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METHODOLOGY

A useful routine for biochemical detection and diagnosis of mucopolysaccharidoses

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ABSTRACT

Mucopolysaccharidoses (MPS) constitute, owing to their biochemical, genetical and clinical characteristics, a large and heterogeneous subgroup among the lysosomal storage diseases (LSD). They are caused by deficiency of specific enzymes, which are responsible for glycosaminoglycan (GAG) breakdown during different steps of its degradation pathway. MPS are responsible for about 32% of inborn errors of metabolism (IEM) and 54% of LSD identified in our laboratory (Regional Laboratory of Inborn Errors of Metabolism (RLIEM), Medical Genetics Unit, Hospital de Clínicas in Porto Alegre), which is a reference center for LSD diagnosis in Brazil. Therefore, we decided to set up a specific laboratory routine for detection and differential diagnosis of MPS in patients with clinical features suggestive of this group of disorders.

INTRODUCTION

Mucopolysaccharidoses (MPS) are a large and heterogeneous subgroup among the lysosomal storage diseases (LSD). It results from a deficiency of one of 10 different lysosomal enzymes (Neufeld and Muenzer, 1995).

Differential diagnosis is important for a correct prognosis, definition of management strategies, genetic counseling, prenatal diagnosis, and prediction of future cases in the family.

Since MPS diseases account for about 32% of inborn errors of metabolism (IEM) and 54% of LSD identified in our laboratory, we decided to set up a specific laboratory routine for detection and differential diagnosis of MPS.

MATERIAL AND METHODS

Sample

The sample consisted of 177 patients referred to our laboratory from pediatric and genetic centers from all over Brazil due to clinical, radiological and/or laboratorial findings suggestive of MPS.

To obtain reference values for quantitative analysis of urinary glycosaminoglycans (GAGs), random urine samples were collected from 40 normal children aged 1 to 10 years. Ranges were established according to age. Reference values for enzyme activities were established from samples collected from normal subjects.

Collection and storage of blood and urine samples

A random urine specimen without preservative (at least 30 ml) and a sample of heparinized blood (10 ml) were obtained from all patients. Plasma and leukocytes (prepared according to Skoog and Beck, 1956) were isolated from blood samples. Samples collected in other centers were sent to our laboratory in Styrofoam containers with ice, arriving generally

up to 48 h after collection. When possible leukocytes were separated immediately after arrival. Otherwise, samples were separated the following day. In these cases, the blood samples were kept at 4ºC. Urine and plasma were kept frozen at -20ºC and leukocytes at -40ºC until analysis.

Routine employed

All patients referred to our laboratory were submitted to cetyltrimetylammonium bromide (CTAB) screening and toluidine blue spot tests for MPS.

When results were positive or doubtful, the investigation was continued through quantitative analysis of urinary GAGs by the alcian blue method proposed by Whiteman (1973). This method was chosen due to its simplicity and value in detection of increased urinary GAGs even in the Morquio syndrome. Results were expressed in mg/g creatinine.

When increased levels of urinary GAGs were found, qualitative analysis of urinary GAGs (after fractionation by the alcian blue method) was performed by thin-layer chromatography (TLC) (Humbel and Chamoles, 1972). Three abnormal patterns were identified by TLC, allowing preliminary classification of patients among subgroups. This step reduced the number of enzyme assays necessary for final diagnosis.

Final diagnosis was performed by detection of specific enzyme deficiency in plasma (α -iduronidase, iduronate-2-sulfatase, α -N-acetylglycosaminidase, β - glucuronidase) or leukocytes (N-acetyl-galactosamine 4-sulfatase, sulfamidase, galactose-6-sulfatase, β -galactosidase). In the present work, some of these assays were carried out at the Paediatric Research Unit, Guys Hospital, London, UK. Figure 1 summarizes the routine used for detection and diagnosis of MPS.

