Future developments will include the design of a small portable system of N.A.A. which will make possible the study of tissue levels in hospitals or places of work. This will comprise a plutonium-beryllium source of neutrons together with a small, modern detector system which should be sufficiently compact to fit into a car.

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POSSIBLE PATHOGENIC ROLE OF ENDOTOXIN IN REYE'S SYNDROME

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Summary Evidence of circulating endotoxin was sought in children with Reye's syndrome, on the thesis that severe hepatic failure is likely to result in loss of capacity to detoxify intestinal endotoxins entering the circulation. A modification of the *Limulus* assay was used to demonstrate high levels of endotoxin-like activity (E.L.A.) in nine comatose patients with Reye's syndrome and in one of the two non-comatose patients. The symptom-free sibling of one patient had raised liver enzymes and a negative *Limulus* test. Plasma E.L.A. correlated significantly with degree of electroencephalographic disturbance early in the course of the illness. E.L.A. was also found in both of two cerebrospinal fluids evaluated. Preliminary in-vitro characterisation of this substance indicated that it resembled endotoxin derived from anaerobic intestinal bacteria. Intestinally derived endotoxin could be one factor in the pathogenesis of encephalopathy and other features of Reye's syndrome.

Introduction

FIRST described in 1963,[1] Reye's syndrome—acute encephalopathy with fatty degeneration of the viscera—is a severe and often fatal disease of unknown ætiology and pathogenesis. Brain damage in this disease may result from failure of a damaged liver to inactivate potentially encephalotoxic substances, such as ammonia.[2] However, no single toxin has been unequivocally implicated as the cause of encephalopathy associated with hepatic failure.[3,4]

Under normal conditions, intestinal bacterial antigens, including endotoxins derived from the gram-negative flora, may regularly be released into the circulation in small quantities; this release increases sharply under a variety of stress conditions.[5,6] The *Limulus* assay has been used to demonstrate endotoxin-like substance in the plasma of patients with fulminant hepatic failure due to conditions other than Reye's syndrome,[7] and we have used this test to search for endotoxin-like activity (E.L.A.) in plasma and cerebrospinal fluid of children with Reye's syndrome.

Patients and Methods

Patients

Reye's syndrome was diagnosed if the patient became comatose or showed greatly altered consciousness after a period of protracted vomiting, lacked focal neurologic signs, had raised serum transaminases and ammonia with little or no jaundice, had normal cerebrospinal-fluid cell-counts and protein, and had no additional diagnosis. E.L.A. was measured in eleven children with Reye's syndrome and in controls. Ten of the patients presented during an

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outbreak of influenza B in February and March, 1974; the eleventh was recovering from varicella when the syndrome developed.

Endotoxin Assay

E.L.A. in plasma was estimated with the *Limulus* test of Levin and Bang.[8] A lysate of the blood-cells of the horseshoe crab (*L. polyphemus*) was prepared according to the method of Yin et al.[9] and buffered with 0·10*M* "tris". Blood-samples were obtained under aseptic conditions with pyrogen-free syringes (Becton-Dickinson Co., Rutherford, New Jersey) and were anticoagulated with 50 units per ml. beef-lung heparin (Upjohn Co., Kalamazoo, Michigan). All glassware was rendered pyrogen-free by heating at 170°C for 4 hours.

A previously unreported method of extracting *Limulus* test inhibitors from plasma was used. Plasma samples were diluted 1/3 in pyrogen-free water (Travenol Laboratories, Deerfield, Illinois) and heated at 100°C for 10 minutes. Comparative preliminary experiments in our laboratory suggested that this method was less cumbersome than chloroform extraction [10] and more effective than a method utilising shifts in pH.[11] After heating, the plasma was further diluted in serial log or half-log increments; 25 µl. samples of these dilutions were incubated with equal volumes of *Limulus* lysate for 4 hours at 37°C and overnight at 22°C, and then examined for the appearance of a viscous gel. Each day's run included a fresh plasma sample from a healthy adult control, pyrogen-free water alone, and serial dilutions of a standard endotoxin (*Escherichia coli* O 26 B6 Boivin endotoxin, control no. 586784, Difco, Detroit). Plasma E.L.A. was estimated by comparing activity of plasma dilutions with the standard endotoxin controls. This method detects 10 pg. per ml. of the standard endotoxin in normal plasma. In some instances, extracted samples were also treated with prolonged heating, with polymyxin B, or with chloroform before testing. Spinal-fluid samples were tested in serial dilution without prior extraction.

