EVALUATION OF PLASMA NEUROTOXIN CONCENTRATION IN URAEMIC POLYNEUROPATHIC PATIENTS

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Summary

Purification of b4-2 sub-peak obtained on DEAE Sephadex A25 chromatography gave us the possibility of quantifying the plasma concentration of the neurotoxin present in uraemic patients with active polyneuropathy. From the purified neurotoxin isolated by kieselguhr and cellulose chromatography we calibrated analytic columns for b4-2 analysis.

Plasma concentration, measured in 6 uraemic neuropathic patients, is between 13 and 19 mg/litre. In 52 uraemic patients without neuropathy, the plasma concentration is between 3 and 9 mg/litre. In 20 healthy subjects the plasma concentration is less than 1 mg/litre.

The weekly neurotoxin removal in uraemic patients without neuropathy, treated by a five hours RP6 session 3 times a week, is of the same order of magnitude as the weekly urinary excretion in healthy subjects.

Preliminary results of a tentative identification of this purified product indicate that it is not a polypeptide but an acid-polyol with carbohydrate structure.

Introduction

We have previously shown that, among the middle molecule (MM) fractions obtained by chromatography from plasma ultrafiltrate (UF) of uraemic patients with active recent polyneuritis, peak b4-2 was at a significantly higher concentration than that obtained from uraemic patients without neuropathy [1]. It has been verified that this b4-2 peak is found in the urine of healthy subjects.

In order to identify the neurotoxic solute contained in this peak, we performed a preparative procedure on urine from healthy subjects to quantify on calibrated analytic columns the humoral and dialysate concentration of this neurotoxic solute and to perform identification procedures.
Material and Methods

Samples

Sixty litres of 24 h urine collected from healthy subjects were used for the purification procedures. Blood samples from 20 healthy subjects, 52 uraemic patients without neuropathy and 6 uraemic patients with active recent polyneuropathy were collected for evaluation of their b4-2 neurotoxin content.

The amounts excreted in the 24 h urine from 7 healthy subjects, in dialysates from 3 uraemic patients without polyneuropathy and in the first dialysate of an uraemic neuropathic patient were also measured.

Purification Procedure

A 100 ml sample from urine lyophilised to 10 times normal concentration is fractionated at pH2 on a Sephadex G15 preparative column (100 x 7.5 cm). pH2 was chosen in order to eliminate polyacids such as citric, isocitric, cisaconitic and tartaric acids where elution volume is the same as that of MM on Sephadex G15 gel chromatography at pH7 [2]. The fraction which contains MM (peaks b, c and d) is collected. The sulphate and phosphate ions, and the solutes corresponding to peaks c and d, are removed from this fraction by QAE Sephadex A25 chromatography (30 x 6 cm column). The b fraction is then submitted at pH7 to ion exchange chromatography on Sephadex DEAE A25 (50 x 3.8 cm column). The sodium contained in the b4-2 collected fraction is removed by SP Sephadex A25 chromatography (30 x 6 cm column). Eluate is then evaporated under low pressure at 40°C, giving a viscous yellow liquid which contains b4-2 solute with acidic contaminants. Finally, this material is purified by partition chromatography on cellulose (80 x 2.5 column).

The degree of purity of the final substance was assessed by thin layer chromatography on silica gel. An in vitro frog sural nerve test [3] was performed at each step of the procedure in order to follow up the preparation of the neurotoxic solute.

Calibration of the Analytical Column

With the purified solute, we prepared standard solutions to calibrate analytical DEAE Sephadex A25 columns [2].

Identification

The solute was identified both by dyeing reagents with Periodic acid/Phenylhydrazine, p-Anisidine/Periodate and by physico-chemical methods with infrared spectrum (IR), proton nuclear magnetic resonance (NMR) spectrum in D6-Dimethylsulfoxide, 13CNMR spectrum in deuterated water and microanalysis.
Results

Purification

From 60 litres of urine from healthy subjects, we obtained 56 mg of material which has the same elution volume as peak b in Sephadex G15 chromatography (Figure 1) and as peak b₄₋₂ in DEAE Sephadex A25 chromatography (Figure 2). On thin layer chromatography, two spots were detected by dyeing
reagents and fluorescence inhibition at 254 nm, one major spot (Rf: 0.33) corresponding to \( b_{4-2} \) and one minor spot (Rf: 0.1) corresponding to a slight amount of a more polar residual contaminant.

**Calibration and Quantification of \( b_{4-2} \) Solute in Normal and Uraemic Body Fluids**

Figure 3 shows a linear relationship between the integrated absorbance given by the surface under the peak (\( \text{cm}^2 \)) obtained with standard solutions and \( b_{4-2} \) concentration (mg/litre) of these solutions.