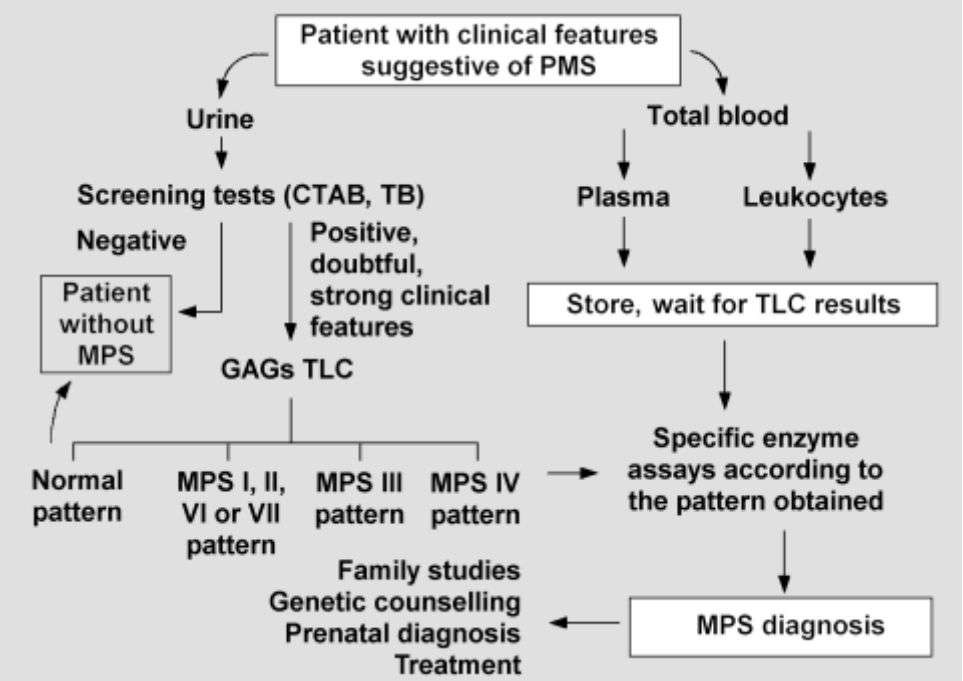

Figure 1 - Proposed laboratorial routine for the detection of mucopolysaccharidoses. MPS, Mucopolysaccharidoses; GAG, glycosaminoglycans; TLC, thin-layer chromatography; CTAB, cetyltrimetylammonium bromide; TB, toluidine blue.

RESULTS

Abnormal results in the screening tests were obtained in 69 of the 177 patients referred to our laboratory with clinical features suggestive of MPS. In 29 of these cases investigation was not completed. The remaining 40 patients were investigated until the enzyme defect was confirmed (Table I).

Table I - Results obtained in the 40 patients who went through the whole MPS routine.

Patient	CTAB	Toluidine blue	Quantitative[#] GAGs	TLC	Enzyme deficiency	Conclusion
01	+	+	796	ds + hs	IDUA	MPS I
02	-	+	632	ks	β-gal	MPS IVB
03	+	+	283	hs	SULF	MPS IIIA

04	±	+	63.2*	ds + hs	IDS	MPS II
05	±	+	780	hs	NAGLU	MPS IIIB
06	+	+	446	hs	SULF	MPS IIIA
07	+	+	976	ds + hs	IDUA	MPS I
08	+	+	805	ds + hs	4S	MPS VI
09	+	+	259	ks	GALNS	MPS IVA
10	+	+	144*	ds + hs	IDS	MPS II
11	-	+	44.5*	ds + hs	IDUA	MPS I
12	+	+	1213	ds + hs	IDS	MPS II
13	-	±	21.6*	ks	β-gal	MPS IVB
14	-	+	20.1*	ks	GALNS	MPS IVA
15	-	+	335	hs	NAGLU	MPS IIIB
16	-	±	2028	ds + hs	IDUA	MPS I
17	+	+	790	ds + hs	IDS	MPS II
18	-	+	450	ds + hs	IDS	MPS II
19	+	+	512	hs	NAGLU	MPS IIIB
20	-	-	334	hs	NAGLU	MPS IIIB
21	+	+	1128	ds	4S	MPS VI

