NEURONAL LESIONS IN THE CEREBELLUM FOLLOWING THE ADMINISTRATION OF EXCESS PHENYLALANINE TO NEONATAL RATS

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ABSTRACT

Conditions necessary to create a laboratory model in the albino rat to approximate the biochemical abnormalities in human phenylketonuria were studied. Such a state could only be achieved during the first postnatal week when activity of hepatic phenylalanine hydroxylase is only 30–50% of normal adult values. During this time, subeutaneous injections of phenylalanine every 12 hours resulted in: (1) sustained 5 to 8 fold elevations of phenylalanine concentrations with maintenance of relatively normal tyrosine levels in blood; (2) maximum phenylalanine/tyrosine ratios of 20:1 approaching levels previously reported for human phenylketonuria; and (3) the existence of the preceding biochemical abnormality during a period when brain, and cerebellum in particular, is undergoing rapid maturation.

Light microscopic study of the brains of 50-day-old rats subjected to chronic neonatal hyperphenylalanemia revealed neuropathologic lesions limited to the cerebellum. The cytoplasm of Purkinje cells was vacuolated and heterochromatin severely decreased in granular cell nuclei. Axons showed different degrees of swelling manifested in formalin-fixed paraffin sections as a sponginess of white matter. Because this latter change was less apparent in perfused, plastic-embedded material, this sponginess may be secondary to osmotic and chemical changes causing myelin disruption in abnormal axons. Although prior biochemical and pathologic studies in both neonatal hyperphenylalanemia in the rat and human phenylketonuria have placed a major emphasis on abnormalities of myelin, the present study reveals a vulnerability of developing neurons. This vulnerability is manifested by cytoplasmic lesions in Purkinje cells and nuclear lesions in granule cells.

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INTRODUCTION

Phenylketonuria is a hereditary disorder of amino acid metabolism, in which the primary defect is an absence of phenylalanine hydroxylase activity in the liver (16). This enzyme deficiency results in elevated levels of serum phenylalanine. Only 10% of ingested phenylalanine is converted to tyrosine and 85% appears in the urine as abnormal metabolites (29). Although moderate to severe mental retardation becomes clinically apparent during early postnatal life (15), little is known about the mechanism by which this selective inborn error of liver metabolism can disrupt the normal sequence of brain maturation. Nevertheless, an improvement of intellectual ability in children treated with low phenylalanine diets from birth (4) suggests that either phenylalanine or one of its abnormal metabolites may exert deleterious effects on the brain during early postnatal development.

The use of pregnant, nursing mother, or weanling rats to create a laboratory model of phenylketonuria has been criticized on the basis that the administration of phenylalanine to animals old enough to have normal phenylalanine hydroxylase activity in liver, results in elevations of tyrosine far in excess of phenylalanine (11). In the present study, an experimental model which more closely approximates the human disease has been created by injecting phenylalanine into newborn rats during the first seven days of postnatal life. The desirable characteristics of this model are: (1) low activity of phenylalanine hydroxylase in the liver of neonatal rats; (2) elevated concentrations of blood phenylalanine in a range similar to that found in the human disease; (3) relatively normal levels of blood tyrosine; and (4) the existence of these biochemical abnormalities during a period in which brain, and in particular cerebellum, is undergoing rapid maturation. This report describes cytologic abnormalities in both Purkinje cells and granular cells of the cerebellum of adult rats subjected to hyperphenylalaninemia during the first week of postnatal life.

MATERIALS AND METHODS

Twelve pregnant rats of the Charles River CD® strain, at 14 to 16 days of gestation, were fed a diet of Purina rat chow and water ad libitum. Parturition occurred on days 21 to 23. Within 8 hours after birth, litters were reduced to 9 pups each and divided into four groups. One hundred and eight animals from 12 litters were used.

Production of neonatal hyperphenylalaninemia and biochemical control of the experimental model. Group I (Neonatal hyperphenylalanemia): This group, consisting of 27 animals from 3 litters, was treated with subcutaneous injections of 0.9 mg of L-phenylalanine per gram body weight. A saturated 2.7% solution of L-phenylalanine (Sigma Chemical Co.) was made in 0.9% sodium chloride and injected as a clear solution after gentle warming. Group II (Saline-injected controls): This group, consisting of 18 animals from 2 litters, was treated with subcutaneous injections of 0.9% solution of sodium chloride. The amounts administered to each age-matched control rat equaled the volume of solution injected into hyperphenylalanemic animals in group I.

