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Effects of Transient Ethanol Exposure on the Incorporation of [^{3}H]Ethanolamine into Plasmalogen in the Differentiating CG-4 Oligodendrocyte Cell Line

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ABSTRACT. We investigated the potential teratogenic effects of ethanol (EtOH) on myelination by monitoring its effects on the labeling of the myelin-typical lipid, ethanolamine plasmalogen (EPl), in the CG-4 cell line of differentiating oligodendrocytes (OLGs). On 5 different days during the first 8 days of OLG development, cells were labeled for 24 hr with [^{3}H]ethanolamine to label EPl and diacyl-ethanolamine phosphoglycerols (diacyl-EPG), and the amount of labeled lipid expressed on each day was determined in the presence and absence of 25–120 mM EtOH. At early stages of development, a lower amount of [^{3}H]EPl per cell was found in cells exposed to EtOH. The ratio of [^{3}H]EPl to [^{3}H]diacyl-EPG in cells exposed to 25, 50, or 120 mM EtOH was decreased by 50% after 4 days of differentiation compared with that in control cells. By adding or withdrawing EtOH at specific days of differentiation, we showed that EtOH inhibited the increased labeling of EPl if it was present for the first 48 hr of differentiation, and subsequent withdrawal failed to relieve the inhibition. Addition of EtOH anytime after the first day of differentiation did not inhibit the increased labeling of EPl. The results show that the increased labeling of EPl in differentiating OLGs resulted from an EtOH-sensitive, developmentally programmed, transient process active only during the first 2 days of differentiation. BIOCHEM PHARMACOL **60**;11:1703–1711, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. plasmalogens; ethanol; oligodendrocyte; myelin; CG-4 cells; differentiation

EtOH† is a potent teratogen for the developing nervous system and causes a variety of abnormal neurological effects in children born of alcoholic mothers, including mental retardation, loss of motor control, and visual impairment. This neurotoxic syndrome is referred to as the FAS or FAE, depending on the degree of neurological impairment [1, 2]. One factor involved in the EtOH-caused neurological defects appears to be abnormal formation of myelin, the lipid-rich membrane wrapped around axons in an arrangement promoting the rapid and efficient conduction of the nerve impulse. In neonatal rats, the effects of prenatal and/or postnatal EtOH exposure on myelination have produced variable results due to differences in the timing and method of EtOH administration; however, in most studies, delayed myelination and altered ultrastructure of the myelin sheath were observed [reviewed in Refs. 3 and 4]. A few studies have examined the molecular basis for the abnormal myelination caused by EtOH consumption. Neonatal rats exposed to EtOH *in utero* or postnatally exhibited delayed expression of mRNAs that code for myelin-specific proteins or enzymes, including specific isoforms of myelin basic protein [5, 6], myelin-associated glycoprotein [5], and 2′,3′-cyclic nucleotide 3′-phosphodiesterase [6]. In primary cultures of OLGs prepared from neonatal rats exposed to EtOH *in utero*, the expression of myelin basic protein and transferrin was delayed [7]. Although 75% of myelin dry weight is from lipids [8] and normal myelin lipid composition is essential for proper nerve function [9, 10], information is lacking on the effects of EtOH on the synthesis of myelin lipids in developing OLGs.

Myelin lipid composition is unique and differs from that of typical plasma membranes by containing higher amounts of myelin-typical lipids: GalCer, SGalCer, and a population of EPG that contains 70–80% EPl [8]. It is well-established that a rapid increase in the synthesis of myelin-associated lipids coincides with the burst of rapid myelin formation observed in neonatal rats between 10 and 17 days after birth [11]. The syntheses of GalCer [12] and SGalCer [13] rapidly increase in brains of rat pups between 10 and 17

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† *Abbreviations*: DoD, days of differentiation; EPG, ethanolamine phosphoglycerol; EPl, ethanolamine plasmalogen; EtOH, ethanol; FAE, fetal alcohol effects; FAS, fetal alcohol syndrome; FBS, fetal bovine serum; GalCer, galacotsylceramide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OLG, oligodendrocyte; PLA_2, phospholipase A_2, and SGalCer, sulfogalactosylceramide.

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days after birth. The amount of EPl shows an initial increase in the brains of 6-day-old rat pups and also displays a rapid increase during the burst in myelin accumulation [14, 15]. A proper myelin EPl composition has been implicated to be important for nerve function by the near absence of a myelin-specific molecular species of EPl [16, 17] in the myelin-deficient mouse mutants *Jimpy* and *Quaking*, whereas the amounts of other EPl molecular species remained at almost normal levels [18]. The potential neurological effects resulting from deficiencies in EPl-synthesizing enzymes are also manifested in children with rhizomelic chondrodysplasia punctata types 2 and 3, each of which is due to deficiency in a different enzyme of EPl synthesis [19, 20].

