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Effect of Experimental *Escherichia coli* Meningitis on Concentrations of Excitatory and Inhibitory Amino Acids in the Rabbit Brain: *In Vivo* Microdialysis Study[1]

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ABSTRACT. Excessive extracellular fluid concentrations of the amino acids glutamate and aspartate play an important role in the pathogenesis of neuronal cell damage during hypoxia, hypoglycemia, and seizure. The purpose of these investigations was to test the hypothesis that bacterial meningitis causes progressive increase in excessive extracellular fluid concentrations of excitatory and inhibitory neurotransmitters. To test this hypothesis, *Escherichia coli* was injected intracisternally in juvenile rabbits after which neurotransmitter concentrations were measured with *in vivo* microdialysis. The data showed significant elevation of the excitatory amino acids aspartate and glutamate, as well as of the inhibitory neurotransmitters γ-amino butyric acid and taurine in the excessive extracellular fluid of animals injected with *E. coli* compared with control animals injected with saline. However, concentrations of these excitatory and inhibitory amino acids rose late in the course of meningitis, at a time when the animals were hypotensive (mean blood pressure ≤40 mm Hg). These data show that the major increase in excitatory neurotransmitters during experimental meningitis occurs in association with the cerebral ischemia produced by septic shock rather than being produced by the meningitis itself. (*Pediatr Res* 34: 187–191, 1993)

Abbreviations

GABA, γ-amino butyric acid
ECF, extracellular fluid
NMDA, *N*-methyl-D-aspartate
CSF, cerebrospinal fluid
CBF, cerebral blood flow

A large body of *in vivo* and *in vitro* experiments already support the notion that excessive concentrations of the excitatory amino acids glutamate and aspartate are an integral link in the chain of events leading to neuronal death (1–4) in a variety of brain insults including ischemia (5–7), hypoglycemia (8), complex seizure (9), and trauma (10). Excessive cell excitation by glutamate or aspartate opens receptor (NMDA) operated calcium channels, allowing neurons to accumulate toxic concentrations of intracellular calcium (1, 2, 11). Further evidence for the "excitotoxic" theory is provided by experiments that show that antagonists to NMDA can prevent cerebral ischemic injury (12–14).

Recently, several investigators proposed that the excitatory amino acids glutamate and aspartate are involved in the pathogenesis of brain injury in bacterial meningitis (15–17). Preliminary experiments by Tunkel *et al.* (18) show that concentrations of glutamate increase substantially in the CSF of rabbits exposed to endotoxin, pneumococcal cell walls, or live pseudomonads. Despite the enthusiasm to include meningitis in the list of brain disorders caused by "excitotoxins," other factors that may play an etiologic role in brain damage in meningitis must be considered; *e.g.* alterations in CBF. Studies of human infants and monkeys and rabbits show reductions in CBF or cerebral blood velocity during bacterial meningitis (19). Cerebral perfusion may be impaired during bacterial meningitis as a consequence of vasculitis, impaired autoregulation (19), or hypotension.

The aim of the present investigations was to test the hypothesis that excessive accumulation of excitatory amino acids occurs during acute bacterial meningitis. To accomplish this, we used cerebral microdialysis, a versatile tool that allows the determination of dynamic changes in concentrations of amino acids in the extracellular space. Through systemic physiologic and metabolic measurements, we hoped also to clarify the relationship of the rise in excitatory amino acids to changes in blood pressure, arterial pH, and blood glucose and lactate. Developing animals were studied because meningitis occurs most frequently in the young human.

MATERIALS AND METHODS

Animal preparation. Juvenile (20- to 30-d-old, mean wt 892 g) New Zealand White rabbits were anesthetized with halothane (induction, 4%; maintenance, 1%) and tracheotomized. An arterial catheter was inserted to monitor blood pressure and blood gases. All sites of incision were initially infiltrated with lidocaine (1%) followed by application of topical lidocaine.

