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Cytochrome P-450 Expression in Sudden Infant Death Syndrome

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ABSTRACT. In the human liver, the major rise of the cytochrome P-450 isoform content occurs during the first months following birth (e.g., the high vulnerability period to sudden infant death syndrome (SIDS), a syndrome frequently associated with viral infection and drug hypersensitivity. We examined the expression of individual P-450 isoforms in liver samples collected postmortem from SIDS infants and compared values with those of control adults and children of the same age suffering from various pathologies. Hepatic microsomes were prepared and examined for their content in total P-450, the level of individual isoforms (CYP1A2, CYP2E1, CYP4A, CYP3A, and CYP2C) determined with specific antibodies and for their enzymatic activities. Total RNA was extracted and probed with several CYP cDNAs and oligomers. The overall hepatic P-450 content was not modified in SIDS infants. Among cytochrome P-450 isoforms, only CYP2C was markedly increased. This rise resulted from an accumulation of RNA encoding CYP2C and was associated with a stimulation of diazepam demethylation. The precocious expression of CYP2C in SIDS could result in a higher production of epoxyeicosatrienoic acids in the neonate, believed to act as relaxant of pulmonary smooth muscles. Its consequence might be the induction of fatal apnea in SIDS. BIOCHEM PHARMACOL 52;3:497–504, 1996.

KEY WORDS. cytochromes P-450; sudden infant death syndrome; epoxyeicosatrienoic acids; CYP2C; diazepam; human liver

During the last decades, unexpected deaths of apparently healthy infants became the first cause of mortality in infants between 1 month and 1 year of age in all developed countries. SIDS[‖] accounted for 50% of postperinatal deaths [1]; only minor lesions were described after full postmortem examinations. These deaths have some well-known characteristics: a peak during autumn and winter [2], a sex distribution with a higher sensitivity for males [3], and an increased risk for babies with a low gestational age or low birth weight [4, 5].

Another feature was the unusual age distribution of SIDS. The risk was rather low during the early neonatal period, increased markedly from the second to the fourth postnatal month, and declined thereafter. More than 90% of SIDS occurred before the age of 6 months [1, 2, 5]. These data supported the hypothesis of an immature period during which infants were highly vulnerable. Increasing risks for SIDS have been associated with viral infections and/or inflammatory responses [6, 7], and drug hypersensitivity to phenothiazines has been reported [8]. In animal models, studies showed repression of drug-metabolizing enzymes by interferon or interferon inducers [9, 10]. This latter point led us to hypothesize a possible modification of drug biotransformation during viral infection accompanying SIDS.

Drugs are hydrophobic compounds that require a first step of activation in the presence of NADPH and molecular oxygen, to be eliminated from the body. This activation is generally carried out by cytochrome P-450-dependent monooxygenases: not only exogenous compounds (drugs, pollutants, carcinogens, cosmetics, etc.) but, also, endogenous molecules, such as steroids, fatty acids, and prostaglandins, are substrates of cytochrome P-450. To cope with the large number of substrates and the wide diversity of their chemical structures, several isoforms of cytochrome P-450 exist and exhibit an overlapping substrate specificity (for review, see [11]). The ontogenesis of hepatic cytochrome P-450 in the human liver has been documented during the last twenty years. It is generally assumed that only CYP3A members are present in the fetal liver [12–14], and others P-450s are present in extremely low concentrations or are totally absent [15, 16] but expected to develop

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‖ *Abbreviations:* CYP, cytochrome P-450 (EC 1.14.14.1); EET, epoxyeicosatrienoic acid; SIDS, sudden infant death syndrome.

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during the weeks or months following birth [15] (i.e. the high vulnerability period to SIDS). The coincidence of these 2 events prompted us to examine carefully the hepatic content of total P-450 and of individual isoforms in SIDS, and to compare these values to those of children of the same age who died of well-identified pathologies, and also to adult control values. Surprisingly, only the CYP2C concentration and activities were augmented in SIDS and were likely associated with a higher expression of CYP2C genes, resulting in an enhanced accumulation of RNA in hepatic samples.