Primary cultures enriched in OLGs isolated from neonatal rats undergo a developmental program forming myelin components similar to that observed *in vivo* [reviewed in Ref. 21]. Isolated preparations contain low numbers of cells, OLGs at different stages of maturity, and up to 30% of cell types other than OLGs [22, 23]. As a result, the direct effects of EtOH on the developmental formation of myelin lipids in OLGs *per se* have not been identified. We recently showed that it may be possible to elude these problems by determining the effects of EtOH on the formation of myelin lipids in the central glial (CG-4) cell line [24], a continuous line of immortalized, non-neoplastic rat OLG progenitor cells established by Louis et al. [25]. The cultured CG-4 cells are virtually free of supporting cells and under proper culture conditions undergo a developmental program similar to that observed in OLG-enriched cultures. A particularly positive incentive for using CG-4 cells is that they are considered to be *bona fide* OLG progenitors, due to their ability to develop into OLGs and myelinate neurons when transplanted into genetically altered, myelin-deficient rats [26] or into areas of the brain permanently depleted of glial cells by x-irradiation [27].

Only limited information is available regarding the generation of myelin typical lipids in developing CG-4 OLGs. In differentiating CG-4 cells labeled with [^{3}H]galactose, the formation of labeled GalCer and SGalCer as determined by autoradiography was increased at a later time of development [28]. Although the formation of the labeled GalCer, SGalCer, and gangliosides in CG-4-derived OLGs showed some differences from primary OLG-enriched cultures, the results showed that CG-4 cells contained the program for inducing the synthesis of myelin galactolipids and were suitable for investigating early development of OLGs [28]. We subsequently showed that in CG-4 cells labeled on different DoD with [^{3}H]ethanolamine or with [^{3}H]galactose, the formation of each labeled lipid, EPls, GalCer, or SGalCer, occurred with its own specific time course and in a defined amount on each DoD [24]. In this study, we have investigated the effects of EtOH on the developmentally regulated temporal and quantitative expression of the labeled EPl in the differentiating CG-4 cell line. We show that EtOH altered the incorporation of [^{3}H]ethanolamine into EPl if it was present only during a specific time window within the differentiation program.

MATERIALS AND METHODS

Culture Conditions for Growing and Differentiating CG-4 Cells

The bipotential CG-4 glial cell line was obtained from the laboratory of Dr. Franca Cambi. The CG-4 cells used in this study were cultured in 100-mm diameter dishes coated with polyornithine, and all media contained 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were cultured and passed as described [25] in serum-free medium comprised of (A) 70% Dulbecco's modified Eagle's medium (high glucose formulation) containing N1 supplements (DMEM-N1): 5 μg/mL of insulin, 5 μg/mL of transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM sodium selenite [29], and supplemented with 10 ng/mL of biotin (Sigma-Aldrich); and (B) 30% conditioned medium from B104 neuroblastoma cells (B104-CM). The B104-CM medium was harvested from B104 neuroblastoma cells that had been cultured in DMEM-N1 for 3 days. The CG-4 cells proliferate in the B104-supplemented DMEM (DMEM-N1-B104). To induce the cells to differentiate into OLGs, the DMEM-N1-B104-CM medium was replaced with DMEM-N1 supplemented with 0.5% FBS (Gibco) with or without EtOH, and it was changed daily. After 48 hr in DMEM-N1–0.5% FBS, the amount of FBS was increased to 2.0%, and the medium was changed daily for an additional 7 days. Cell viability was determined using the MTT (Sigma-Aldrich) assay as described [30]. Viable cells with active mitochondria cleaved the tetrazolium ring into a dark blue formazan reaction product, and the viable cells were counted by bright field microscopy.

Labeling of Cells with [^{3}H]Ethanolamine

Progenitor cells were labeled in DMEM-B104-CM at a starting density of 30 cells/mm^2 for 24 hr with 15 μCi/dish of [1-^{3}H]ethan-1-ol-2-amine, 28 Ci/mmol (Amersham), to label the EPG. These proliferating progenitor cells are referred to as cells at 0 DoD in this study. After the cells were transferred to the differentiating medium, they were labeled for 24 hr at specific days of differentiation. The number of DoD in this study refers to the number of days the cells had been in the differentiation media at the end of the 24-hr labeling period.