In both groups, injections were begun 12 hours after birth and continued every 12 hours for 7 days. On the 1st, 3rd, and 7th day of postnatal life, tail blood was assayed for phenylalanine and tyrosine, prior to and at 1, 2, 3, 4, 5, 6, 7, 8, and 12 hours after injection in the
morning. It was impractical to remove hourly samples of blood from a single newborn rat because of their relatively small total blood volume. Different pups in each litter therefore were used for each time interval. Phenylalanine was assayed using a filter-paper modification of the fluorometric method described by McCamen and Robins (23, 25). Tyrosine was determined by a filter-paper modification of the fluorometric method described by Waalkes and Udenfriend (31, 32).

Animals in both groups were nursed for 21 days after birth by mothers given unrestricted access to a standard laboratory rodent diet. After weaning, the animals were placed in separate cages and fed on a diet similar to their mothers. In no instance was supplemental phenylalanine added to the rations. The pups were weighed at birth, daily for the first week, weekly thereafter, and killed at 50 days.

Neuropathologic examination. Eight animals from group I and 4 animals from group II were killed by intravascular perfusion of 4% glutaraldehyde in 0.1M cacodylate buffered to pH 7.3. Samples of cerebellum and cerebrum were post-fixed in osmium, dehydrated in alcohol, and embedded in epoxy. One micron sections were stained with toluidine blue.

The remainder of the animals in groups I and II were decapitated. The brains were removed immediately, fixed in 10% formalin and routinely processed for paraffin embedding. Sections were stained with hematoxylin and eosin, luxol fast blue and cresyl violet, and impregnated with silver by Bodian's method.

Group III (Blood phenylalanine tolerance tests in older animals): Members of this group, consisting of 36 animals from 4 litters, were randomly chosen at 8, 13, 15, 20, 30, and 50 postnatal days, to receive either a single subcutaneous injection of 0.9 mg of L-phenylalanine per gram body weight, or an equivalent volume of 0.9% sodium chloride. Prior to and at 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours after injection, phenylalanine was assayed (23, 25) in tail blood from phenylalanine and saline-injected rats.

Group IV (Hepatic phenylalanine hydroxylase activity): This group consisted of 27 additional treated and untreated animals studied at various ages from birth to 50 postnatal days. The activity of hepatic phenylalanine hydroxylase was determined by the method of Udenfriend and Cooper (30) as modified by Freedland, Krakowski, and Waisman (13); protein was assayed by the method of Lowry, Rosebrough, Farr and Randall (20).

RESULTS

A. Biochemical Studies

Groups I and II: Sustained elevations of blood phenylalanine concentrations for the first week of postnatal life, had a mortality rate of 9%. All deaths occurred during the period of injection. An identical mortality rate during the first 7 postnatal days was found in saline-injected controls, indicating that deaths could not be attributed to the excess phenylalanine administered. In control animals, the most rapid increase of body weight occurred between 10 and 40 days of age. The rate of body growth of rats subjected to neonatal hyperphenylalanemia was identical to that of control animals, and the apparent, lag between 7 and 20 days was not statistically significant (Figure 1).

In vivo evidence for a relative deficiency of liver phenylalanine hydroxylase during the first postnatal week was obtained by assessing blood phenylalanine and tyrosine levels as a function of time following phenylalanine injections at 1, 3, and 7 postnatal days. Subcutaneous injections administered every 12 hours for 7 days resulted in increased blood concentrations of phenylalanine to resting levels of 14.0 and 12.5 mg% in 3 and 7 day old rats, respectively. In comparison, saline-injected controls had levels of 2.7 to 3.8 mg%. In all instances, peak
Fig. 1. Growth curve of saline-injected control albino rats compared to those subjected to the administration of excess phenylalanine during early postnatal life. Heavy bar and arrow denote the injection period from birth to 7 postnatal days. Each point is the mean of 18-27 animals. Bracketed vertical lines on control curve represent standard errors of the means.