MATERIALS AND METHODS

Tissue Collection

All protocols were conducted following the recommendations of the Ethical Committee of INSERM. Adult liver samples were obtained from donors for kidney transplantation. Donors had no severe chronic pathology and had generally died following traffic accidents. They had no known repeated drug consumption; no information was available regarding their smoking and drinking habits. Liver samples were collected postmortem from children aged ½ hr to 9 years, with parental informed consent, and were classified according to their postnatal age. Causes of death for controls were hypertension, hydro- and microcephaly, malformation, or severe bacterial infection. Unexpected, but fully explained, deaths of infants with congenital defects and infants treated with drugs during the days before death were excluded from the study, but were occasionally used for comparison. Liver samples were excised, usually within the hour following death, for controls, or admission to hospital, for SIDS (less than 4 hr after death), frozen in liquid nitrogen, and kept at −80°C until use.

Microsome Preparation and Determination of Individual P-450 Protein Content

After thawing in ice-cold isotonic saline to remove the excess hemoglobin, tissues were homogenized and microsomes prepared as previously described [17]. The overall cytochrome P-450 content was assayed by its capacity to spectrally absorb at 450 nm after addition of carbon monoxide and chemical reduction by sodium dithionite [18], and the protein concentration was estimated by the procedure of Lowry *et al.* [19]. Individual cytochrome P-450 isoforms were immunochemically determined in liver microsomes with antibodies raised against purified isoforms: microsomal proteins (60 μg) were applied to SDS-9% polyacrylamide gel [20]. After migration, proteins were blotted overnight onto nitrocellulose sheets and further detected with corresponding antibodies. The amount of reacting material was measured by densitometric scanning of nitrocellulose membranes and a computerized program from Imstar (France) [21]. Under these conditions, signals were proportional to the amount of cross-reacting material loaded onto the gel. Reference adult samples were routinely incorporated in each experiment to calibrate determinations. Results were expressed as optical density units per mg microsomal protein.

Origin of Antibodies

Rat CYP1A1 was prepared as previously detailed [22] and antibodies raised in rabbits. Antirat CYP1A recognized both rat and human CYP1A1 and 1A2. CYP2C9 (formerly P-450-8) and CYP3A4 (formerly P-450NF or P-450-5) were purified from adult human liver, and polyclonal antibodies raised in rabbits [23]. Under our electrophoretic conditions, the antiCYP2C9 sera recognized a unique band in