Analysis of Lipids

The labeled cells were washed twice with Dulbecco's phosphate-buffered salt solution (Gibco; Ca^{2+}- and Mg^{2+}-free), treated briefly with trypsin (custom made, ATV), harvested, and centrifuged twice to remove FBS and trypsin; the cell pellets were resuspended in 0.2 mL water. After freezing and thawing, the cells were extracted twice with 2

mL of chloroform/methanol (C/M) (1:1, v/v), the extracts were combined, and the ratio of C/M was adjusted to 2:1 by the addition of 2 mL chloroform. Polar gangliosides were partitioned into the aqueous phase with the addition of 1.6 mL of 0.1 M KCl. The organic phase containing the total lipids was collected and washed twice with Folch theoretical upper phase (C/M/0.1 M KCl, 3:48:47, by vol.) [31]. To minimize oxidation of the lipids, all solvents were flushed with N_2, lipids were concentrated by evaporation under N_2, all lipids were loaded onto TLC plates under a stream of N_2, and TLC tanks were flushed with N_2 prior to developing the plates.

The total lipid extract from [^{3}H]ethanolamine-labeled cells was directly used for TLC. The lipids were separated on the LK6D plates using C/M/acetic acid/H_2O (100:60:12:6, by vol.), and the area containing the total EPG fraction was scraped from the plates and extracted twice with 2.5 mL of methanol. To determine the amount of label in the EPl and the diacyl fractions, the extracted lipids and a standard bovine brain EPG fraction containing 60% EPl (Type I, Sigma) were loaded onto Silica gel G 60 HPTLC plates (Merck). The areas containing the lipids were sprayed with 1% $HgCl_2$ in glacial acetic acid to cleave the acid-labile vinyl-ether bonds in EPls [32], and after 20 min, the plates were developed in C/M/H_2O (65:25:4, by vol.). The procedure was shown to hydrolyze the EPl-containing standard but not a standard containing only diacyl-EPG. The areas with the unhydrolyzed diacyl-EPG and the lyso-EPG formed by the hydrolysis of the vinyl ether bond of the EPls were located with I_2, scraped from the plates, and analyzed for radioactivity. The areas containing the EPGs typically contained 25,000–50,000 cpm.

RESULTS

Effects of EtOH on the Developmental Changes in the Morphology of Differentiating CG-4 Cells

The effects of EtOH on the developmentally regulated changes in morphology during differentiation were identified by examining the CG-4 cells cultured with and without EtOH by phase contrast microscopy. In the absence of EtOH, the CG-4 progenitor cells and the developing OLGs displayed the same morphology as described for the original characterization of the cell line [25]. The continuous presence of 120 mM EtOH in the medium throughout the differentiation period did not alter the gross morphology at any stage of development (Fig. 1) and did not result in an increased amount of type-2 astrocytes. When cells at a starting density of 25–70 cells/mm^2 were grown for 24 hr without or with EtOH in the B104 conditioned medium, the progenitors had a bipolar morphology (Fig. 1, a and b). After the cells were transferred to the B104-free, low serum medium for 2 days, most of the cells (>90%) acquired a multipolar phenotype typical of immature OLGs and displayed several long branched processes (Fig. 1, c and d). After 4 DoD, the control and EtOH-exposed cells displayed an OLG phenotype with multiple interconnections between the long branched processes (Fig. 1, e and f).

Previous immunocytochemical characterization showed that proliferating CG-4 bipolar progenitor cells with the morphology shown in panels a and b of Fig. 1 were positive for the progenitor marker detected by the anti-A2B5 antibody and were negative for the OLG markers, GalCer and myelin basic protein [25]. Other investigators have shown that when the B104 mitogenic source is removed and the CG-4 cells are cultured in the presence of low serum, they undergo differentiation and display the typical morphologies characteristic of cultured OLGs shown in panels c–f of Fig. 1 [25, 28, 33]. Cells with the morphologies in panels c–f of Fig. 1 were shown previously to be positive for the various OLG markers: myelin basic protein [25, 33], myelin-associated glycoprotein [24], and proteolipid protein [33]. In addition, at 2 and 6 DoD, only 2–3% of the cells display bipolar morphology and are positive for the progenitor A2B5 marker, and less than 1% display the star-shaped astrocyte morphology and are positive for the astrocyte marker, glial fibrillary acidic protein [25, 33]. In agreement with those studies, we also found that less than 5% of the cells exhibited a non-OLG morphology in cultures of both control and EtOH-exposed cells at 2–8 DoD. Although immunocytochemical studies were not done by us, the CG-4 cells in our cultures displayed the same morphologies as those analyzed immunocytochemically. However, previous biochemical analyses by us [24] showed that, in our cultures of GG-4 cells, the synthesis of myelin-associated galactolipids increases during differentiation. The cells cultured with and without EtOH were counted at each DoD in all of the studies presented here. The cell count was the same in the control and the EtOH-exposed cells at each day for all concentrations of EtOH used and showed a similar increase between 2 and 4 DoD as reported in the original characterization of CG-4 cells [25]. For example, the numbers of cells for control and EtOH-exposed cells at 2 DoD (expressed as millions of cells per culture dish) were 3.5 ± 0.8 and 3.5 ± 0.8 (± SD, N = 4), respectively; at 4 DoD, they were 14 ± 0.8 and 14.5 ± 0.4, and at 6 DoD they were 15.1 ± 1.5 and 15.7 ± 1.0. Thus, CG-4 cells cultured with or without EtOH displayed the established morphology and biochemistry. During the first 8 DoD, cells were not released from the polyornithine substratum into the medium, and at 8 DoD 95% or more of the control and EtOH-exposed cells were viable as determined by the MTT assay.