Results

Nine patients became fully comatose during their illness. Among twenty plasma samples obtained from these patients during the first 5 days of central-nervous-system illness, E.L.A. ranged between 1 and 3000 ng. per ml. with a mean of 30 ng. per ml. Five of these patients were studied sequentially (fig. 1).

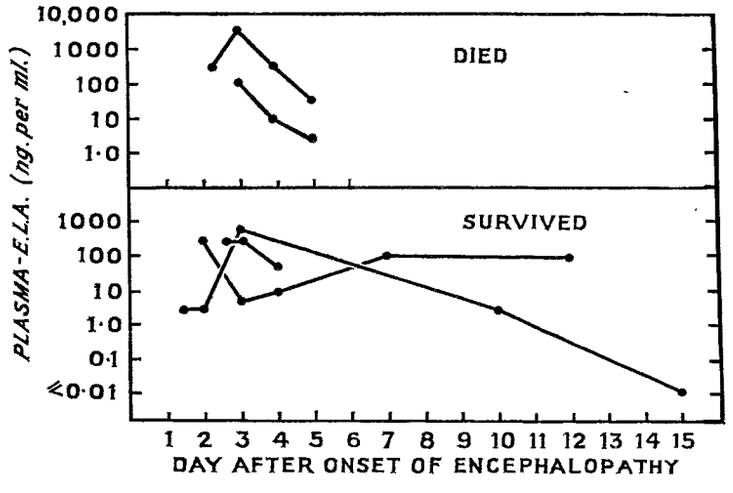

Fig. 1—Sequential plasma E.L.A. in five patients with Reye's syndrome.

E.L.A. fell during the first few days after onset of encephalopathy. In two patients, E.L.A. remained detectable long after resolution of coma.

Declining levels were associated with clinical improvement in three. In the two fatal cases, E.L.A. decreased only on appearance of ante-mortem E.E.G. patterns (electrocerebral silence or near-silence). The E.L.A. concentration in one survivor rose again for several days without clinical signs of relapse.

Two patients exhibited delirium and ataxia but did not become comatose. Both were sampled during the first day of improvement in central-nervous-system symptoms. Plasma-E.L.A. levels were 30 ng. per ml.

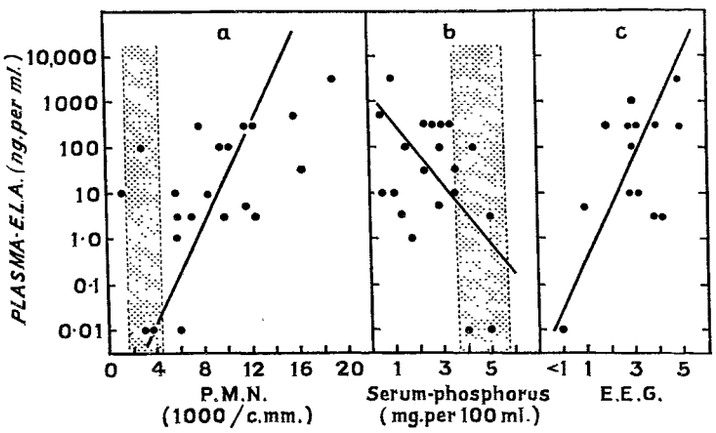

Fig. 2—Correlates of plasma E.L.A. in children with Reye's syndrome.

E.L.A. was significantly correlated with: (*a*) Polymorphonuclear-cell count (P.M.N.) ($n=21$, $r=0{\cdot}58$, $P<0{\cdot}01$). (*b*) Serum-phosphorus depression ($n=20$, $r=0{\cdot}43$, $P<0{\cdot}05$). (*c*) Electroencephalographic (E.E.G.) stage (Aoki and Lombroso) in patients tested during the first 4 days of central-nervous-system illness who had not reached E.E.G. silence ($n=14$, $r=0{\cdot}63$, $P<0{\cdot}01$). Shaded areas show normal ranges.

and undetectable (<0·01 ng. per ml.). In addition, the sibling of one of our patients had raised liver enzymes but no clinical symptoms; his plasma *Limulus* test was also negative. Control samples were donated by ward personnel each day that patient samples were drawn; these were run simultaneously with patient samples and were uniformly negative.

Plasma *Limulus* activity was not correlated with body temperature, liver-enzyme levels, or platelet-count. No patient had significant hypotension when studied. Polymorphonuclear neutrophil-count was significantly related to E.L.A., however (fig. 2a). E.L.A. was also related to serum-phosphorus depression (fig. 2b).

