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Hepatic Carbamyl Phosphate Synthetase and Ornithine Transcarbamylase in Mouse Influenza A and Influenza B Infection[1] (39330)

DUANE PIERSON,[2] VERNON KNIGHT, PAUL HANSARD, AND ELAINE CHAN

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77025

Reye's syndrome, an acute encephalopathy accompanied by alterations in liver structure and function has been recognized for a number of years as an infrequent complication of varicella-zoster infection, measles, influenza, and other virus infections (1-4). Neurological and hepatic abnormalities were also described in an influenza A epidemic in India in 1958 by Kapila (5). Recently, however, Reye's syndrome has been most prominently associated with influenza B infection, and there are only scattered reports of its occurrence after influenza A infection (4, 6-8).

Typically, the syndrome develops abruptly after partial recovery from the prodromal virus infection that is usually not severe. It is characterized by high fever, nausea and vomiting, abdominal pain with rapid onset of cough, seizures, coma, and frequently death. Despite severe cerebral dysfunction with increased spinal fluid pressure, cerebrospinal fluid cell counts and protein are usually not increased (1, 2).

The etiology of the disease is obscure, but it appears that the encephalopathy may result from ammonia and possibly other metabolites of the urea cycle that accumulate in elevated concentrations in blood and tissue because of impaired function of one or more urea cycle enzymes in liver (9, 10). Thaler and his associates (10) found that activity of the hepatic mitochondrial enzyme, ornithine transcarbamylase (OTC), was only 20.7% of normal in a patient with Reye's syndrome, and that there was an 18-fold reduction in the affinity of this enzyme for ornithine. Brown *et al.* (11) observed a reduction in carbamyl phosphate synthetase (CPS) as well as OTC in patients with Reye's syndrome. Tang and his associates (9) report a reduction to 10% of normal OTC activity in a patient with Reye's syndrome. The suggestion was made that Reye's syndrome may occur in persons having a genetically determined deficiency of an enzyme(s) of ammonia metabolism that is triggered by a variety of acute virus infections (10).

The apparent more frequent occurrence of Reye's syndrome with influenza B infection than with influenza A suggested the possibility, however, that some circumstance associated with influenza B infection might interfere with the activity of one or more urea cycle enzymes, resulting in hyperammonemia. This possibility was emphasized by the fact that influenza A occurs more frequently and is more severe than influenza B. Therefore, the hepatic levels of CPS and OTC were determined in mice during experimental infection with the two types of influenza.

Materials and methods. Virus. Experimental infection was produced by 0.05-ml intranasal instillation of a suspension of virus in mice anesthetized with ether. Control mice were treated with intranasal instillation of 0.85% saline following ether anesthesia. Influenza A/PR/8/34 (HONI), Ferret 8, Mouse 593, Egg 170, Mouse 1, Egg 1 ($10^{7.5}$ $TCID_{50}$/0.2 ml, rhesus monkey kidney cell culture) was used in a dilution of 1:10,000, and influenza B/Lee/40, Ferret 8, Mouse 137, Ferret 127, Mouse 23, Egg 1 (10^{5} $TCID_{50}$/0.2 ml, rhesus monkey kidney cell culture) in a 1:100 dilution. Both virus strains were obtained from Dr. Walter Dowdle, Center for Disease Control, Atlanta, Georgia. Subsequent passages of both viruses were reidentified by hemagglutination inhibition with antiserum provided by CDC and found to be authentic.

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[2] Author to whom reprint requests should be addressed.

Mice. Female white Swiss mice (CDI type) weighing approx 20 g were obtained from Charles River Breeding Farms. Neonatal mice were obtained from Microbiological Associates, Washington, D.C. For liver enzyme assays, mice were sacrificed on Day 5 of influenza A infection, and on Day 6 with influenza B infection. A few mice with influenza A infection died spontaneously before the time of sacrifice and were included. Assays of livers from these animals were not appreciably different from those of sacrificed animals. Livers were weighed, visually inspected, and frozen in Dry Ice immediately after autopsy. The lungs of all mice were examined for gross pathology at the time of sacrifice or death.

Human liver was obtained within 8 hr of death from six patients, none of whom had liver disease. Their age, sex, and diagnoses were as follows:

Age	Sex	Diagnosis
49	F	Cerebral hemorrhage
49	F	Left midcerebral arterial occlusion, hypertension
20	M	Accident
52	M	Coronary artery occlusion
50	M	Gun shot wound–head
46	M	Cerebral hemorrhage

Enzyme assays. The whole livers were kept frozen at −70° until immediately before assay. No appreciable differences in the levels of the enzymes measured occurred due to the freezing procedure. The livers (approx 1.0 g) were suspended individually in 19 ml of 4° water and homogenized using a Ten Broeck tissue grinder (10 strokes). The homogenate was centrifuged for 5 min at 600 rpm to remove unbroken cells and cell debris. The supernatant was used as the enzyme preparation.