Effects of EtOH on the Distribution of [^{3}H]Ethanolamine between Diacyl- and Alkenyl-acyl-EPGs at Different Times of Development

Over 95% of the [^{3}H]ethanolamine was incorporated into the fraction containing diacyl-EPG and alkenyl-acyl-EPG, and no increase in the amount of lyso-EPG was detected at any stage of development or by EtOH exposure. The lipid extract from the EtOH-exposed cells did not contain any

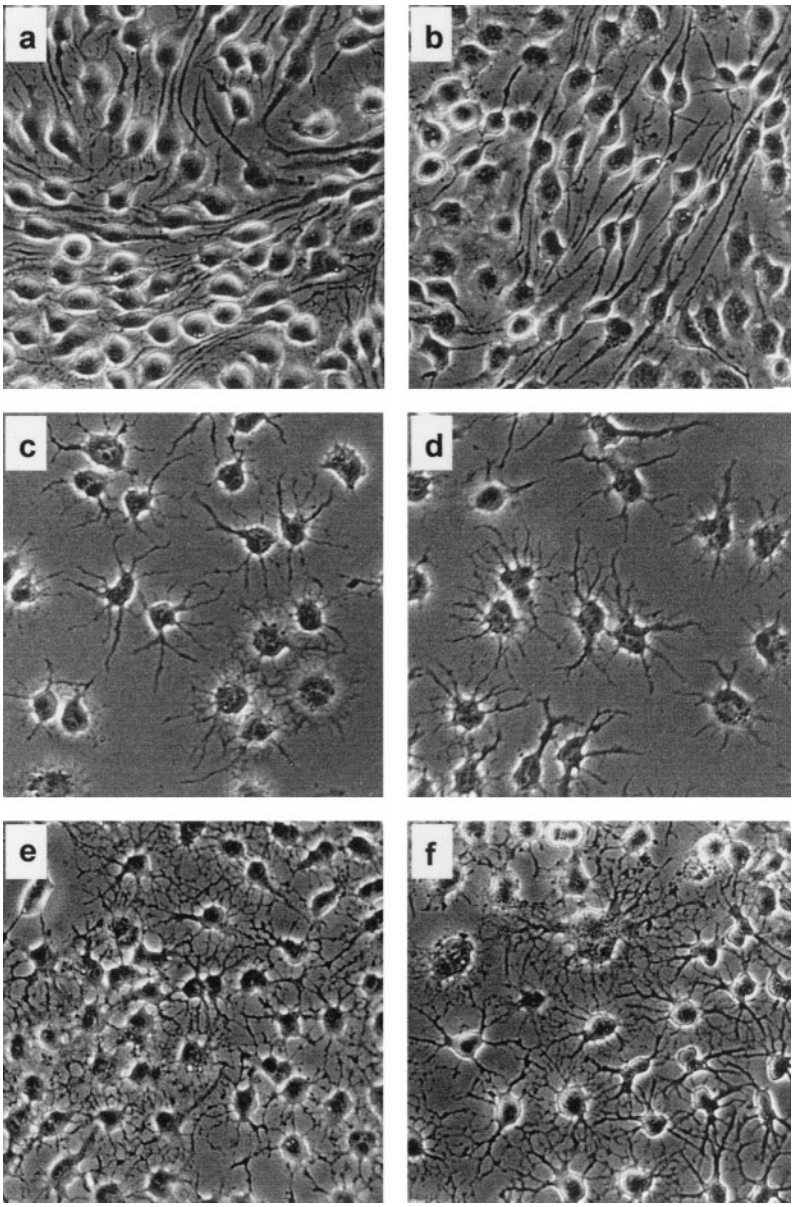

FIG. 1. Differentiating CG-4 cells cultured without (a, c, e) and with (b, d, f) 120 mM EtOH. (a, b) CG-4 progenitor cells grown for 24 hr. (c, d) Cells after 2 DoD. (e, f) Cells after 4 DoD. Magnification, 200X.