Concentrations in blood were achieved one hour after subcutaneous injection with levels increased 13 to 19 fold above those of saline-injected controls. Mean phenylalanine concentrations in 1, 3, and 7 day old rats for the eight hour post-injection period were 28.3 mg%, 29.8 mg% and 21.0 mg%, respectively. In spite of these elevations, animals showed a relatively small rise of tyrosine in blood, with mean levels of 3.0 to 4.3 mg%. The ratios of phenylalanine to tyrosine in blood averaged 6.6, 9.9, and 5.7 during the 8 hour post-injection interval at 1, 3, and 7 postnatal days, respectively, with maximum values of 13 to 21 achieved 2 to 4 hours following injection (Figure 2).

Group III: After the first week of postnatal life, phenylalanine tolerance tests were performed in previously untreated Group III animals. Blood concentrations of phenylalanine were measured as a function of time after a single
subcutaneous injection of 0.9 mg of phenylalanine per gram body weight. Between 8 and 15 days, blood phenylalanine concentrations rose only 6 fold above saline-injected control values, decreasing to only slightly greater than pre-injection levels in 4 hours. Between 16 and 50 postnatal days, blood levels rose only 2 fold, declining to values that were only slightly above control levels 2 hours after injection (Figure 3). Hence, after the first week of postnatal life, in association with an increase of phenylalanine hydroxylase activity in normally developing rat liver, there was a progressive increase in the ability to maintain normal phenylalanine concentrations in blood after subcutaneous injection of a loading dose.

Group IV: At birth, the activity of phenylalanine hydroxylase in the liver of control animals was found to be less than 30% of normal values for 50 day old rats. With rapid postnatal maturation of the liver parenchyma, the enzyme activity was approximately 60% of normal adult values by 7 days of age, and greater than 90% by 15 postnatal days (Figure 4). In rats injected with phenylalanine during the first week of postnatal life, hepatic enzyme activity at 1, 3, and 7 days was identical to that found in control specimens, suggesting that neither inhibition nor induction of enzyme activity during liver development had occurred in response to raised blood levels of phenylalanine.

B. Pathological Studies (Groups I and II)

Paraffin sections of the cerebellum of animals subjected to neonatal hyperphenylalaninemia showed Purkinje cells which were often shrunken, with deeply
Fig. 3. Mean phenylalanine concentrations in blood, expressed as milligrams per 100 ml, in 8-15 day old rats (upper graph) and 16-50 day old rats (lower graph) as a function of time following a single subcutaneous injection of 0.5 mg phenylalanine per gram body weight and compared with saline-injected, age-matched controls. Arrow denotes time of injection.

Fig. 4. Phenylalanine hydroxylase activity, expressed as μMoles of tyrosine formed/hour/milligram protein, in liver of normal rats during the first 50 days of postnatal development.
basophilic cytoplasm. The cerebellar white matter was spongy; innumerable small zones of pallor separated the myelin sheaths when stained with luxol fast blue (Figure 5). In contrast, the neurons of the cerebral cortex and basal ganglia were normal, and the white matter of the cerebrum showed few of the spongy changes. The neurons and white matter of the brain stem were similarly unaffected.

In the semi-thin sections of perfused, osmium-fixed, plastic-embedded specimens from the cerebellum, severe vacuolization of the Purkinje cell cytoplasm was seen (Figure 6). In some Purkinje cells, only one or two small vacuoles were seen, but in others, numerous large vacuoles distorted the cell. Groups of affected Purkinje cells alternated with groups of morphologically normal cells. Changes in the internal granular layer were seen in relation to both vacuolated and normal Purkinje cells. In affected regions, the nuclei of the granule cells showed an almost total loss of heterochromatin. Between the altered granule cells, the glomeruli or "cerebellar islands" had lost their usual sharp borders and internal structure, and appeared as homogeneous masses with ill-defined edges. Within these relatively homogeneous glomeruli, numerous tiny vacuoles were scattered (Figure 7).