CPS. The substrate mixture (12) contained (μmole/ml): NH_4HCO_3, 100; ATP, 10; L-ornithine, 10; *N*-acetyl-L-glutamate, 10; and $MgSO_4$, 20. The substrate solution was stored at −70° to prevent deterioration. Prior to use, the substrate mixture was saturated with CO_2. The reaction mixture consisted of 0.40 ml of the substrate mixture (preincubated at 37° for 3 min), 0.25 ml of 50 m*M* NH_4HCO_3 (containing 100 units of OTC-bovine), and 0.15 ml of the liver homogenate was added to initiate the reaction. The complete reaction mixture was allowed to incubate at 37° for 15 min, at which time the reaction was terminated by the addition of 0.2 ml of 15% perchloric acid. The resulting precipitate was removed by centrifugation (4000 rpm for 5 min). A 0.1-ml aliquot of the supernatant was used for the determination of citrulline as described by Hunninghake and Grisolia (13).

OTC. The reaction mixture contained (μmole/ml): carbamyl phosphate, 20; L-ornithine, 15; NH_4HCO_3 (pH 7.8), 50; and 0.01 ml of liver homogenate in a total volume of 0.8 ml. The reaction was initiated by the addition of the homogenate to the preincubated (3 min at 37°) substrate mixture. The reaction mixture was incubated at 37° for 15 min and then terminated by the addition of 1.2 ml of 15% perchloric acid. The mixture was centrifuged for 5 min at 4000 rpm, and 0.06 ml of the supernatant was utilized for citrulline determination.

It was determined for both CPS and OTC that product formation was linear over the time interval utilized and for the concentrations of enzymes and substrates used in the reaction mixtures. The protein concentration was estimated by the method of Lowry *et al.* (14) and specific activity is expressed as micromoles of citrulline produced per minute per milligram of protein. Carbamyl phosphate (dilithium salt) and L-ornithine were purchased from Sigma Chemical Company and all other chemicals were of highest purity commercially available. The bovine OTC was provided by Dr. Steve Bishop (Department of Biochemistry, Baylor College of Medicine).

Results. Characteristics of influenza A and B infection in the mouse. In Fig. 1 is shown the mortality of two groups of 10 mice following intranasal inoculation with influenza A/PR/8/34 (HONI) or influenza B/Lee/40. Deaths from influenza A infection began on Day 4 and by Day 8, 9 of 10 mice were dead. Deaths from influenza B infection began on Day 5 and by Day 8, 8 of 10 mice had died. In Fig. 2A is shown daily food consumption of the mice described in Fig. 1, with sham-inoculated uninfected mice serving as controls. In mice infected with influenza A, food consumption dropped progressively from 4 g to 0.25 g per day per mouse by Day 5, while food consumption of mice

infected with influenza B dropped to about 1.5 g per day in this period of time.

In Fig. 2B is shown the daily weight of mice during infection with influenza A and B. During influenza A infection, the average weight dropped from 20 g to 15 g by Day 6 while with influenza B infection it dropped to 18 g. Uninfected mice gained weight, averaging 22 g by Day 6.

The lungs of all mice were examined for gross pathology. Pneumonia was extensive in almost all animals and was most severe in those inoculated with influenza A.

CPS and OTC activity in livers of infected mice, neonatal mice, and in man. Values for the two hepatic enzymes in adult mice infected with influenza A and B are shown in Table I. Only in mice infected with influenza A were levels of enzyme activity significantly reduced. A 12 and 17% reduction was observed in CPS and OTC activities, respectively. Intraperitoneal or intravenous administration of the influenza A or influenza B did not result in a fatal disease, and

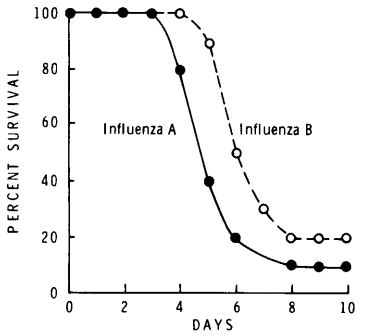

FIG. 1. Mortality from influenza A and influenza B infection (see Methods). There were 10 mice in each group.