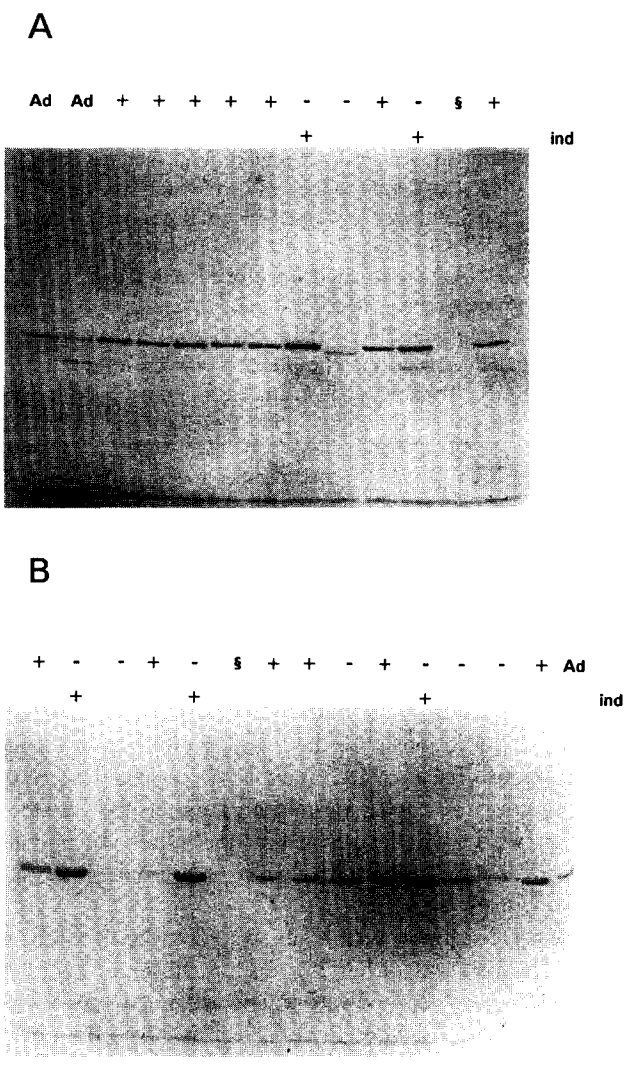


FIG. 1. Western blot analysis of liver microsomes. 60 μg of microsomal proteins were electrophoresed on 9% SDS-polyacrylamide gel, transferred onto a nitrocellulose sheet, and probed with polyclonal antibodies to CYP2C9 (A) or CYP3A (B). Human liver microsomes from adults [Ad], from nonSIDS [−], or from SIDS [+] newborns were migrated. § denotes proteins from degraded microsomes deprived of any CO binding spectrum and enzymatic activities, and used as a negative standard. Liver microsomes isolated from nonSIDS children treated with inducers (noted as ind +) were excluded from control groups.

adult liver microsomes, believed to be composed of all CYP2C subfamily members even at a higher protein concentration (120 μg) ([12] and Fig. 1). Antibodies against rat liver CYP4A1 were prepared and kindly provided by Dr. C. Célier [24]; a single band was detected in human liver microsomes in the 40–60 kDa range. Antirat CYP2E1 was purchased from Oxygene (Dallas, TX, U.S.A.), and recognized a single band in human liver microsomes.

Monooxygenase Activities

Several substrates were used to probe P-450 isoforms. Monooxygenase activities were measured with 0.3 nmoles P-450 as reported elsewhere. Methoxyresorufin and ethoxyresorufin dealkylations (CYP1A1 and 1A2) were estimated by spectrofluorometry according to Burke *et al.* [25]. The formation of 6-hydroxychlorzoxazone (CYP2E1) was quantified after separation by HPLC [26], and diazepam demethylation (CYP2C and CYP3A) was assayed essentially as described by Reilly *et al.* [27]. The metabolism of tolbutamide was assayed following the procedure of Knodell *et al.* [28], with some minor modifications. Briefly, microsomal proteins corresponding to 0.1 nmole P-450 were incubated with 0.6 mM tolbutamide. Tolbutamide and its metabolite were extracted with 1 mL of CH_2Cl_2 and separated by HPLC on a Dupont Zorbax C18 column (150 × 4.6 mm, 5 μm) with a mobile phase consisting of acetonitrile: 0.05% H_3PO_4 (28:72, v:v) at a flow rate of 1 mL/min. Retention times for hydroxytolbutamide and the parent drug were, respectively, 3.9 and 10.9 min. Effluents were monitored at 230 nm and the hydroxyderivative was quantified with authentic hydroxytolbutamide as standard (Ultrafine Chemicals, Manchester, U.K.).

RNA Preparation and Analysis

RNA was isolated from frozen tissues by the procedure of Moroy *et al.* [29]. The RNA pellet was resuspended in diethylpyrocarbonate-treated water and the quality of the RNA preparation checked in formaldehyde-denaturing electrophoresis. The degradation of 18 and 28S ribosomal RNA should have been minimal, and the sample was discarded if any trace of smear was visible on the gel. For quantitative purposes, total RNA (5 to 10 μg) was analyzed in slot-blots as detailed elsewhere [30]. Hybridization temperatures were 42°C and 50°C, respectively, for the 19-base oligomer designed for CYP3A and the 37-base oligomer for CYP2E1. Washes were performed at room temperature in 5 × SSC, 0.1% SDS. Relative to the time of exposure, the signal intensity was a function of the amount of RNA applied to nitrocellulose, permitting quantitation of RNAs reacting with probes. Hybridizations were conducted with P-450 probes and with a β-actin probe as reference; results were expressed as the P-450 RNA/actin RNA ratio.