After tracheostomy and arterial catheterization, halothane was discontinued, and the animals were mechanically ventilated (Harvard rodent ventilator; Harvard Apparatus, So. Natick, MA) with a gas mixture containing 70% N_2O/30% O_2 (N_2O induces an amnestic state). The animals were paralyzed with pancuronium (0.5 mL/h) to prevent dislodgement of intracerebral and intracisternal catheters (see below). The ventilator was adjusted to ensure normoxia [arterial oxygen tension >100 mm Hg (>13.3 kPa)] and normocarbia [arterial carbon dioxide tension, 30–40 mm Hg (4.0–5.3 kPa)]. Blood glucose and blood lactate were monitored with a Beckman Glucose Analyzer II (Beckman In-

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struments, Palo Alto, CA) and Yellow Springs Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH), respectively. Because neuromuscular blockade was used, body temperature was maintained at 36°C by means of a rectal probe and servo-controlled heating unit (Yellow Springs Instruments). The electroencephalogram was recorded with subdermal electrodes and a Grass model 79D polygraphic recorder (Grass Instruments, Quincy, MA).

Studies were approved by the Yale Animal Care and Use Committee and were performed in accordance with federal guidelines for the care and use of laboratory animals.

In vivo cerebral microdialysis. Microdialysis probes of a concentric design were fabricated from vitreous silica fibers (Polymicro Technologies, Phoenix, AZ) arranged side by side and inserted into a dialysis sack (300-μm outside diameter Caprophan, 5000-kD cutoff; 4-mm exposed membrane surface; Fig. 1) (20). This design reduced the probe dimensions, minimized brain trauma, and minimized the dead space between the dialyzed brain area and the sampling tube. The microdialysis probe was stereotactically lowered into the posterior frontal cerebral cortex (coordinates from the bregma: 3 mm posterior, 2 mm lateral, and 4 mm inferior to the skull surface) according to the atlas of Urban and Richard (21). Probe location was verified visually *post-mortem*. No samples were obtained for 2.5 h after probe placement to avoid traumatic artifacts. Previous studies have confirmed that the presence of the probe does not cause significant changes in amino acid levels in ECF 2 to 3 h after probe placement.

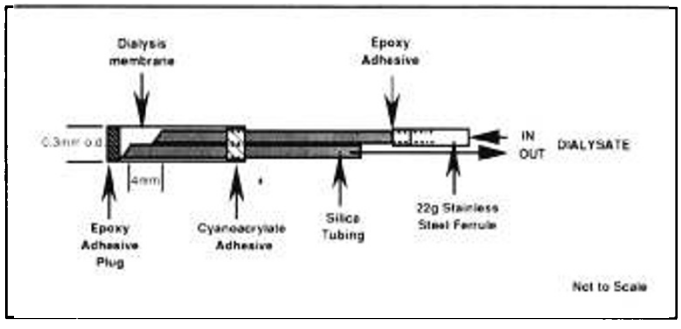

Fig. 1. Microdialysis probe. The probe consists of a pair of vitreous silica fibers mounted in a dialysis membrane. Molecules, driven by a concentration gradient, cross the dialysis membrane.