				+ hs		
22	+	+	282	hs	NAGLU	MPS IIIB
23	+	+	510	ds + hs	IDUA	MPS I
24	-	+	588	ks	GALNS	MPS IVA
25	-	+	970	ds + hs	4S	MPS VI
26	+	+	657	ds + hs	IDS	MPS II
27	-	±	399	ks	GALNS	MPS IVA
28	+	+	1333	ds + hs	4S	MPS VI
29	+	+	593	ds + hs	IDS	MPS II
30	na	-	1306	ds + hs	IDUA	MPS I
31	-	+	453	hs	SULF	MPS IIIA
32	-	-	563	ks	GALNS	MPS IVA
33	+	±	1547	ds + hs	4S	MPS VI
34	+	+	1112	ds + hs	IDUA	MPS I
35	+	+	129*	ds + hs	IDS	MPS II
36	+	+	773	ds + hs	IDS	MPS II

37	±	+	7.8*	ds + hs	IDUA	MPS I/S
38	±	+	82*	hs	NAGLU	MPS IIIB
39	-	+	416	ks	GALNS	MPS IVA
40	±	±	251	ks	GALNS	MPS IVA

IDUA = α -Iduronidase GALNS = N-Acetylgalactosamine 6-sulfatase
IDS = Iduronate 2-sulfatase
SULF = Sulfamidase
NAGLU = α -N-Acetylglucosaminidase
*quantitative GAG analysis by the carbazol method
higher compared to age-related reference values
β -gal = β -Galactosidase
4S = N-Acetylgalactosamine 4-sulfatase
na = not analyzed
ds, hs, ks = dermatan, heparan and keratan sulfates
+, ±, - = positive, doubtful, negative

Of the 39 patients who had a CTAB test performed, 20 displayed positive results and 19 (most with MPS III or MPS IV) showed doubtful (5) or negative (14) results.

Toluidine blue test was performed in all 40 patients and was positive in 32, doubtful in 5 (4 with MPS IV) and negative in 3 (1 with MPS III and 1 with MPS IV).

Quantitative analysis of urinary GAGs showed increased levels in all 40 cases, with no false negative results. The lowest values were detected in MPS III and MPS IV patients (although 2 or 3 times greater than the normal age-related values), while the highest values were seen in MPS I and MPS II patients (values between 10 to 40 times higher than normal age-related values).

In the qualitative analysis of urinary GAGs, we detected the presence of both dermatan and heparan sulfates in 22 cases, which may occur in MPS I, MPS II or MPS VII. This pattern was also observed in MPS VI, because a second band of dermatan sulfate overlaps the heparan sulfate band. In these 22 patients we found 8 patients with α-iduronidase deficiency (MPS I), 9 with iduronate sulfatase deficiency (MPS II) and 5 with N-acetylgalactosamine 4-sulfatase deficiency (MPS VI).

In 9 patients the chromatographic pattern showed a marked excretion of heparan sulfate, which suggests Sanfilippo syndrome. In 3 patients we detected sulfamidase deficiency (MPS IIIA). The remaining 6 had N-acetyl-α-D-glucosaminidase deficiency (MPS IIIB).

In the remaining 9 patients the qualitative analysis detected increased amounts of keratan sulfate by TLC, suggesting Morquio syndrome. We found galactose-6-sulfatase deficiency in 7 patients (MPS IVA) and ß-galactosidase deficiency (MPS IVB) in 2 patients.

DISCUSSION

Efficiency of screening tests for MPS detection

According to Renuart (1966), the CTAB test shows positive results only in urine samples with high levels of GAGs. This fact was not observed in our patients, since CTAB results were not related to the results observed in the quantitative analysis. In the CTAB positive samples, urinary excretion of GAGs was approximately 7 times higher than age-related values. The same occurred in samples with negative or doubtful CTAB results. The CTAB test not only is difficult to interpret (Renuart, 1966), but also can be affected by ionic strength, pH and urinary concentration. For these reasons we expected false negative results, and indeed 14 false negative cases (35.9%) were observed. These patients would not have been detected if the CTAB test was the only one for MPS screening. If we consider the doubtful results as negative, we increase our percentage of false negative to 48.7%.