Probes consisted of a genomic clone pAC.H8H supplied by Dr. Battula (NCI, Bethesda, MD, U.S.A.) for β-actin [31] and by Dr. C. Ged for CYP2C [32]. The latter clone hybridized with RNAs encoding CYP2C8, CYP2C9, CYP2C10, CYP2C18, and CYP2C19, and data collected from these experiments represented the sum of the entire subfamily. Oligomers for CYP3A4 (5′-AGAATGGATC-CAAAAAATC-3′) and for CYP2E1 (5′-AAACTCTGT-GTCATTCCCCGCTCATGAGTGTGTGGAG-3′) were 5′-end labeled with γ-^{32}P-ATP and polynucleotide kinase.

Statistical Analysis

Results were compared among groups and significance was calculated according to the Student's *t*-test, using a computerized program (NCSS, version 5.0).

RESULTS

Composition of Groups

The number of hepatic samples and the mean gestational age of children included in the different groups are listed in Table 1. Control groups were mostly constituted of premature babies with gestational ages lower than in SIDS groups (especially for group 3). However, this should have no influence because it has been shown previously that the gestational age did not modify the postnatal evolution of cytochrome P-450 ([25] and unpublished data). The major group of SIDS (group 4) included 35 children: 32 were aged

TABLE 1. Composition and mean gestational age of each group

		Controls		Sudden Infant Death Syndrome	
Group	Postnatal age	*n*	gestational age	*n*	gestational age
1	<24 hr	11	30 ± 3.6	–	–
2	1–7 days	15	31.2 ± 3.8	–	–
3	8–28 days	12	31.3 ± 4.8	6	40
4	1–9 months	6	35 ± 7	35	38 ± 2.5
5	>9 months	5	n.a.		
6	adults	13	n.a.		

Results are the mean ± SEM and are expressed in weeks. n.a. not available.

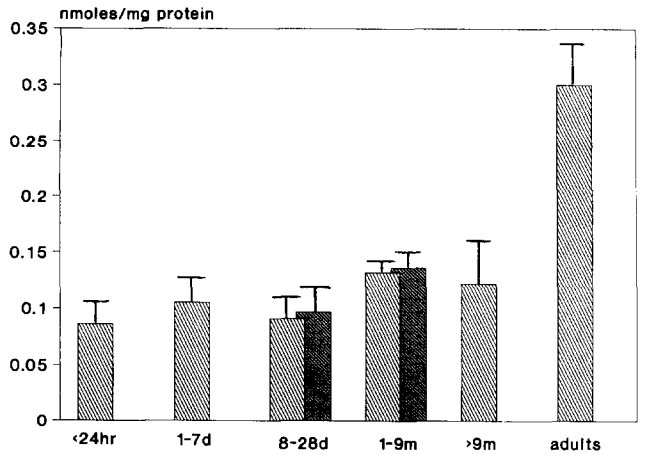


FIG. 2. Evolution of total cytochrome P-450 content in control and SIDS human liver microsomes from birth to adulthood. Results are the mean ± SEM for samples classified according to Table 1. Darker columns correspond to SIDS samples.

less than 6 months (90%), 21 were male and 14 female (sex ratio 1.5), 5 were premature (14%), 7 were small-for-date (20%), and 4 suffered from birth injuries (11%). The mean postnatal age was 101 ± 11 days in the SIDS group vs 144 ± 43 in the corresponding group of controls.

Cytochrome P-450 Content

Basically, the total P-450 concentration remained fairly stable during the period studied, to about 50% of the adult value (0.300 ± 0.037 nmol · mg^{-1} protein, $n = 13$) consistent with previous data from our laboratory [12]. When the total cytochrome P-450 content was compared between control and SIDS groups, no significant difference ($P > 0.3$) could be noticed (Fig. 2).