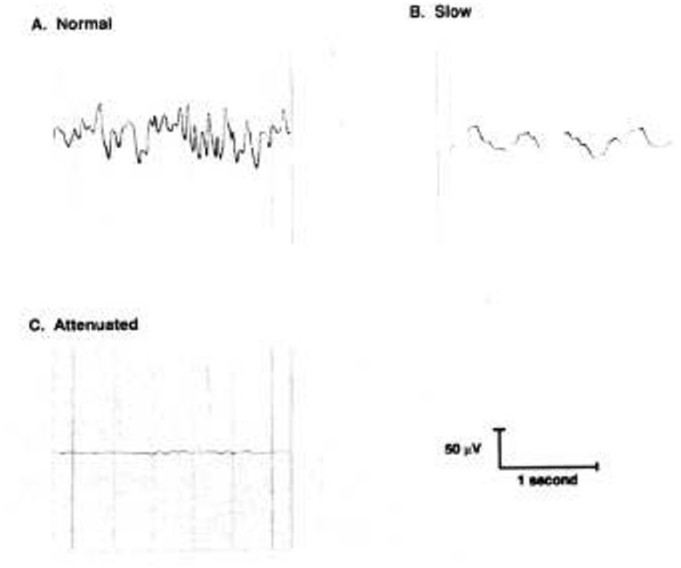

Fig. 2. EEG changes in experimental meningitis. EEG activity was characterized as normal (*A*), slow (*B*), or attenuated (*C*). During the baseline period, EEG activity consisted of asynchronous 5- to 10-Hz activity (*A*). Within 2 h of administration of *E. coli*, there was substantial slowing of background rhythms (*B*) followed by progressive attenuation (*C*).

The dialysate was delivered through the probe at 2 μL/min with a microinfusion pump (Harvard Apparatus). The ionic composition of the dialysate consisted of 147 mM Na^+, 129 mM Cl^-, 1.2 mM Ca^{++}, 3.5 mM K^+, 1.0 mM Mg^{++}, 1 mM phosphate, and 25 mM HCO_3^- at pH 7.4. Probe efficiency was determined by placing probes in 10^{-6} and 10^{-7} M solutions of amino acids. Recovery of amino acids ranged from 20 to 30%. Collected samples were frozen and later analyzed with a BAS-200A Ternary Gradient HPLC system (Bioanalytical Systems Inc., W. Lafayette, IN) with a CM-200 autoinjector. The amino acids were derivatized using the autoinjector before injection. The derivatizing reagent consisted of 100 mg of O-phthaldehyde in 2.5 mL methanol, 2.5 mL 0.2 M borate buffer, pH 9.6, with 22.5 μL of tert-butylthiol. An 8:1 sample to reagent ratio was used. After a 60-s reaction, the samples were injected into the BAS 200 HPLC using a BAS phase 2 100 × 3.2 mm 3-μm outside diameter column. The mobile phase used to achieve separation was 0.1 M acetic acid, pH 5.8, with an increasing gradient of acetonitrile from 12 to 30% and tetrahydrofuran from 1.2 to 15%.

Chromatograms were usually complete within 16 min with separation of the major transmitter amino acids. Combination dual electrochemical, 600-mV (detector 1) and 700-mV (detector 2) *versus* an Ag/AgCl reference electrode [and UV (330-nm) detectors in series in selected samples] were used with peak heights recorded on a two-channel chart recorder and compared with standards. We determined the concentrations of the excitatory amino acids glutamate and aspartate and the inhibitory amino acids GABA, glycine, and taurine. Alanine was measured because concentrations of this amino acid rise during impaired oxidative metabolism (6, 9, 22).

Bacteriology and cisternal cannulation. E. coli, originally isolated from a clinical specimen (courtesy of Col. Alan Cross, Walter Reed Army Medical Center), was grown in flasks of trypticase soy broth at 37°C to a concentration of 10^6 organisms/mL. The organisms were then centrifuged, washed, and resuspended in saline. The actual titer of the innoculum was determined by quantitative cultures on blood agar plates.

The model of experimental meningitis in rabbits described by Dacey and Sande (23) was used in these studies. The skin overlying the cisternal space was shaved and scrubbed with povidine iodine. The posterior atlantooccipital membrane was then exposed through sharp dissection, and a 22-gauge stainless steel cannula was stereotactically lowered through the membrane to obtain CSF.

Preliminary studies were conducted in pentobarbital-anesthetized, but not paralyzed, rabbits to determine the size of inoculum needed to produce meningitis. These studies disclosed that intracisternal injection of 10^6 *E. coli* organisms produced opisthotonos, pupillary dilatation, and coma after 1 to 2 h (25).