The toluidine blue spot test seemed to be more sensitive, with 20% false negatives (including negatives and doubtful results). This proportion decreased when we used a combination of the two tests (CTAB and toluidine blue). In this case we had 15% false negatives (negative and doubtful) in screening tests. These results show the importance of using the two tests together as recommended by

Caportori and Ventura (1966). If this is not possible, the toluidine blue test is preferred.

Efficiency of quantitative and qualitative analysis of GAGs

Quantitative analysis of urinary GAGs showed, in all 40 patients with confirmed MPS, a level at least two times higher than normal age-related individuals. We did not register any false negative with this method. The lowest values were seen in MPS III and MPS IV patients. A possible explanation is the presence of endoheparinases in many tissues which cleave the heparan sulfate into smaller fragments. The low molecular weight fragments produced by this "endocleavage" can be detected in urine, but sometimes can be lost in the current analysis directed at the largest polymers. In MPS IV, the residual compounds found in keratan sulfate are galactose instead of uronic acid, and the majority of the techniques used are directed at the precipitation of uronic acid. Moreover, GAGs excretion in MPS IV decreases significantly with age (Neufeld and Muenzer, 1995).

Because GAGs excretion decreases with age, care should be taken with neonates as they can display apparent high levels of GAGs (Pennok, 1971). The opposite is also true, and care should be taken when interpreting as normal the apparent low levels observed in adolescents or adults.

We were able to classify the patients through TLC into three major groups (see Results) focusing the enzyme assays to be performed and consequently saving time and costs.

Although the literature reports that patients with MPS I excrete more dermatan than heparan sulfate, patients with MPS II excrete more heparan than dermatan sulfate, and MPS VI patients excrete exclusively dermatan sulfate (Neufeld and Muenzer, 1995), we couldnt observe specific patterns for these diseases with the TLC method employed here. This does not seem to be a problem, because the ultimate diagnosis is based solely on the specific enzyme assay.

Specific enzyme diagnosis

The final MPS diagnosis was made only after specific enzyme assays, which were determined by clinical features and urinary analysis. The task of establishing almost all the assays used in the present work in the RLIEM showed that it is feasible, even in a third world country like Brazil, to set up a reference laboratory for MPS diagnosis.

Even if there is no efficient treatment so far, for most cases, diagnostic confirmation of MPS by specific enzyme deficiency detection is valuable for the prevention of such diseases, through genetic counseling, prenatal diagnosis and carrier detection. Nowadays, with the advent of DNA technology, the characterization of the disease causing mutation is of immeasurable importance for carrier detection, specially for X-linked diseases like Hunter syndrome, where enzyme assays are not reliable due to non-random X-inactivation. Furthermore, gene therapy relies on the identification of each individual mutation, and it may be a suitable approach in the near future for treatment of this group of disorders, where bone marrow transplantation has shown little, if any, improvement.

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RESUMO

As mucopolissacaridoses (MPS) constituem, devido às suas características bioquímicas, genéticas e clínicas, um grupo grande e heterogêneo dentro das doenças lisossômicas de depósito (LSD), e são causadas pela

deficiência de enzimas específicas que são responsáveis pela quebra de glicosaminoglicanos (GAGs) em passos diferentes da sua rota de degradação. Sendo as MPS responsáveis por aproximadamente 32% dos erros inatos do metabolismo (EIM) e 54% das LSD identificadas em nosso laboratório (Laboratório Regional dos Erros Inatos do Metabolismo (RLIEM), Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre), que é um centro de referência para o diagnóstico de LSD no Brasil, nós decidimos implantar uma rotina para a detecção e o diagnóstico diferencial de MPS em pacientes com características clínicas sugestivas deste grupo de doenças.

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