Individual Cytochrome P-450 Isoforms

The developmental profile of individual cytochrome P-450 varied from isoform to isoform. The content of CYP2E1 is shown in Fig. 3a; it steadily increased over the perinatal period, and no modification was shown to occur in SIDS compared with control groups ($P > 0.5$). A similar lack of difference was observed for the content of CYP1A2 (data not shown) and CYP3A4 (Fig. 1). The hepatic content of CYP4A1 remained fairly stable during the postnatal period in control groups and declined slightly at adulthood. In SIDS groups, a moderate but significant decrease was noticed (Fig. 3b). Conversely, the CYP2C content increased progressively during the perinatal period, to reach adult values. In SIDS, the CYP2C value rose sharply and exceeded control and adult values as early as 1 month of age (Fig. 3c). Between 1 and 9 months, the CYP2C content remained constant when examined in SIDS infants.

Monooxygenase Activities

Methoxyresorufin demethylase and ethoxyresorufin deethylase activities and the 6-hydroxylation of chlorzoxazone mediated, respectively, by CYP1A2 and CYP2E1, remained similar in SIDS and control groups (data not shown).

The rate of diazepam demethylation followed the CYP2C content of liver microsomes from children aged 1–9 months; it was consistently higher in SIDS infants than in controls (70.3 ± 13.1 vs 31.7 ± 8.2 pmoles of desmethyldiazepam · min^{-1} · mg^{-1} protein; $P < 0.02$) and even exceeded activity measured in adults (Fig. 4a). The hydroxylation of tolbutamide exhibited the same behaviour. A significantly higher activity was reported in SIDS than in controls (18.0 ± 2.7 vs 8.2 ± 2.8 pmoles of hydroxy tolbutamide · min^{-1} · mg^{-1} protein; $P < 0.01$) and was close to the adult value (Fig. 4b). On the other hand, the hydroxylation rate of diazepam into temazepam (CYP3A-dependent) was augmented, but not significantly: 69.5 ± 14.9 vs 40.0 ± 28.9 ($P > 0.1$) in SIDS. In newborns aged 8–28 days, no difference was noticed between SIDS and control groups in diazepam demethylation or tolbutamide hydroxylation, but this group included a very limited number of samples.

Determination of RNA Levels

SIDS values for CYP2C RNA were 3–4 times higher than either control neonatal or adult values (Fig. 5). The same blots hybridized with CYP3A and CYP2E1 oligonucleotides did not exhibit any significant difference between control and SIDS samples ($P > 0.5$) (data not shown).

All together, these data clearly suggest that the expression of CYP2C members is specifically affected in the sudden death infant syndrome.

DISCUSSION

In this study, we explored the cytochrome P-450 system of human microsomes prepared from neonatal livers, in relation to pathology and postnatal age. We took advantage of the presence of a National Centre for Study and Prevention of Infant Death, where samples from Sudden Death Infant Syndrome were collected. In this respect, we were concerned about the composition of groups. In the SIDS group are children who died in cot without previous disease (except cough with light fever) and with no classifiable cause of death after a full anatomohistological examination. When the characteristics of this group were compared to those of a larger SIDS group, the data, including age distribution, sex ratio, and percentage of prematures or low birth weights, were comparable and indicated that this group was a good representative of SIDS [5].

"Control" groups were more questionable: actually, they included pediatric patients hospitalized for well-defined diseases (no hepatic disease), not having received drugs with known inhibitory or inductive capacities for cytochrome P-450. Samples from children given these inductive drugs showed a higher content of certain P-450 isoforms (mainly CYP3A, but also CYP2C) and dependent-monooxygenase activities. Consequently, these samples were discarded; this partly explains the relatively low number of samples included in the control group of children aged 1 to 9 months.

The results clearly showed that the overall P-450 content, the levels of CYP3A, CYP2E, and CYP1A2, and of