Experimental protocol. After insertion of the microdialysis probe, a 2-h period was allowed for probe equilibration and prevention of traumatic artifacts. After this equilibration period, baseline measurements of blood pressure, pH, P_{CO_2}, P_{O_2}, glucose, and lactate were made. A sample of CSF was obtained, and concentrations of glucose and lactate were measured. An aliquot of dialysate was obtained from the microdialysis probe to determine ECF concentrations of excitatory and inhibitory amino acids.

The animals were then randomized to a control group or to the experimental (meningitis) group. Animals in the former group were injected intracisternally with 0.3 mL of sterile normal saline (control animals), whereas those in the latter group received 10^6 *E. coli* organisms suspended in 0.3 mL of saline. Samples of arterial blood, CSF, and dialysate were then obtained hourly for 6 h in the control animals, after which the control animals were killed with a bolus of pentobarbital (50 mg/kg) and

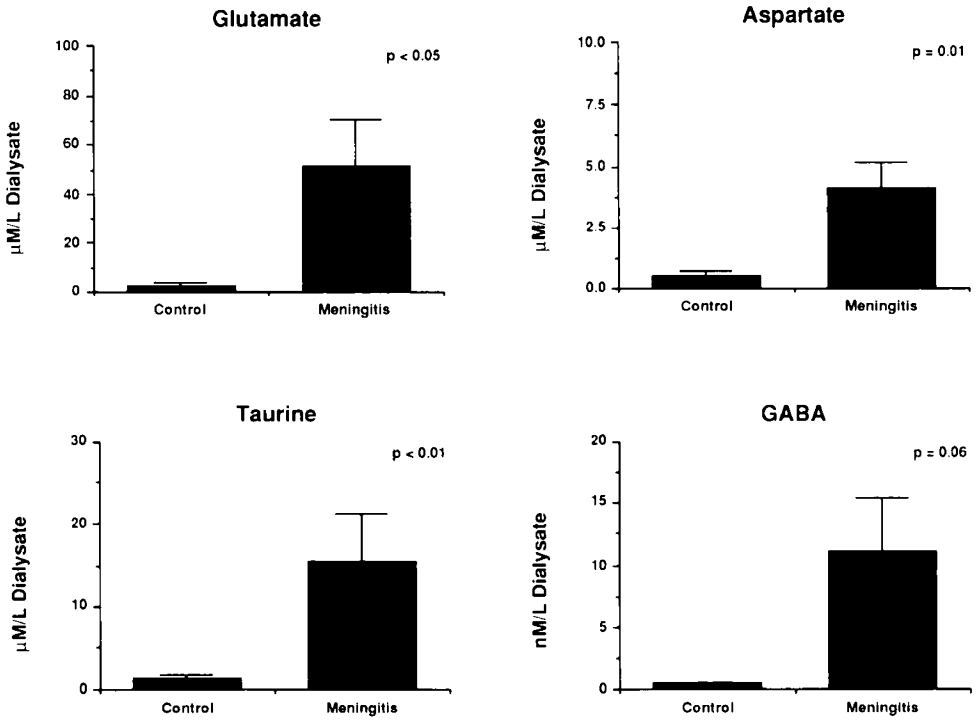

Fig. 3. Excitatory and inhibitory neurotransmitters in experimental meningitis. Concentrations of the excitatory amino acids, glutamate and aspartate (μmol/L dialysate), and inhibitory amino acid, taurine, were significantly elevated after 6 h of *E. coli* meningitis compared with saline-treated control animals. The rise in GABA (nmol/L dialysate) in meningitic animals was nearly significant; $n = 10$ (controls, 4; meningitis, 6); statistical analysis by t test.