additional radioactive areas not observed in the extracts from control cells. To determine whether EtOH altered the amount of [^{3}H]ethanolamine that was directed to the net synthesis of EPls compared with that directed to the net formation of the diacyl-EPG fraction as the cells differentiated, we determined the ratio of [^{3}H]ethanolamine incorporated into the EPl to that incorporated into the fraction of diacyl-EPG (hereafter referred to as the ratio) every 2 days of development. The ratio was lower in cells exposed to 120 mM EtOH throughout the developmental period (Fig. 2). The ratio increased during the first 4 DoD in cells not exposed to EtOH, whereas in the presence of 120 mM EtOH, the increase in the ratio was delayed and was decreased by 56 and 50%, at 2 and 4 DoD, respectively. In EtOH-exposed cells, the relatively high ratio was not reached until 6 DoD, and its value was limited to 70% of that attained in control cells. At 8 DoD, the ratios in the control and EtOH-exposed cells were no longer significantly different.

Although 120 mM EtOH did not cause cell death or inhibit the acquisition of the morphological characteristics of the differentiating CG-4 cells, we examined the effect of lower, more clinically relevant EtOH concentrations on the labeling of EPl. Exposure of four different sets of cells to 25, 50, and 120 mM EtOH caused the same 47–53% decease in the ratio at 4 DoD in the EtOH-exposed cells. Statistical analysis of the difference between the ratios in control and EtOH-exposed cells was determined by the two-tailed, paired Student's *t*-test. The ratios at 4 DoD were 1.9 ± 0.1 (± SD) in the absence of EtOH, 0.9 ± 0.0 in 25 mM EtOH ($P \leq 0.001$), 1.0 ± 0.1 in 50 mM EtOH ($P \leq 0.005$), and 0.9 ± 0.0 in 100 mM EtOH ($P \leq 0.001$). Once

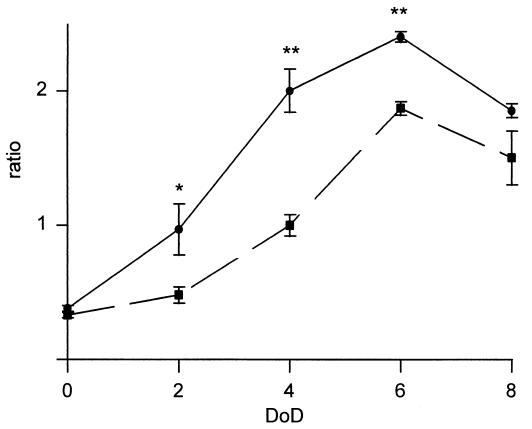

FIG. 2. Effects of EtOH on the ratio of [^{3}H]ethanolamine incorporated into EPl to that incorporated into diacyl-EPG in CG-4 cells at different DoD. Cells underwent differentiation in the medium without EtOH (●—●) or with 120 mM EtOH (■—■). The data are presented as the average ± SD (N = 3) of the ratios of the cpm in EPl to the cpm in diacyl-EPG per million cells. Statistical analysis of the values for control and EtOH-exposed cells was determined by the two-tailed paired Student's *t*-test. Key: (*) $P \leq 0.05$; and (**) $P \leq 0.005$.

the inhibition of the increased ratio by lower concentrations of EtOH was established, we determined their effects on the ratio at different DoD and found that 25 and 50 mM EtOH caused essentially the same temporal delay in the increased ratio as 120 mM EtOH (Fig. 3). Although 25 mM EtOH exhibited the same quantitative inhibition of [^{3}H]EPl labeling at 4 DoD in four different experiments, the studies presented in this report were carried out with 120 mM EtOH to ensure that the maximum effect would be observed.

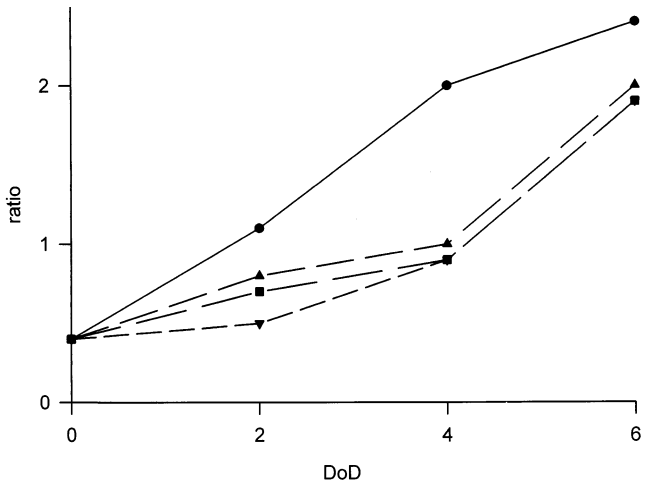

FIG. 3. Effects of different concentrations of EtOH on the ratio of [^{3}H]ethanolamine incorporated into EPl to that incorporated into diacyl-EPG in CG-4 cells at different DoD. The same set of progenitor cells underwent differentiation in the medium without EtOH (●—●), or with 25 mM EtOH (■—■), 50 mM EtOH (▲—▲), or 120 mM EtOH (▼—▼). The data are from a typical experiment out of two and are presented as the ratios of the cpm in EPl to the cpm in diacyl-EPG per million cells.