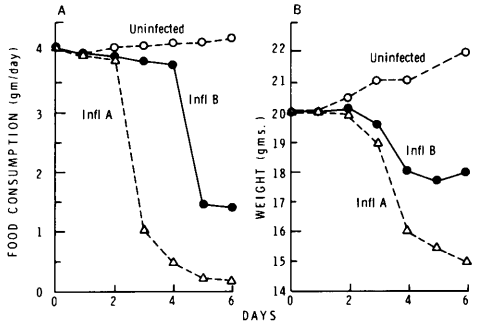

FIG. 2. (A) Reduction in food consumption in mice infected with influenza A and B infection. Values represent mean consumption by mice surviving each day. There were two cages of five mice at the start of the experiment. (B) Reduction in body weight during course of infection with influenza A and B.

CPS and OTC activities were unaltered.

Livers from 10 neonatal mice were also assayed (Table I) for the activity of these enzymes. CPS values were about 60% of the level of young adult mice (controls in Table I) while OTC values in neonates were 40% of those in normal adult mice. There was a 5–10% reduction in both CPS and OTC activities in neonatal mouse livers during influenza A or influenza B infections (not shown). For purposes of comparison, assays for CPS in livers obtained at autopsy from five adult human patients without known liver disease were 1.6 times greater than those in adult mice. However, OTC values in the human livers were only 30% of the values for adult mice. The values for both enzymes in livers of human adults were comparable to most published values for human neonates and adults (10, 15–19).

Discussion. The principal finding of this

TABLE I. SPECIFIC ACTIVITY OF HEPATIC CARBAMYLPHOSPHATE SYNTHETASE AND ORNITHINE TRANSCARBAMYLASE IN ADULT MICE INFECTED WITH INFLUENZA A AND B, IN NEONATAL MICE, AND IN HUMAN ADULTS WITHOUT LIVER DISEASE.[a]

	Number	Carbamylphosphate synthetase ($\times 10^2$)	Ornithine transcarbamylase
Adult Mice			
Controls	20	3.03 ± 0.21	1.90 ± 0.09
Influenza A	20	2.66 ± 0.13	1.58 ± 0.20
Influenza B	20	2.97 ± 0.20	1.79 ± 0.23
Neonatal mice	10	1.77 ± 0.16	0.718 ± 0.25
Human adults	6	5.02 ± 2.37	0.568 ± 0.166

[a] Specific activity is expressed as micromoles of product produced per minute per milligram of protein. Mice were inoculated (intranasal) with either saline (control) or influenza A or B as indicated.

Statistical significance: One-tailed *t*-test, 38 *df*. CPS, control vs influenza A, $t = 1.96$, $P < 0.05$. OTC, control vs influenza A, $t = 2.76$, $P < 0.005$. No other pairs involving infected mice were significantly different.

study is that highly lethal infections in mice following intranasal inoculation with influenza A or influenza B were associated with only a moderate reduction in activity of two hepatic enzymes of the urea cycle, CPS and OTC. The activity of the two enzymes in both infections ranged from 83 to 98% of normal. Since the CPS and OTC levels of neonatal mice were intrinsically lower than adult levels, it was thought that small reductions in CPS and/or OTC activities could induce hyperammonemia. However, CPS and OTC activities of neonatal mice infected with influenza resulted in no more than a 10% reduction of either enzymatic activity. In the few human patients with Reye's syndrome in whom these measurements have been made, enzyme activity has been in the range of 10 to 20% of normal. The reductions of enzyme activity in the present study are, therefore, probably a nonspecific result of the debilitating effect of illness.

Thus, influenza infection in mice does not result in a sufficient reduction in CPS or OTC activities to produce hyperammonemia. If the foregoing thesis is correct, then the search for the pathogenetic mechanism of the syndrome must turn toward factors related to the patients or features of the epidemiology of the various diseases that predispose to development of Reye's syndrome. The injury from virus infection responsible for reduced enzyme activity may result directly from a dissemination of virus to the viscera including the liver (4, 5, 9), despite an apparent mildness of the prodromal illness. Other hepatic enzymes and functions are also affected in Reye's syndrome, and the relationship among these various alterations cannot be clearly defined at present.

From an epidemiological point of view, the apparent paradox of relative absence of Reye's syndrome with influenza A and its more frequent presence with influenza B attracts attention, but the present study did not afford an explanation for this difference.

Summary. In mice infected with mouse-adapted influenza A/PR/8/34, hepatic carbamyl phosphate synthetase (CPS) activity was reduced to 88%, and ornithine transcarbamylase (OTC) was reduced to 83% of control values. In mice infected with mouse-adapted B/Lee/40, CPS activity was 98% and OTC was 94% of control values. These limited reductions in enzyme activity were attributed to a nonspecific debilitating effect of acute influenzal pneumonia. These findings suggest that the pronounced reduction of CPS and OTC activities reported in Reye's syndrome in man are not a general manifestation of the severity of influenza infection.

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