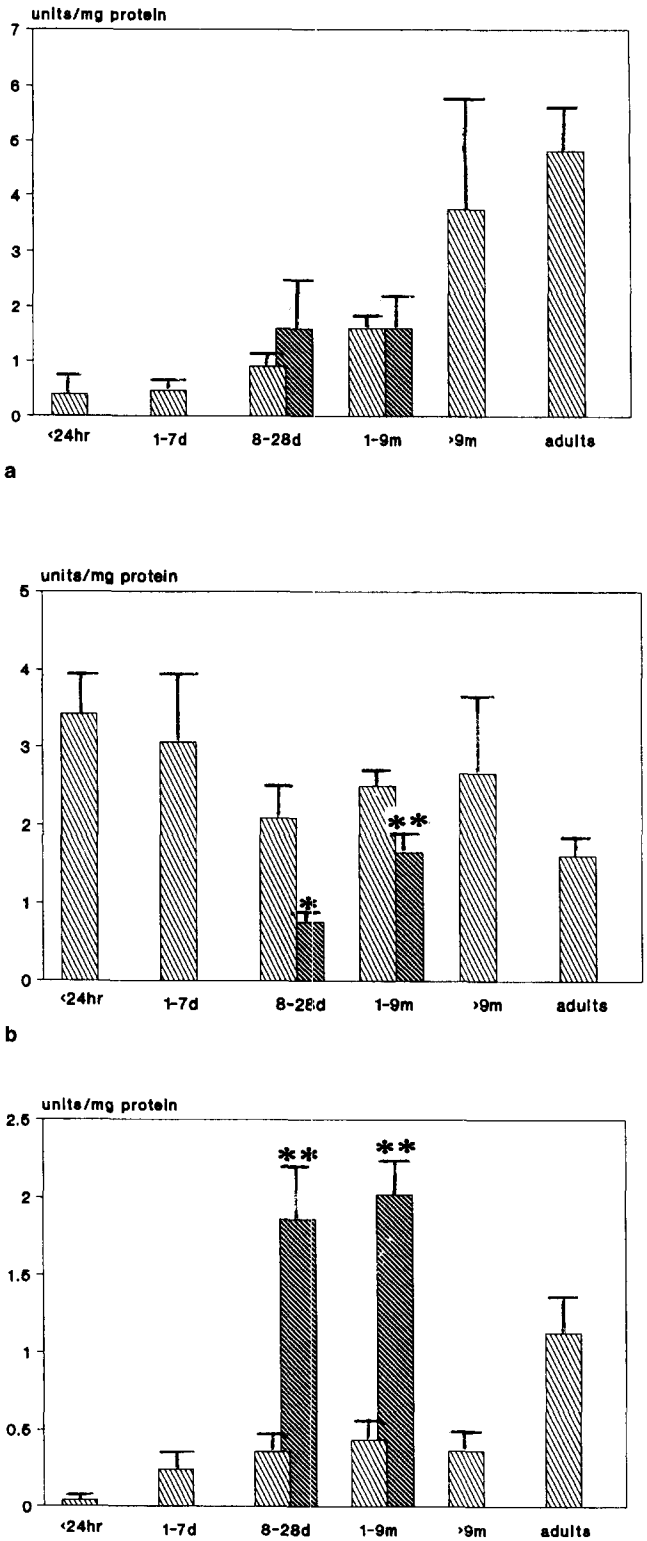


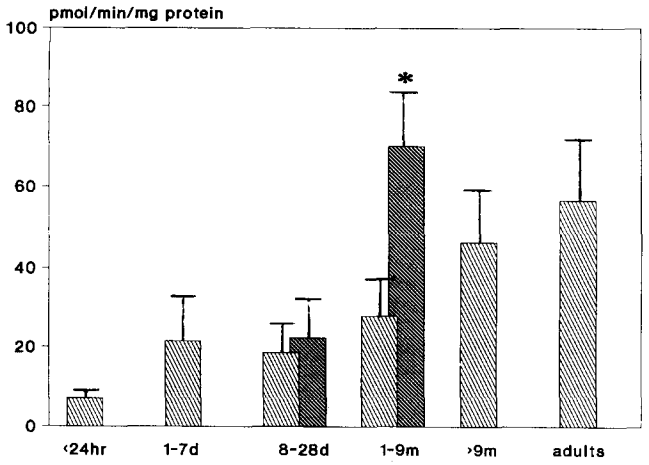
FIG. 3. Evolution of CYP2E1, CYP4A, and CYP2C members in control and SIDS human hepatic microsomes from birth to adulthood. Microsomal proteins (60 μg) were applied onto 9% SDS-polyacrylamide gel electrophoresis. After migration, proteins were transferred onto nitrocellulose sheets and probed with either antirat CYP2E (a), antirat CYP4A (b) or antihuman CYP2C (c) and visualized with 4-chloronaphthol. Immunoreacting material was quantified after scanning of immunoblots. Results are the mean ± SEM of quantifications. Darker columns correspond to SIDS. *P < 0.05; **P < 0.01 compared to controls.

