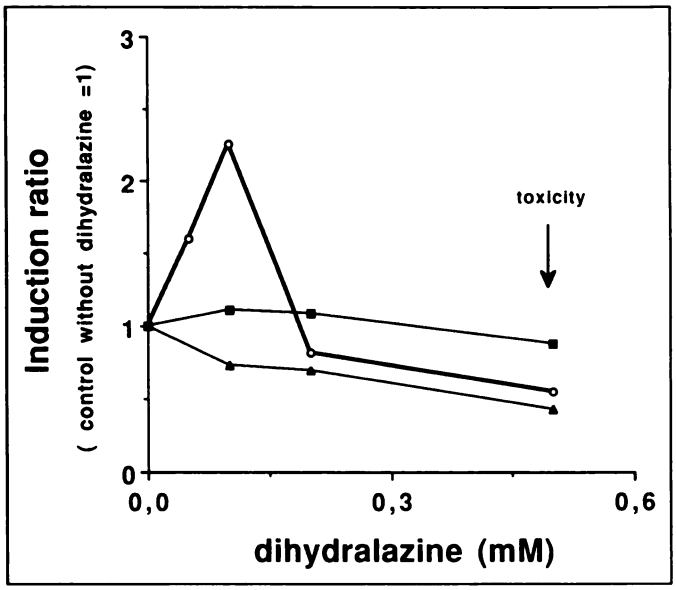

Fig. 8. Effect of increasing concentrations of dihydralazine on the induction of P450s in human hepatocyte cultures. Immunoquantification of the P4502C subfamily (▲), P4501A2 (○), and P4503A4 (■). Results shown are mean of experiments from two or three different cultures.

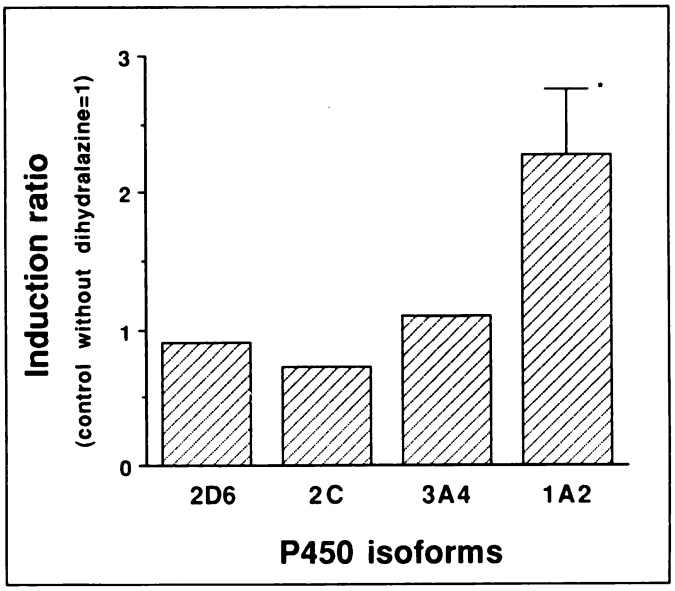

Fig. 9. Immunoquantification of P450s in primary cultures of human hepatocytes treated either with the vehicle (0.2% DMSO) or with dihydralazine (0.1 mM). Results shown are mean of experiments from two or three different cultures; *, $p < 0.05$.

P4501A1 (expressed in yeast or in bacteria), whereas antibodies developed in rabbits against rat P4501A1/2 recognized both antigens. These results were confirmed by immunoinhibition of a monooxygenase activity supported by both P4501A1 and P4501A2, namely MROD; anti-LM antibodies more strongly inhibited MROD activity in microsomes from yeast expressing P4501A2 than from yeast expressing P4501A1. The amount necessary for 50% inhibition was 1 order of magnitude higher for P4501A1 than for P4501A2. Such a difference was also observed in sera from patients and from controls in tienilic acid-induced hepatitis (9), indicating that immunoinhibition of P4501A1 may not be very specific, in spite of the fact that control sera were not inhibitory at all (Fig. 3). Human P4501A2

and P4501A1 show >80% similarities. Recognition of P4501A2 alone indicates that the autoantibodies are probably oligoclonal or monoclonal, because they react only with rare epitopes that are not shared by the two proteins. Moreover, such epitopes should be located either close to the active site or close to the reductase binding site, because the autoantibodies were able to inhibit monooxygenase activities. It is puzzling that, in another disease, atypical chronic active hepatitis (7), the autoantibodies were quite similar, i.e., recognizing human P4501A2 and not P4501A1. In that disease, no toxic substance or drug has been implicated, but it is possible that such a compound, unidentified until now, might be involved in triggering of the disease. It will be interesting to map the epitope(s) recognized by these anti-LM and to compare them with the epitope(s) recognized by the autoantibodies described by Manns *et al.* (7).