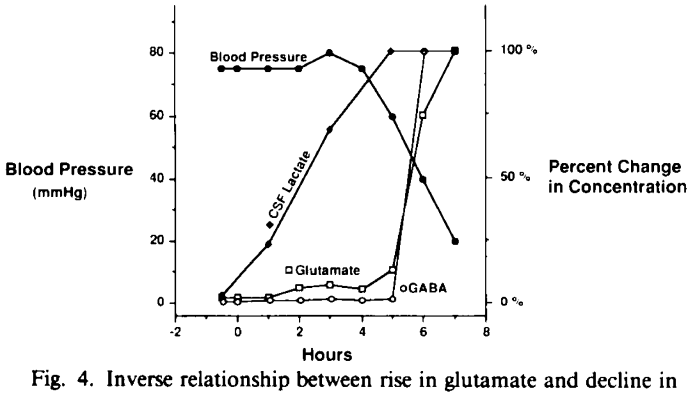

Fig. 4. Inverse relationship between rise in glutamate and decline in blood pressure in experimental meningitis. Blood pressure is stable for the first 4 h of meningitis. As the blood pressure begins to fail, there is a rise in concentrations of the brain's principal excitatory amino acid, glutamate, and inhibitory amino acid, GABA. Elevation of these amino acids is greatest when blood pressure falls below the lower end of the autoregulatory plateau. Note that CSF lactate, an indicator of meningitis, rises well in advance of the increase in glutamate.

KCl (2 cc). Animals subjected to meningitis were observed until they died or became profoundly hypotensive (blood pressure <20 mm Hg). After death, the brain was removed, fixed in 10% formalin, embedded in paraffin, sectioned, and examined by light microscopy.

Statistical analysis. A total of 10 animals were studied (four control animals, six experimental animals). Values are reported as mean ± SEM. Measurements of excitatory and inhibitory amino acids, plasma and CSF glucose and lactate, and blood pressure in control and experimental animals were analyzed with the two-tailed t test (24). Values were deemed significant at $p < 0.05$.

RESULTS

Systemic, CSF, and EEG measurements. Control animals showed no significant alteration of blood pressure, plasma glucose or lactate, or CSF glucose or lactate during any of the six hourly measurements. The EEG in these animals showed moderate voltage (<200 μV), 8 to 10 Hz activity.

In contrast, animals subjected to experimental *E. coli* meningitis developed significant hypotension after 6 h (controls, 68 ± 9 mm Hg; meningitis, 21 ± 5; $p < 0.0001$, t test). The duration of survival for meningitic animals was 6.3 ± 0.8 h. Blood glucose after 6 h was 3.78 ± 0.56 mM in control animals and 3.28 ± 0.56 in meningitic animals. Blood lactate was 0.37 ± 0.08 mM in control animals after 6 h *versus* 1.83 ± 0.64 mM in animals subjected to meningitis. However, at the end of the period of observation, CSF glucose was 25% lower (at 6 h: controls, 4.83 ± 0.94 mM; meningitis, 3.67 ± 0.39), and CSF lactate was 4-fold greater in the meningitic animals (controls, 1.4 ± 0.67 mM; meningitis, 5.4 ± 1.7; $p = 0.05$). Examination of the EEG showed substantial slowing of background rhythms (Fig. 2) within 2 h of injection of *E. coli*. Thereafter, the electrical activity became severely attenuated.

Microdialysis measurements. Control animals showed no significant alteration in concentrations of glutamate, aspartate, GABA, glycine, taurine, or alanine during the 6 h of observation. In contrast, there was significant increase in the excitatory amino acids glutamate and aspartate and in the inhibitory amino acids GABA and taurine in animals exposed to *E. coli* meningitis (Fig. 3). After 6 h, concentrations of alanine and glycine were also increased in animals subjected to meningitis compared with control animals (alanine: control, 2.5 ± 0.63 μM/L; meningitis, 13.57 ± 2.65; $p < 0.01$; glycine, control, 5.36 ± 1.08; meningitis, 18.4 ± 2.74; $p < 0.01$). There was an inverse rise in glutamate and GABA and decline in blood pressure in animals subjected to experimental meningitis (Fig. 4).