Although the myelin sheaths in plastic embedded specimens stained by tolui-
Fig. 6. Plastic-embedded sections of cerebellum of age-matched control (A), and 50-day-old experimental (B) animals. Purkinje cells of the experimental animal are vacuolated. Cells of the internal granule cell layer have lost their heterochromatin, and the usually well-defined “glomeruli” (arrows in 6A & B) are poorly defined. The axons in the experimental animal are more variable in size than those in the control animal. Toluidine blue; × 252.
Fig. 7. Higher magnification of junction between Purkinje cell layer and internal granule cell layer in control (A) and experimental (B) animals. Purkinje cell vacuolization and loss of heterochromatin of internal granule layer cells is apparent in the experimental animal. Degeneration of "glomeruli" can also be seen (arrows). Plastic section stained with toluidine blue; × 630.
dine blue, appeared normal in the cerebellar white matter, there was mild swelling of the axons. This change made the white matter of experimental animals stain less deeply than that of the controls, but it could not be characterized as the spongy change seen in the paraffin sections. The change in the white matter was diffuse, and was not related to the areas of altered Purkinje cells nor to changes in the internal granular layer.

In the plastic-embedded specimens of the cerebral cortex, no neurons were found which exhibited changes similar to that seen in the cerebellar neurons. The subcortical white matter was also unremarkable.

**DISCUSSION**

In an effort to study the pathogenesis of central nervous system dysfunction in human phenylketonuria, several investigators have attempted to create a laboratory model of this disease in the albino rat. Numerous obstacles have been encountered in these efforts, the major one resulting from an inability to sustain hyperphenylalaninemia in association with a high phenylalanine/tyro-sine ratio. In prior studies, pregnant rats (21), mothers of nursing rats (5), or weanling rats (6), have been given diets supplemented with high amounts of phenylalanine. This diet has resulted in transient elevations of serum phenylalanine, rapidly fluctuating as a function of changing dietary intake. Although this variable can be circumvented by parenteral administration of phenylalanine, the relative insolubility of phenylalanine in water has necessitated the use of either much lower doses in relation to adult body weight, or an unreliable delivery of the amino acid resulting from injection of an emulsion. Regardless of the method of administration, phenylalanine loading in weanling or adult rats produces levels of tyrosine in blood which greatly exceed phenylalanine concentrations (11, 26). This observation indicates that the administered phenylalanine in rats older than 21 postnatal days is efficiently metabolized by the normal tyrosine pathway, creating a biochemical abnormality that is dissimilar from the condition in human beings with phenylketonuria (11). Hence, prior studies using pregnant rats, mothers of nursing rats, or weanling rats, all with well developed activity of liver phenylalanine hydroxylase, have produced inconclusive results leading to a false concept that the rat is of little use in providing a laboratory model for study of human phenylketonuria (24, 26).

The present study shows that rats subjected to subcutaneous injections of phenylalanine during the first week of postnatal life can be used to study the pathogenesis of neural lesions occurring under biochemical conditions approximating those found in human phenylketonuria. The liver of the normal rat at birth contains poorly differentiated hepatic cells which undergo rapid cytoplasmic differentiation during the first two weeks of postnatal life (10). Our study of Group IV animals confirms a prior report (12) showing that the activity of phenylalanine hydroxylase in the liver of the newborn rat is less than 30% of adult values, reaching 60% of normal adult activity by 7 days of age, and 90% of adult values by 15 days of age. Moreover, chronic treatment of newborn rats with high doses of phenylalanine does not influence the rate of
development of enzyme activity. As a result of this relative deficiency in the activity of phenylalanine hydroxylase during the first week of postnatal life, chronic administration of phenylalanine results in an average 5 to 8 fold increase of blood levels; ratios of phenylalanine to tyrosine at their maxima approach the 20:1 serum ratio of human phenylketonurics as demonstrated by Group I animals. After the first postnatal week, injections of phenylalanine produce progressively diminishing and evanescent elevations of blood phenylalanine levels which may be exceeded by levels of blood tyrosine (Group III). Thus phenylalanine loading in the rat can produce phenylalanine/tyrosine ratios approximating those found in human phenylketonuria only during the first postnatal week.