CYP1A2- and CYP2E-dependent activities remained unchanged in the SIDS group. In these samples, the CYP4A content was moderately reduced, whereas the CYP2C content was remarkably increased and precociously reached adult values. It is generally assumed that CYP2C9 is the more abundantly expressed member of the 2C subfamily in the adult human liver, and that CYP2C18 and 2C19 are present in much lower concentrations [33–34]. To examine the effects of SIDS on the balance between the individual CYP2C members, electrophoretic and enzymatic analysis could be performed. For Goldstein *et al.* [35], CYP2C migrated with different mobilities but all were recognized by a polyclonal antibody to CYP2C9. In our conditions, the antibody preparation directed against the former P-450-8 (2C9) gave a unique band with adult liver microsomes and did not allow such discrimination. Similar results were reported by Shimada *et al.* [36]. In only one sample, not discussed in this study, (5th band from the right in Fig. 1A) this band was virtually absent, and a protein with greater electrophoretic mobility was visualized that could represent either the CYP2C19 or, alternatively, a truncated 2C9.

Enzymatically, several activities have been attributed to CYP2C proteins: mephenytoin hydroxylase and the low K_M (≈20 μM) component of diazepam demethylase exhibit a common genetic polymorphism and have been assigned to CYP2C19 [35, 37], but the high K_m (≈350 μM) component of diazepam demethylase was not clearly attributed to a single isoform [38]. The situation was less complex with tolbutamide, which did not coseggregate with mephenytoin hydroxylase in genetic studies and was unequivocally ascribed to CYP2C9 and, to a lesser extent, CYP2C8 proteins [39]. In SIDS, tolbutamide hydroxylase and diazepam demethylase activities were affected identically: the precocious rise in CYP2C resulted in an elevated capacity to biotransform both diazepam and tolbutamide, and suggested that the expression of CYP2C9 and 2C19 could be coregulated in SIDS. RT-PCR are currently in progress to further examine the content of individual RNAs encoding CYP2C members in SIDS.

Increased expression of CYP2C was not likely due to better efficiency in protein synthesis or to a longer half-life of the protein but was, rather, regulated at the RNA level by stabilization of existing mRNAs or by activation of transcription rates, whereas other CYP RNA levels remained unchanged. All together, these data would indicate that CYP2C expression was selectively and precociously activated to the adult level in SIDS.

Only highly speculative explanations could be conceived regarding the involvement of CYP2C in SIDS because the actual cause of death was still unknown and, probably, multifactorial. To date, the most likely explanation has been based on impaired ventilation in SIDS, which can be partly prevented by a supine position during sleeping. Any event causing prolonged apnea could increase the risk of SIDS: among worsening factors, viral infection has been associated with obstruction of the upper respiratory tract and hypoxia. In a study performed at the Royal Hospital for Sick Children in Glasgow, viruses have been positively identi-



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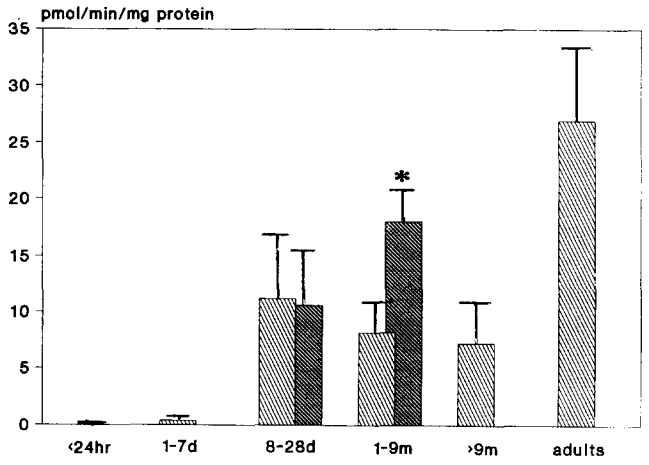

