Characterization of the storage material of peripheral lymphocytes in aspartylglycosaminuria

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(Received 10 September 1979; accepted 9 October 1979)

Summary

1. Aspartylglycosaminuria is a neurovisceral storage disease characterized by reduced or absent activity of the lysosomal enzyme N-aspartyl-β-glucosaminidase. Although vacuolization of peripheral lymphocytes is a well-documented feature of aspartylglycosaminuria, the chemical composition of the lymphocyte storage material is not known. In this paper we describe the results of glycopeptide analyses of peripheral lymphocytes isolated from the blood of a 29-year-old female patient with aspartylglycosaminuria and from a control subject.

2. By use of a highly specific and sensitive gas-chromatographic-mass-spectrometric technique, 4-N-2-acetamido-2-deoxy-β-D-glucopyranosyl-L-asparagine (N-acetylglucosaminylasparagine) was found to accumulate in the patient's lymphocytes, but not in those of the control subject.

3. The amount of this compound stored in the diseased lymphocytes was found to be approximately 2.3 nmol/100 μg of soluble protein.

4. We suggest that this compound is the main storage substance in the peripheral lymphocytes in aspartylglycosaminuria and that it is probably responsible for the cytoplasmic vacuolization of these cells.

Key words: aspartylglycosamine, aspartylglycosaminuria, lymphocytes, lysosomes, storage material.

Introduction

Vacuolization of lymphocytes is an unusual finding limited to some hereditary storage diseases affecting the metabolism of polysaccharides, sphingolipids and glycopeptides, and to a few other conditions, such as lymphatic leukaemia, lymphomas and infectious mononucleosis (Bruning, 1970; Schuurmans Stekhoven, van Haelst, Joosten & Loonen, 1977). Although morphologically well defined, chemical characterization of the lymphocyte inclusions has in most cases not been accomplished.

In aspartylglycosaminuria, a storage disease characterized by reduced or absent activity of lysosomal N-aspartyl-β-glucosaminidase (Pollitt, Jenner & Merskey, 1968; Palo, Riekkinen, Arstila, Autio & Kivimäki, 1972; Aula, Näättö, Laipio & Autio, 1973), 16–28% of the peripheral lymphocytes contain cytoplasmic vacuoles (Aula, Rapola & Andersson, 1975). The chemical nature of the lymphocyte storage material is unknown.

In this paper we report the results of glycopeptide analyses of peripheral lymphocytes from a patient with aspartylglycosaminuria and from a normal subject. It is shown that the main accumulating metabolite in the lymphocytes in aspartylglycosaminuria is 4-N-acetamido-2-deoxy-β-D-glucopyranosyl-L-asparagine (GlcNAc-Asn), which is also the main metabolite excreted in the urine in this disease (Jenner & Pollitt, 1967; Maury, 1979a).

Methods

Materials

4-N-2-Acetamido-2-deoxy-β-D-glucopyranosyl-L-asparagine was obtained from Vega-Fox Biochemicals, Tucson, U.S.A. Melibiitol was prepared from melibiose (Eastman Organic Chemicals,
Rochester, U.S.A.) by borohydride reduction (Kärkkäinen, 1969). Reference monosaccharides were obtained from commercial sources and used as such. Solvents were of analytical grade and redistilled before use.

Lymphocytes were prepared from blood of a 29-year-old female patient and a female control of the same age by the Isopaque–Ficoll (Lymphoprep) method (Bayum, 1974). The preparations contained 96–98% lymphocytes and 2–4% monocytes, but no granulocytes.

Analytical methods

Monosaccharides were analysed by gas chromatography as described by Bhatti, Chambers & Clamp (1970). Protein was measured by a modified Lowry method (Hartree, 1972). Anion-exchange chromatography was carried out on Dowex-1 (acetate) columns. Permethylated of the samples was carried out with methyl iodide in dimethyl sulphoxide in the presence of dimethyl sulphinyl carbanion (Hakomori, 1964; Rauvala, 1979). Gas chromatography was performed on a Perkin-Elmer model 900 gas chromatograph equipped with hydrogen flame ionization detectors. Glass columns, 2 m × 3 mm (internal diameter), packed with 2.2% OV-101 and 2.2% SE-30 Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania, U.S.A.) were utilized. Nitrogen was used as carrier gas. Gas chromatography–mass spectrometry was carried out on a Variant 1700 gas chromatograph coupled to a Varian MAT CH-7 mass spectrometer equipped with a Spectro System 100 MS data-processing system. The ionization current was 300 μA and the ionization potential was 70 eV. Mass fragmentographic detection of methylated GlcNAc-Asn was carried out by monitoring ions at m/e 186, 238 and 260 (Maury & Kärkkäinen, 1979).

Isolation of lymphocyte storage material

The purified lymphocyte preparations were homogenized in water and centrifuged at 1500 g for 25 min. The supernatant (0·96 mg of soluble protein) was subjected to anion-exchange chromatography on a Dowex-1 (acetate) resin column 0·9 cm × 3·0 cm. The water eluate was dried in vacuo, and analysed for glycopeptides after permethylation (Maury & Kärkkäinen, 1979). The pellet obtained at centrifugation and part of the supernatant were dried and subjected to methanolysis and analysed for monosaccharides according to Bhatti et al. (1970).