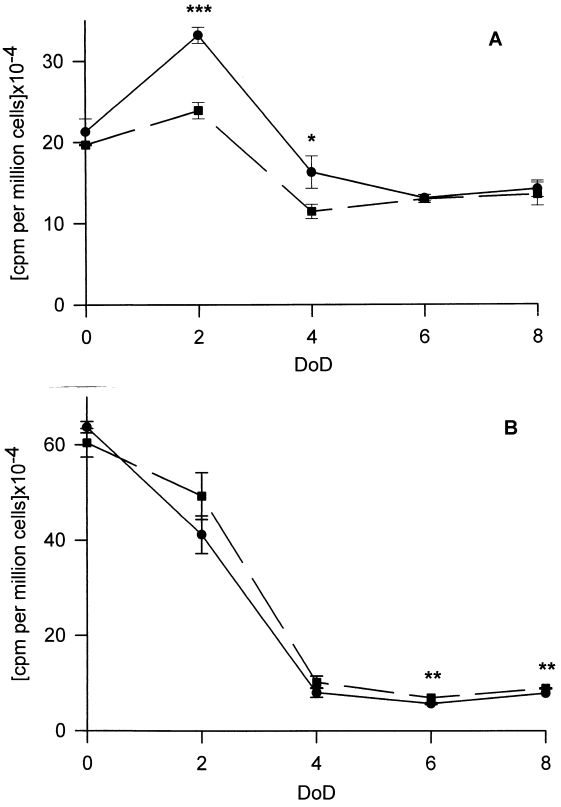

FIG. 4. Effects of EtOH on the amounts of [^{3}H]EPl and [^{3}H]diacyl-EPG per cell at different DoD. The data are presented as the average cpm ± SD (N = 3) incorporated into (A) [^{3}H]EPl and (B) [^{3}H]diacyl-EPG per million cells on the DoD indicated with 120 mM EtOH (■—■) or without EtOH (●—●) in the medium. Statistical analysis of the values for control and EtOH-exposed cells was determined by the two-tailed paired Student's *t*-test. Key: (*) $P \leq 0.03$; (**) $P \leq 0.01$; and (***) $P \leq 0.005$.

Effects of EtOH on the Incorporation of [^{3}H]Ethanolamine into Diacyl- and Alkenyl-acyl-EPGs at Different Times of Development

The EtOH-caused changes in the ratio of the [^{3}H]EPl to [^{3}H]diacyl-EPG did not reveal whether the altered ratio was due to a decreased net synthesis of EPl or to a stimulation in the net synthesis of diacyl-EPG. Incorporation of ethanolamine into diacyl-EPG is considered to be a measure of *de novo* synthesis of diacyl-EPG. In this study, the cells were labeled for 24 hr, and, consequently, the amount of label in a lipid was the net synthesis resulting from the synthesis and turnover of the lipid during the 24-hr period. To determine the time course of the net synthesis of the EPls and the diacyl-EPG in the cells, the amount of radioactivity incorporated into [^{3}H]EPl and [^{3}H]diacyl-EPG on a per cell basis was determined at different stages of development. In control cells, the amount of [^{3}H]EPl per million cells increased significantly between 0 and 2 DoD (Fig. 4A), and exposure to EtOH inhibited the increase. The amount of [^{3}H]EPl per million cells between 2 and 4 DoD remained significantly higher in control cells compared with that in EtOH-exposed cells. During the first 4 DoD, the amount of

TABLE 1. Effect on plasmalogen labeling of the addition or withdrawal of EtOH on different days of differentiation

Culture conditions	DoD with 120 mM EtOH				Ratio (% of control)
	1	2	3	4	
A	+	−	−	−	94
B	+	+	−	−	49
C	+	+	+	−	47
D	+	+	+	+	49
E	−	+	+	+	100
F	−	−	+	+	100
G	−	−	−	+	100

Progenitor cells underwent differentiation in the presence of EtOH on a specific DoD, indicated by (+), or the absence of EtOH, indicated by (−). The cells were labeled with 15 μCi/dish of [^{3}H]ethanolamine for 24 hr; the data are the percentage of the ratio of the cpm in [^{3}H]EP1 to the cpm in [^{3}H]diacyl-EPG per million cells at 4 DoD for each condition compared with the ratio in the control cells (ratio = 2.2 ± 0.1) cultured without EtOH.