A second advantage of injecting phenylalanine during the first postnatal week is that the sustained elevation of the phenylalanine/tyrosine ratio in blood is produced during a period in which the brain is rapidly developing. At birth, the brain of the normal rat weighs 0.32 grams. It increases 4 fold to 1.26 grams by the 20th postnatal day, achieving 50% of adult brain weight (12). During this developmental interval, the rate of maturation of different regions is asynchronous; the weight of the cerebral cortex only doubles in comparison with a 6-fold increment of cerebellar weight (12). Furthermore, while the process of neuronal formation in the cerebral cortex is completed in both rat and man during intrauterine life (3), active neurogenesis occurs postnatally in the cerebellum (1). The rate of neural development is much more rapid in the rat than in man, making inferences to human brain uncertain. It may nevertheless be hypothesized that the metabolic abnormalities induced by sustained elevation in the ratio of phenylalanine to tyrosine in blood may have its most severe effect on the most rapidly developing neurons. Indeed, in our Group I rats subjected to neonatal hyperphenylalanemia and killed at 50 days of age, neuronal lesions are limited to the cerebellum. Because impaired development of the cerebellum results from neonatal undernutrition (7), litters were reduced to 9 pups at birth and evidence for maternal rejection of the chronically injected nursing pups was not apparent; their rate of body growth was similar to saline-injected control animals, and to normal rats (3).

In experimental hyperphenylalaninemia induced from 13 to 21 days of life, biochemical abnormalities are most severe in the cerebellum (27). Animals sacrificed on day 21, showed a 21% decrease in cerebellar wet weight compared to only a 12% decline for whole brain, in addition to selective decreases of DNA, RNA, and protein. When phenylalanine was injected during the first 18–20 days of postnatal life, even more dramatic biochemical changes were found in whole brain (8, 9). Brain weight, myelin lipids including the incorporation of 35S-sulfate into galactolipids, total protein and DNA were all decreased.

In spite of the widespread chemical abnormalities reported in these studies, major pathologic emphasis in the past has been placed on abnormalities of myelin formation in experimental hyperphenylalaninemia. One neuropathologic study has shown abnormalities in the white matter of hyperphenylalaninemia
rats which included foci of pallor secondary to degeneration of myelin sheaths (17). This morphologic observation was comparable to pathologic reports of white matter lesions in the brains of children with phenylketonuria (22, 28). However, no evidence has been obtained to suggest whether the lesion was related to either (a) a direct effect on the myelin membrane; (b) a selective vulnerability of satellite oligodendroglia, the myelin forming cells; or (c) an impairment of neuronal metabolism associated with axonal degeneration and secondary myelin destruction (33).

In the present study, paraffin sections of cerebellum from adult rats subjected to neonatal hyperphenylalaninemia, showed pallor of white matter similar to that previously described (17). In addition, a suggestion of a primary neuronal lesion was indicated by the presence of numerous shrunken Purkinje cells with deeply basophilic cytoplasm. However, with more immediate and better preservation obtained by intravascular perfusion and improved histologic preparations made possible by thin plastic sections, a more detailed and clearer view of the cerebellar lesion was obtained.

Firstly, the sponginess of white matter, seen in paraffin sections, was represented in plastic embedded material only by mild axonal swelling. A reasonable explanation that may be offered for the spongy appearance of the white matter in paraffin sections is that the abnormally swollen axons are more sensitive to osmotic and chemical changes of formalin fixation and subsequent embedding resulting in disruption of their myelin sheaths.

Secondly, a cytoplasmic lesion involving Purkinje cells is clearly seen only in the perfused, plastic-embedded specimens. In these cells, vacuoles are seen in the cytoplasm which are similar to those previously described in Purkinje cells of cerebellar explants grown in tissue cultures with high concentrations of phenylalanine in the media (19). Although it may be hypothesized that vacuolation of Purkinje cell cytoplasm may represent abnormal or swollen endoplasmic reticulum associated with defective protein synthesis necessary for the elaboration of axodendritic processes, future electron microscopic and biochemical studies are planned to further explore this hypothesis.

Thirdly, a nuclear lesion involving granule cells was seen in which almost total loss of heterochromatin occurred with preservation of only a thin rim of chromatin beneath the nuclear membrane. This may represent a residual lesion from an abnormality of mitosis or migration of these micro-neurons from the external granular layer (1). The present neuropathologic observations in adult rats subjected to chronic hyperphenylalaninemia during the first postnatal week, suggest that neurons of the cerebellum undergoing mitosis and differentiation are selectively vulnerable to metabolic abnormalities initiated by sustained elevation of the phenylalanine/tyrosine ratio in blood.

REFERENCES