The plasma concentration in 20 healthy subjects is lower than 1 mg/litre. The mean plasma concentration (± SEM) in 52 dialysed patients without neuropathy is 4.7 ± 0.23 mg/litre (range 3–9 mg/litre). In 6 dialysed patients

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Figure 3. Ion exchange chromatograms of \( b_{4-2} \) standard solution and calibration curve
Figure 4. Plasma neurotoxin b$_{4-2}$ concentration (mg/litre) in 52 uraemic patients without neuropathy and in 6 uraemic patients with neuropathy

with active recent neuropathy, the plasma concentration was between 13 and 19 mg/litre (Figure 4).

When the neurological status of these patients improved after 3 months of adequate dialysis, the plasma concentration returned to within the range observed in uraemic patients without neuropathy (Table I).

TABLE I. Plasma b$_{4-2}$ Concentration at the Onset of Polyneuropathy and after 3 months of Adequate Dialysis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma b$_{4-2}$ concentration (mg/litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_0$</td>
</tr>
<tr>
<td>1. MP</td>
<td>13.0</td>
</tr>
<tr>
<td>2. ML</td>
<td>19.0</td>
</tr>
<tr>
<td>3. GA</td>
<td>13.0</td>
</tr>
<tr>
<td>4. AD</td>
<td>13.5</td>
</tr>
<tr>
<td>5. FN</td>
<td>15.5</td>
</tr>
<tr>
<td>6. PR</td>
<td>16.0</td>
</tr>
</tbody>
</table>

In patient PR, the plasma concentration fell rapidly from 15.8 mg/litre to 6.2 mg/litre after six dialysis sessions on a PAN membrane.

The amount of b$_{4-2}$ solute excreted in 24 h urine was 9–18 mg. The amount of b$_{4-2}$ solute removed from RP6 dialysis in our patients was 39–45 mg per session. It attained 156 mg in patient AD, whose uraemic neuropathy was severe.

Identification

An acid vicinal polyol function (–CHOH–CHOH–COOH) was detected by dyeing reactions on silica gel. Infra red spectrum, proton and $^{13}$C nuclear magnetic resonance spectrum confirmed the existence of this function.
Preliminary microanalysis determination suggests a carbohydrate structure whose formula might be: $C_{22}H_{45}O_{30-40}$. More precise identification is hindered because the purification recovery rate (20%) does not presently provide a great amount of pure material.

Discussion

Whatever the origin of $b_{4-2}$ (plasma, urine or dialysate), the chromatographic pattern is the same in gel chromatography, ion exchange chromatography and adsorption chromatography. Since the processes involved in these three methods differ (molecular size, ionic charges and polarity respectively) it is likely that the same solute is detected in plasma, urine and dialysate.

Weekly removal of neurotoxin (117–135 mg) in uraemic patients without neuropathy treated by five-hour RP6 sessions three times a week, is similar to the weekly urinary excretion (63–126 mg) in healthy subjects. Inadequate dialysis leads to excessive retention of the solute and results in neuropathy.

With Fürst and Bergström's method of fractionation [4], peak b corresponds to the same elution volume as that of peak 8. But with chromatography on Centriflo ultrafiltrate, discrimination for this peak between uraemic patients with and without neuropathy becomes impossible [5]. The physical and chemical identification procedures used in this study demonstrate the carbohydrate structure of the uraemic neurotoxin. This leads us to consider the relationship between the $b_{4-2}$ solute and other carbohydrate metabolites such as myoinositol or D-glucaric acid with respect to their neuronal toxicity.

Myoinositol has been proposed as a possible uraemic neurotoxin because of its high blood level in uraemic patients [6] and its neuronal toxicity on root ganglion cells in organotypic cultures [7]. Several assays have indicated that $b_{4-2}$ solute is not myoinositol.

Urinary excretion of D-glucaric acid is increased in alcoholic subjects [8]. Excessively high plasma concentration of some carbohydrate metabolites might account for the nerve fibre degeneration which has been found by Dyck et al in both uraemic and alcoholic neuropathies [9].

Conclusion

1. The preparative procedure developed for the urine of healthy subjects produces a neurotoxin solute with a high degree of purity.

2. Our results show a striking similarity between $b_{4-2}$ solute in plasma and in urine. The uraemic patient exhibits a plasma retention of this solute. A high level of plasma retention of this solute in uraemic patients always correlates with active recent neuropathy.

3. Preliminary identification of this neurotoxic solute indicates that it is not a polypeptide but an acid-polyol with a carbohydrate structure.
References

6 Clements, RS Jr., Dejesus, PV Jr and Winegrad, AI (1973) Lancet, i, 1137
7 Liveson, JA, Gardner, J and Bornstein, M (1977) Kidney Int., 12, 131

Open Discussion

PARSONS (London) Have you studied patients with diabetes with and without renal failure, because this substance is reminiscent of the polyol sorbitol type of substances which the diabetic physicians have been interested in for some years?

MAN No, we have no studies on diabetics in renal failure but in our patients, although we have not determined the plasma concentration of myoinositol we are sure from several assays that it is not myoinositol.