Percentages are the averages of three experiments ± ≤5%.

[^{3}H]diacyl-EPG per million cells decreased rapidly, and EtOH did not cause any significant difference in the amounts observed (Fig. 4B). Consequently, the decreased ratio of [^{3}H]EPl to [^{3}H]diacyl-EPG at 2 DoD in EtOH-exposed cells resulted from a 33% decrease in the accumulation [^{3}H]EPl on a per cell basis (Fig. 4A).

Determination of the Days during Differentiation When the Increase in [^{3}H]Plasmalogen Labeling Is Sensitive to EtOH

The CG-4 cells undergo a developmental program as they differentiate into OLGs and develop the ability to produce myelin components. The early increased labeling of [^{3}H]EPl per cell is most likely to be part of a sequential program of transient induction processes during differentiation, and, consequently, the transient process may be active only during a specified time window during development. We tested this possibility by determining whether the presence of EtOH was required for the entire developmental period to inhibit the increased labeling of [^{3}H]EPl, whether it was required on only specified "critical" days, whether it would inhibit the labeling if added at any time during development, or whether the inhibition would be relieved if EtOH were withdrawn.

First we determined the number of days of EtOH exposure required to inhibit the increased labeling of EPl. This was accomplished by exposing the differentiating CG-4 cells to EtOH for an increasing number of consecutive days of development starting with the first DoD, and then allowing the cells to continue development after the removal of EtOH from the medium. The effect of the EtOH exposure was assessed by comparing the ratio of [^{3}H]EPl to [^{3}H]diacyl-EPG at 4 DoD in control and EtOH-exposed cells. The results in Table 1 show that if EtOH was present for only the first DoD (condition A) and then removed, the labeling of EPl was not inhibited. If EtOH was present for only the first 2 DoD (condition B) and then withdrawn, the labeling was inhibited to the same extent as when EtOH was present for all 4 DoD (condition D).

We next determined if the inhibition of the EPl labeling required the presence of EtOH when the progenitors started to differentiate into OLGs, or if it could be added at a later time and still inhibit the EPl labeling. The time for initial exposure of the cells to EtOH was determined by sequentially delaying the addition of EtOH until the cells had differentiated for 1, 2, or 3 days. The information in Table 1 also shows that if EtOH was not present on the first DoD, the labeling was not inhibited, even if EtOH was added on the second DoD (condition E) or the third DoD (condition F) and continued to be present through 4 DoD. If the growing OLG progenitor cells were exposed to 120 mM EtOH for 24 hr before initiating the OLG developmental program, no additional effects on the ratio other than those shown in Table 1 were observed (data not shown).

DISCUSSION

In studies where myelination was altered by EtOH administration to developing rat fetuses *in utero* and/or to neonatal rats or to OLG-enriched cultures [3, 4], it was not possible to determine whether the effects of EtOH were due to direct effects on the OLGs or to indirect effects on non-OLG cell types. In our cultures, 5% or less of the cells were of a non-OLG nature; consequently, the delayed labeling of EPl was apparently due to an action of EtOH on the developing OLGs *per se*. The effects of EtOH on early myelination events observed in cultured CG-4 cells are most likely relevant to the *in vivo* stimulation of EPl synthesis in neonatal rats [14, 15] for a number of reasons. The CG-4 cells are *bona fide* OLG precursors and retain the ability to myelinate nerve fibers when transplanted into myelin-deficient animals or myelin-deficient areas of brains [26, 27]. The CG-4 cells undergo a developmental program in which they developmentally express OLG markers [25, 33], myelin-associated proteins [25, 28, 33], and myelin-associated lipids [24, 28] in a temporal and quantitative pattern similar to that observed *in vivo* [24].

The sequential addition and withdrawal studies shown in Table 1 indicated that the increased EPl labeling is caused by a transient process, which is activated only during a developmentally programmed window within the first 2 DoD. The transient EtOH-sensitive period apparently began during the first DoD and continued into the second DoD, a mechanism which accounts for the requirement that EtOH be present on both the first and second DoD to inhibit the increased labeling of [^{3}H]EPl. Exposure of the proliferating progenitor cells to EtOH for 24 hr prior to the initiation of the differentiation program did not inhibit the EPl labeling. Once the processes responsible for increased labeling of EPl have been inhibited by EtOH during the first 2 DoD, the inhibition appears to be permanent, as labeling of EPl was not rapidly reactivated if the EtOH was withdrawn. The results show that if EtOH was not present during a "critical" EtOH-sensitive period, subsequent expo-