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FIG. 4. Evolution of diazepam demethylation and tolbutamide hydroxylation in control and SIDS human hepatic microsomes from birth to adulthood. (a) Microsomes (0.3 nmol total P-450) were incubated with diazepam as detailed in Materials and Methods. Desmethyldiazepam and 3-hydroxydiazepam were separated by HPLC and quantified at 256 nm during elution. Results, expressed as nmol of desmethyldiazepam formed in 1 min by 1 mg protein, are the mean ± SEM of determinations performed in duplicate for each sample. (b) Microsomes (0.1 nmoles total P-450) were incubated with tolbutamide as detailed in Materials and Methods. Tolbutamide and its hydroxylated metabolite were separated by HPLC and quantified at 230 nm during elution. Results, expressed as nmol of hydroxytolbutamide formed in 1 min by 1 mg protein, are the mean ± SEM of determinations preformed in duplicate for each sample. Darker columns correspond to SIDS samples. *P < 0.01 compared to controls.

fied in 40% of infants with SIDS and were probably underestimated [40]. Viral infection was also implicated in two thirds of SIDS infants in a Danish report [41]. Finally, evidence has been provided for an abnormal pulmonary inflammation response in SIDS [42]. This

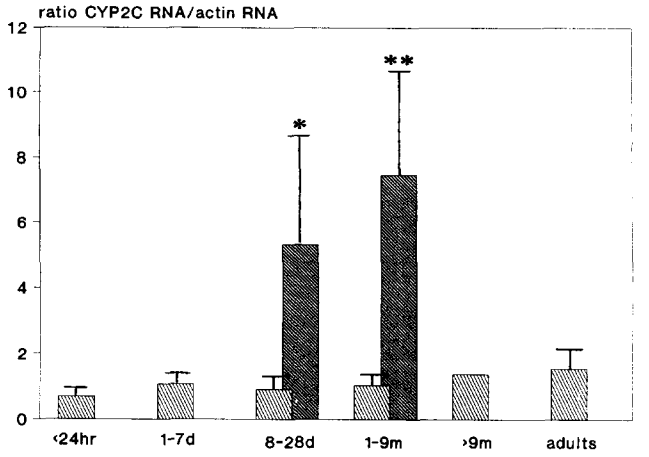

FIG. 5. Evolution of CYP2C RNA in control and SIDS human hepatic microsomes from birth to adulthood. Total RNA was extracted from liver and 5 to 10 µg were blotted on nitrocellulose sheets. Slot-blots were hybridized with ^{32}P-labelled CYP2C cDNA, washed as indicated in Materials and Methods and films were exposed. After stripping, membranes were hybridized with a β-actin genomic probe. Films were scanned, and results expressed as the ratio CYP2C RNA/actin RNA. Darker columns correspond to SIDS samples. *P < 0.02; **P < 0.01 compared to controls.

clearly indicates that the lung is a major target, but does not permit excluding the participation of other tissues in respiratory control dysfunction in SIDS.

Here, we address a question about the direct implication of CYP2C in SIDS etiology. We can hypothesize that CYP2C could be responsible for the biotransformation of endogenous substrates that could play a role in SIDS. Therefore, epoxyeicosatrienoic acids produced by CYP2C8 and CYP2C9 [43] have demonstrated activity in the relaxation of bovine coronary and rabbit pulmonary arteries [44, 45]. Furthermore, EET have been implicated in the delayed recovery after ischemia in isolated guinea pig heart or rabbit lung [46, 47]. In humans, severe apnea or respiratory depression was reported in infants of low birth weight treated with prostaglandins, which limited their use during the postnatal period [48]. In SIDS, the high content of CYP2C9 might stimulate the production of EET, leading to a relaxation of pulmonary vessels resulting in respiratory depression or apnea. Our next study will be a comparison of the rate of formation of EET by lung and liver microsomes in SIDS and control patients to confirm this hypothesis.

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