Fourteen electroencephalograms were done within 24 hours of E.L.A. sampling. These were scored by the criteria of Aoki and Lombroso [12] without knowledge of patients' clinical condition or E.L.A. These criteria include five grades of abnormality in addition to electrocerebral silence, and were shown to have prognostic significance in patients with Reye's syndrome. There was a positive relationship between plasma E.L.A. and electroencephalographic abnormality among patients in the first 4 days of central-nervous-system illness who had not reached electrocerebral silence (fig. 2c).

Two spinal-fluid samples were obtained for study; both had E.L.A. The first was from a conscious,

recuperating patient and contained 0·01 ng. per ml. activity; a simultaneous plasma sample was not obtained. The second was from a comatose patient and contained 1·0 ng. per ml., while simultaneous plasma level was 300 ng. per ml. In both instances spinal-fluid glucose, protein, and cell-count were normal.

Preliminary attempts to characterise the E.L.A. were made on a few samples. Activity was unaltered by heating at 100°C for 60 minutes, by vigorous extraction of the samples with chloroform for 15 minutes, or by incubation at 37°C for 15 minutes in the presence of 10 μg. per ml. polymyxin B. Simultaneous experiments demonstrated that these findings were also characteristic of *Bacteroides fragilis* s.sp. *vulgatus* endotoxin (kindly provided by V. R. Dowell, Center for Disease Control, Atlanta, Georgia).

Discussion

We found the new method of extracting plasma inhibitors in the *Limulus* test to be effective and convenient, and it should improve the utility of the test as a clinical research tool. The test can be done on as little as 50 μl. plasma.

We found E.L.A. in the plasma and cerebrospinal fluid of children with Reye's syndrome, and endotoxin-like activity was related to clinical and electroencephalographic indices of central-nervous-system disability early in the course of the disease, suggesting that the endotoxin may be encephalopathogenic during this phase. The brain may be very sensitive to small amounts of endotoxin reaching it [13,14]; one prominent early response to intraventricular endotoxin is hyperventilation with respiratory alkalosis.[15] Other manifestations of endotoxin activity include the release of endogenous vasoactive mediators,[16] fever,[17] hæmorrhagic effects,[18] granulocytosis,[18] mitochondrial swelling with paralysis of oxidative phosphorylation,[19] and increase of free fatty acids with hepatic dysfunction and triglyceride accumulation.[20] These features, demonstrated largely in animals and animal tissues, have all been theoretically implicated in the pathogenesis of Reye's syndrome.

We have measured endotoxin activity in twelve patients with gram-negative septicæmia but no evidence of severe hepatic disease (unpublished). This group, which included six babies, had a mean peak level of 1 ng. per ml. (range 0–100) compared with 30 ng. per ml. (range 1–3000) found among comatose patients with Reye's syndrome. This suggests that the levels found in children with Reye's syndrome are very high, although the two groups are not strictly comparable since different extraction techniques were used for many plasma samples in the earlier study.

There is no irrefutable evidence that E.L.A. in patients with Reye's syndrome or hepatic failure [7] is due to endotoxin. However, our preliminary in-vitro experiments demonstrating its chloroform insolubility and resistance to chloroform or 100°C heat denaturation are compatible with that possibility. Resistance to polymyxin was also a property of this substance; the only known endotoxin with this characteristic is that produced by *B. fragilis*,[21] a finding we have confirmed. The correlation of E.L.A. with neutrophil-count is compatible with bone-marrow response to bacterial endotoxin.[18] *Limulus* reactivity of bioactive substances such as polyribonucleotides has been claimed.[22]

To our knowledge, hypophosphatæmia with Reye's syndrome has not previously been described. In our series, endotoxæmia, hypophosphatæmia, and electroencephalographic abnormality were statistically correlated. A renal tubular loss of phosphate secondary to the proximal tubular lesion described in Reye's syndrome [1] could be postulated.

We do not know whether the encephalopathy in children with Reye's syndrome is primary or results from severe hepatic failure. However, our findings suggest the latter, since positive *Limulus* tests, like raised blood-ammonia levels, are linked with the encephalopathy of fulminant hepatic failure from causes other than Reye's syndrome.[7] In addition, the E.L.A., which is very possibly due to enteric endotoxin, could contribute to the encephalopathy as well as other features of Reye's syndrome, and deserves further evaluation as such.

Our findings support the view that efforts to control gut flora in patients with acute, reversible hepatic failure should be directed towards anaerobic as well as aerobic intestinal flora. Anaerobic organisms are largely resistant to aminoglycosides such as neomycin,[23] yet they are potentially a source of many toxic substances, including ammonia and endotoxin.

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