Results

Identification of unbound GlcNAc-Asn in the diseased lymphocytes

The supernatants obtained at centrifugation of the lymphocyte preparations from the control subject and the patient with aspartylglycosaminuria were subjected to anion-exchange chromatography. The water eluate was dried and permethylated with methyl iodide in dimethyl sulphoxide in the presence of dimethyl sulphinyl carbanion. The completeness of methylation was tested with triphenylmethane (Rauvala, 1979).

Gas chromatography and mass fragmentography of the permethylated fraction revealed a peak with identical retention time with authentic methylated free GlcNAc-Asn in the preparation obtained from the diseased lymphocytes, but not from that of the normal lymphocytes (Fig. 1).

The identity of the compound stored in the lymphocytes from the patient with aspartylglycosaminuria with authentic unbound GlcNAc-Asn was confirmed by combined gas chromatography–mass spectrometry. In the low-mass range the peaks were the same as in the spectrum of methylated N-acetylglucosamine (Stoffyn, Stoffyn & Orr, 1972), but the relative intensities were different, which indicates substitution at C-1 of the sugar moiety. The heaviest fragments of the spectrum were found at m/e 403 (M-44) and 402 (M-45) (Maury & Kärkkäinen, 1979).

Fig. 1. Mass fragmentograms of methylated GlcNAc-Asn (a), methylated purified fraction from lymphocytes of a patient with aspartylglycosaminuria (b) and a methylated corresponding fraction from normal lymphocytes (c). Detection was at m/e 186. Conditions: 2·2% OV-101, 255°C.
**Quantitative aspects on the storage compound of the diseased lymphocytes**

For quantitative purposes 0.058 μmol (20 μg) of melibiitol was added as internal standard to the supernatant fraction (0.96 mg of protein) prepared from the lymphocytes of the patient with aspartylglycosaminuria. After anion-exchange chromatography, the water eluate was dried, permethylated and subjected to gas chromatography. By comparing the peak area of methylated GlcNAc-Asn with that for methylated melibiitol, the concentration of GlcNAc-Asn in the diseased lymphocytes can be estimated, as there exists linear relationship between peak area ratios and mass ratios of methylated GlcNAc-Asn and methylated melibiitol (Maury, 1979a). From the diseased lymphocytes were found to contain approximately 2.3 nmol of GlcNAc-Asn/100 μg of soluble protein.

**Discussion**

Cytoplasmic vacuolization of epithelial and mesenchymal cells is a constant feature of aspartylglycosaminuria. Some 20% of the peripheral lymphocytes in aspartylglycosaminuria are vacuolized, T-cells being significantly more vacuolized than B-cells (Aula et al., 1975). Morphologically these cytoplasmic vacuoles are similar to abnormal lysosomes seen in visceral organs, but chemical identification of the storage material has not been achieved. Failure to detect the glycopeptide GlcNAc-Asn by electrophoretic or histochemical methods in peripheral lymphocytes in aspartylglycosaminuria has led to the assumption that a different mechanism might be responsible for the vacuolization in these cells (Palo & Savolainen, 1973). However, in this paper we have been able to demonstrate that GlcNAc-Asn accumulates in peripheral lymphocytes in aspartylglycosaminuria. Thus, it seems obvious that the same storage phenomenon takes place in lymphocytes as in other tissues. This finding might have significance in other storage diseases as well. In addition to GlcNAc-Asn, higher-molecular-weight glycoasparagines accumulate in the urine (Pollitt & Pretty, 1974; Lundblad, Masson, Norden, Svensson, Ökerman & Palo, 1976; Sugakara, Funakoshi, Funakoshi, Aula & Yamashina, 1976) and liver (Maury, 1979b) of patients with aspartylglycosaminuria. However, their relative amount is low compared with GlcNAc-Asn. In liver, Man₂-GlcNAc₂-Asn comprises 7% of the content of the major storage substance, GlcNAc-Asn (Maury, 1979b). If such a glycoasparagine were present in the diseased lymphocytes in the same relative amount, it would not have been detected by the methods used in this study. Thus it is possible that other minor storage substances are stored in lymphocytes in aspartylglycosaminuria in addition to GlcNAc-Asn. However, monosaccharide analyses by gas chromatography (Bhatti et al., 1970) of the crude lymphocyte fractions revealed no major differences in mannose, galactose, N-acetylgalactosamine or sialic acid content between the normal lymphocytes and the lymphocytes from patients with aspartylglycosaminuria (P. Maury & J. Palo, unpublished work), which suggests that there are no other major glycoconjugate storage products present in the diseased lymphocytes.

Without isolating the pathological lysosomes of lymphocytes in aspartylglycosaminuria, no definite proof of their chemical composition can be provided. However, the results of this study suggest that the storage substance of the lymphocytes isolated from the patient with aspartylglycosaminuria is mainly GlcNAc-Asn, and thus it seems probable that the pathological lysosomes contain that compound. Although a low-molecular-weight compound, GlcNAc-Asn will in the absence of the degrading enzyme N-aspartyl-β-glucosaminidase probably induce cytoplasmic vacuolization in the same way as non-metabolizable disaccharides (Cohn & Ehrenreich, 1969) and some peptides (Ehrenreich & Cohn, 1969) do under experimental conditions.

**Acknowledgments**

The skilful technical assistance of Mrs Maire Ojala and Mrs Hilikka Rönkkö is gratefully acknowledged. This investigation was supported by the National Research Council for Medical Sciences and the Finska Läkaresällskapet.

**References**