sure to EtOH could not block the increased labeling of EPl. These results with an *in vitro* system complement the recent demonstration of a transient, EtOH-vulnerable period *in vivo* for the EtOH-caused apoptotic neurodegeneration in prenatal and neonatal rats [34]; however, the critical period varied for different brain regions. If the EtOH-vulnerable time for inhibiting the accumulation of EPl observed *in vitro* also extends to humans, then a pregnant mother who consumes a sufficient amount of EtOH during the short vulnerability period could impair myelination processes in the developing fetus. The morphological and biochemical changes associated with myelination occur during the brain growth spurt period [11, 35], which occurs mainly during the last trimester of pregnancy in the human fetus [36, 37]. Consequently, the EtOH-vulnerable period would not be limited to only one short period, but rather a series of critical periods would extend throughout the last trimester. The potent inhibition of EPl labeling by EtOH at the clinically relevant concentration of 25 mM further indicates that it may be one mechanism contributing to FAE or FAS neurological defects (for reference, 22 mM EtOH is the maximum legal amount of blood alcohol for driving in the United States).

The possible effects of altered EPl content in myelin have been implicated in *Jimpy* and *Quaking* mice [18] and in children with rhizomelic chondrodysplasia punctata types 2 and 3 [19, 20]. The abnormal nerve conduction, loss of motor control, and early death observed in knockout mice deficient in the GalCer galactosyl transferase [9] also reveal the consequences of an altered myelin lipid composition. The studies showing that EtOH delays the expression of myelin-associated proteins [5–7] along with our studies showing delayed and deficient plasmalogen labeling all indicate that altered expression of myelin proteins and lipids contribute to abnormal brain development. Although the ratio of labeled EPl to diacyl-EPG recovered at later stages of CG-4 OLG development, the adverse effects on early myelin formation may not be reversible due to the slow turnover of myelin lipids. Permanently impaired vision has been observed in children of alcoholic mothers [38]. Studies in neonatal rats exposed to EtOH *in utero* or both postnatally and *in utero* showed that EtOH caused a delay in myelination [39–41] and an altered myelin ultrastructure in the optic nerve [41]. In humans, the optic nerve is myelinated before birth, and if the rat studies can be extended to humans, then EtOH-caused dysmyelination may be one factor contributing to the impaired vision observed in the children of alcoholic mothers. The effect of EtOH on EPl formation in the developing brain is not limited to OLGs, as the EPl content in synaptosomes was decreased by 30 and 52%, respectively, in 17- and 24-day-old neonatal rats exposed to EtOH *in utero* [42]. The EtOH-altered metabolism of EPl observed *in vitro* and *in vivo* in OLGs and neurons may cause some of the neurological defects associated with FAE/FAS.

The results do not reveal the biochemical mechanisms by which EtOH exposure delays the labeling of EPl. Phospholipid metabolism is complex, and EtOH may inhibit the induction of EPl-synthesizing enzymes, stimulate up-regulation of EPl-hydrolyzing enzymes, or change the activity of existing enzymes. The altered pattern of [^{3}H]EPl accumulation may be due to changes in the rates of hydrolysis of EPGs. EtOH exposure causes an increase in the activity of Ca^{2+}-dependent PLA_2 in mouse brain [43]. However, the effects that such an activation may have on the labeling of EPl are difficult to assess because several isoforms of PLA_2 exist, they have different substrate specificities, and they may be localized in different cells or membranes [reviewed in Ref. 44]. A Ca^{2+}-independent, EPl-selective PLA_2 has been purified from bovine brain [45], but its response to EtOH exposure is not known. EtOH might also affect the turnover by activating or increasing the expression of the enzyme that hydrolyzes the vinyl ether bond of EPl, also producing a lyso-EPG. The effect of any process forming lyso-EPG on the ratio observed would depend on whether the lyso-EPG is reacylated or further hydrolyzed. Although we did not observe any increase in the amount of labeled lyso-EPG in the EtOH-exposed cells, transient, EtOH-caused changes in the activities of the various phospholipases cannot be ruled out.

The decreased labeling apparently is not due to an inhibition of the EPl-synthesizing enzymes by a reversible direct interaction between EtOH and the enzyme protein or the phospholipid environment of the enzyme, since the inhibition could not be achieved by randomly adding or withdrawing EtOH at any time during development. During myelination in neonatal rats, the plasmalogen content of the brain [14, 15] and the activities of enzymes in the EPl synthesis pathway [46, 47] increase. A high rate of conversion of exogenously added alkylether-EPG to EPl occurs in brain microsomes from 12- to 14-day-old rats and significantly decreases by 19 days [47]. Therefore, it is plausible that EtOH may specifically inhibit the increased induction or the activation of one or more enzymes involved in the synthesis of EPl. Investigations are currently underway to determine whether EtOH causes the delayed labeling of EPl by decreasing the activities of EPl-synthesizing enzymes in differentiating CG-4 cells.

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