In an earlier paper, we showed that the autoantibodies were specific to the disease (6). Indeed, they were found in all tested patient sera and were absent in all controls, including patients receiving dihydralazine who did not have hepatitis. In the present paper, we show a time-dependent relationship between the disease and the antibody titer. In the three cases tested, it was shown that the antibodies were present at high titers during the disease; the titers decreased upon recovery or the autoantibodies became undetectable after a few months of recovery. This does not mean that the autoantibodies were the cause of the disease. Nevertheless, these results complement other evidence in favor of the immunologic origin of the disease, i.e., fever, eosinophilia, the absence of a dose-response relationship, lymphocyte infiltration in liver, and increased response in the case of rechallenge (4, 5). However, it was shown only that the relationship between the disease and the autoantibodies was time dependent, meaning that autoantibodies may simply be a marker of the disease. Nonetheless, it would be of interest to search for them, to detect the onset of the immunoreaction before hepatitis becomes severe.

In the second part of this work, we looked for a relationship between dihydralazine and the P4501A subfamily; the first question that arose concerned the inductive capacity of this compound. In rats treated *in vivo* and human hepatocytes treated *in vitro* with dihydralazine, we found a 2-fold increase in P4501A2 and in monooxygenase activities specific to the P4501A subfamily. Treatment with this compound resulted in the appearance of P4501A1 in rat liver microsomes. This induction was quite weak, compared with the effects of classical P4501A inducers such as dioxin, β-naphthoflavone, or 3-methylcholanthrene (25). In human cultures, P4501A1 could not be detected, because of the technique used and because of the lack of specificity of our antibodies. The use of anti-LM would not have provided further information, because they do not recognize P4501A1. Dihydralazine decreased the P4502C11 in rat liver. This result is in agreement with the fact that down-regulation of P4502C proteins was observed when liver microsomes from rats were treated with agents that induced the P4501A subfamily (12). The levels of other P450 isoforms tested in this work were unchanged during *in vivo* treatment of rats or *in vitro* cultures of human hepatocytes. A modified P450 enzymatic pattern was observed in liver upon dihydralazine treatment, leading to an overall modified metabolic capacity. In human hepatocytes, dihydralazine produced a dose-dependent increase in the level of P4501A up to 0.1 mM; at 0.2 mM induction disappeared, and at 0.5 mM P4501A levels were lower than the control. Induction of the P4501A subfamily was obtained with a high dose, compared with that given to patients, but it is possible that the treatment of patients on a long term basis with a low dose might have the same effect as treatment of human hepatocytes for a short time with a high dose. Such findings were observed with phenobarbital treatment (26). The inductive effect was also weak in human hepatocytes, compared with that of 3-methylcholanthrene, which produced the same effect with much lower concentrations (24) (this paper). In patients with dihydralazine-induced hepatitis, a lower activity of P450-catalyzed biotransformation reactions was observed (27). This is not in contradiction to an inductive effect of dihydralazine; the decrease in the P450 concentration was the consequence of the disease but not the cause of it. It is logical that P450 concentrations decreased in patients suffering from severe hepatitis; in a group with mixed liver disease, P450 activities were shown to be significantly impaired, compared with normal controls (28). Contact, by patients treated with dihydralazine, with another inducer of the P4501A subfamily, such as polycyclic hydrocarbons (prominent components of cigarette smoke) (Ref. 25 and references herein), may increase the risk of hepatotoxicity by enhancing the metabolism of dihydralazine and, therefore, the formation of reactive metabolite(s) and by enhancing the target antigen of the autoantibodies, both in endoplasmic reticulum and in plasma membrane (29). Several studies suggest a >40-fold interpatient variation in the liver content and catalytic activity of P4501A enzymes in the liver (30, 31). The strong heterogeneity in the expression of P4501A enzymes in humans and the interpatient differences in the catalytic activities of the P450s or detoxifying enzymes indicate that some subjects may be more prone to produce reactive metabolites.

In conclusion, we have shown that (a) autoantibodies (anti-LM) specifically recognized P4501A2 and not P4501A1, (b) because of the decreased titer upon recovery of dihydralazine, anti-LM could be both a marker of disease and a cause of it, and (c) dihydralazine was a specific inducer of the P4501A subfamily, and this induction might increase the risk of hepatotoxicity by causing formation of reactive metabolites and enhancing the target antigen level of autoantibodies and T cells.

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Send reprint requests to: Philippe H. Beaune, INSERM U-75, CHU Necker-Enfants-Malades, 156 Rue de Vaugirard, F-75730 Paris, Cedex 15, France.