Neuropathology. Neuropathologic examination showed widespread accumulation of polymorphonuclear leukocytes in the Virchow-Robin space of the cortex of animals injected with *E. coli*.

DISCUSSION

The most striking finding in this study was the marked increase in concentrations of both excitatory and inhibitory amino acids

in the animals subjected to *E. coli* meningitis compared with control animals. However, careful inspection of the time course of the rise in glutamate in experimental meningitis and the decline in blood pressure showed an inverse relationship with abrupt increase in these amino acids when the blood pressure fell below 40 mm Hg. It is at this point that the animal's cerebral perfusion would likely fail and ischemia occurs. CBF fell to negligible levels (<20 mL/100 g/min) when blood pressure was <45 mm Hg in rabbits subjected to pneumococcal meningitis (26). Our laboratory previously demonstrated that CBF falls regardless of whether hypotension is caused by hemorrhage or by *E. coli* endotoxin (27). Experiments to demonstrate that hypotension induced by *E. coli* endotoxin will cause similar alterations in levels of extracellular neurotransmitters are in progress.

Focal or global cerebral ischemia frequently complicates bacterial meningitis for a number of reasons: impaired cerebral autoregulation, septic shock due to bacteria or bacterial cell products, vasculitis due to inflammation in the Virchow-Robin space, and polymorphonuclear leukocyte obstruction of the microcirculation (28).

The magnitude of the rise in excitatory and inhibitory amino acids in our experimental animals was consistent with that observed by other investigators in studies of cerebral ischemia. Ten min of ischemia in fetal sheep induced by cord compression produced marked increase in glutamate (maximum, 11-fold), aspartate (maximum, 7-fold), GABA (maximum, 5-fold), and taurine (maximum, 18-fold) in cerebral cortex (5). Marked increase in glutamate and aspartate (8- and 3-fold, respectively) was noted in the hippocampus of adult rats during 10 min of complete transient cerebral ischemia (6). Alanine levels were also severely increased in the meningitic animals. Alanine production increases by transamination of pyruvate formed during increased glycolysis. An increase in alanine was also observed in rabbits that became hypotensive during seizure (28).

Could the delayed rise in glutamate and GABA be attributable to the fact that meningitis developed only after many hours? This is an unlikely explanation, inasmuch as the animals became symptomatic from meningitis hours before the rise in glutamate and GABA. Our preliminary studies in nonparalyzed animals disclosed the development of opisthotonos and coma within 1 h after injection of bacteria. In addition, the rise in CSF lactate and slowing of EEG activity preceded the rise in excitatory and inhibitory neurotransmitters. These data suggest that if there is increased release of excitatory neurotransmitters during the early phase of meningitis, concomitant mechanisms for removal exist such that no net accumulation occurs in the ECF.

Could the development of seizure activity play a role in the rise in excitatory and inhibitory amino acids in meningitis? This is not a tenable explanation, because studies in our laboratory show that uncomplicated seizures (no preceding hypoxia or hypotension) do not produce alterations of excitatory or inhibitory amino acids. Other investigators have also demonstrated that neither bicuculine- nor kainic acid–induced seizure produces an increase in glutamate or GABA (22, 29). From this, we conclude that in meningitis, as with seizure, as long as CBF and levels of ATP are maintained (3), any increase in levels of excitatory amino acids is counterbalanced by uptake.

A similar parallel may be drawn between rise in excitatory neurotransmitters during hypoxia and hypoglycemia. Hypoxia that is too brief to cause a decrease in ATP levels does not result in a significant increase in glutamate. Similarly, glutamate does not increase during hypoglycemia until blood glucose falls below 1.1 mM (8).

In summary, there is excessive accumulation of excitatory and inhibitory amino acids in bacterial meningitis. However, the stimulus triggering the release of glutamate and aspartate may be the hypotension of septic shock rather than bacterial infection itself. This finding is consistent with the clinical observation that hypotension during meningitis is a poor prognostic sign.

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