PARTIAL DEFICIENCY OF GLYCOPROTEIN NEURAMINIDASE
IN SOME PATIENTS WITH MORQUIO DISEASE TYPE A

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SUMMARY

A deficiency of glycoprotein neuraminidase (sialidase,
acylneuraminyl hydrolase, EC 3.2.1.18) activity was found in
fibroblasts from a patient with the clinical symptoms of Morquio
disease type A (mucopolysaccharidosis IV A). Residual neurami-
nidase activity was about 5 % of the mean normal activity. N-Acetyl-
galactosamine-6-sulfate (GalNAc-6-S) sulfatase activity was
reduced to less than 1 % of normal with a pH-optimum of 3.0 as
expected for the severe form of Morquio disease. In peripheral
leucocytes of the patient, however, neuraminidase activity but
not GalNAc-6-S sulfatase activity was in the normal range.
Mixing experiments excluded the presence of excessive amounts of
inhibitors of neuraminidase activity.

In the urine of the patient, the content of bound sialic acid
was with 0.357 $\mu\text{mol}/\text{mg}$ creatinine increased, but amounted for only
about 10 % of that of a sialidosis patient.

Somatic cell hybridization of the patient's fibroblasts with
those from a Morquio A patient did not result in complementation
of GalNAc-6-S sulfatase activity. Fusion with sialidosis fibro-
blasts, on the other hand, yielded a significant increase in
neuraminidase activity.

Reinvestigation of 10 different Morquio A cell lines revealed
two of them to have neuraminidase activities below the normal
range, whereas the activity of 6 cell lines was in the low normal
range.

CONCLUSION AND SPECULATION

If neuraminidase deficiency should be detected in fibroblasts,
additional enzyme activities should be determined to exclude
combined β -galactosidase/neuraminidase deficiency, mucopolidoses
II and III, and Morquio disease type A. The reason for deficiency
of neuraminidase in some patients with the latter disorder
remains speculative at present.

INTRODUCTION

In mammalian cells, there is evidence for the existence of at
least two types of neuraminidases. One of them is responsible for
the hydrolysis of gangliosides, the other one attacks glyco-
proteins and sialyloligosaccharides (3, 12).

A glycoprotein neuraminidase deficiency has been described in
several clinically distinct genetic disorders. In the sialidoses,
glycoprotein neuraminidase deficiency is the primary defect (for
review see 12). A further group of patients has been described
with coexistent deficiencies of neuraminidase and β -galactosidase
(8, 17). It is proposed that this combined β -galactosidase/neu-
raminidase deficiency is caused by a defective 32 000-dalton glyco-
protein which is normally required to protect these enzymes
against excessive intralysosomal degradation and to give these
enzymes their full hydrolytic activity (1). In I-cell disease
(mucopolidosis II) and in pseudo-Hurler polydystrophy (mucop-
lipidosis III) neuraminidase deficiency is one of several acid
hydrolase deficiencies caused by insufficient intracellular
retention of these enzymes due to deficient UDP-N-acetylglucos-
amine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase
activity (7, 20).

This is the first report describing a partial neuraminidase
deficiency coexistent with a deficiency of N-acetylgalactosamine-
6-sulfate (GalNAc-6-S) sulfatase. An isolated defect of this
sulfatase is characteristic of Morquio disease type A (mucopoly-
saccharidosis IV A) (13, 22). Morquio disease is a rare inherited
disorder of glycosaminoglycan catabolism characterized clinically
by disproportionate dwarfism, spondyloepiphyseal dysplasia, dental
anomalies, corneal clouding, but normal intelligence (for reviews
see 10, 14). In most cases the patients excrete elevated amounts
of keratan sulfate in their urine (18) and store this poly-
saccharide in cartilage (19) and liver (16).

CLINICAL DESCRIPTION

M.Q., a boy of Austrian origin, was born in 1977 from healthy,
unrelated parents by a Caesarian sectio. The birth weight was

3410 g, the body length 54 cm. The first clinical symptom, pectus
carinatum, was observed by the parents soon after birth. At the
age of 1 8/12 y he was diagnosed as suffering from Hunter disease.
However, on the occasion of a prenatal diagnosis during a further
pregnancy of his mother, the enzymatic analysis of his fibroblasts
revealed normal activity of iduronide 2-sulfate sulfatase, the
enzyme deficient in Hunter's disease, but deficiencies of glyco-
protein neuraminidase and GalNAc-6-S sulfatase. Clinical
examination at the age of 4 2/12 y revealed an alert child with
normal behaviour and normal intelligence. The stature was short
(body length 88.5 cm, below the 3rd percentile), the weight was
13.5 kg (below the 3rd percentile). Head circumference and
configuration were normal in accordance with age, but appeared
too large with respect to body length. The neck and trunk were
short, the sternum protruded, and there was a scoliosis of the
spine and a thoracolumbar kyphosis. The hands appeared clumsy.
Motility was moderately limited in the hip and the finger
phalanges.

The roentgenologic examination at the age of 1 8/12 y revealed
signs of dysostosis multiplex with general platyspondyly,
tonguelike protrusions of the vertebral bodies, dislocation of
the first lumbar vertebra, dysplasia of the acetabular roofs and
conical appearance of the proximal phalangeal ends. The sella
turcica appeared normal. Ophthalmologic investigations at the
age of 1 8/12 y showed moderate strabismus of the left eye,
miosis of both eyes, diffuse clouding of both corneas and
essentially normal fundi.

At no occasion typical signs of sialidosis like myoclonus,
cherry-red spots of the macula and neurological changes were
observed. A younger sister appears healthy.

A normal 46,XY karyotype was found by Giemsa staining,
Q-banding, constitutive heterochromatin, R-banding and NOR
techniques.

MATERIALS AND METHODS

Fibroblast cultures from patients and from normal individuals
were established from skin biopsies and cultured in modified
Eagle's minimum essential medium (MEM) as described (2, 25). From
the present patient, skin biopsies were taken at two occasions.
For cell hybridization 5×10^5 cells of both parental cell lines
were mixed one day before fusion and plated in a 25 cm² Falcon
plastic flask. The fusion procedure was as follows: after rinsing
the cells with Hanks' Balanced Salt Solution (BSS), 1.5 ml of 41 %
(w/w) polyethylene glycol (PEG) 4000 (Merck, Darmstadt) in MEM
with 10 % (w/w) DMSO were added. The solution was rocked for
90 sec, 1.5 ml 41 % PEG in MEM were added, rocked again for 90 sec
and then 12 ml Hanks' BSS were added. After 2 min the mixture was
removed and the cells were washed twice with Hanks' BSS. After
7 days of cultivation in MEM containing 10 % fetal calf serum
the cells were harvested by trypsinization. The cell pellet was
washed twice with 0.15 M NaCl, suspended in 0.2 ml water for the
determination of GalNAc-6-S sulfatase or in 0.8 ml of 50 mM sodium
acetate buffer, pH 4.2, for the determination of neuraminidase,
and homogenized by ultrasonication for 3 x 5 sec at 25 W
(Labsonic 1510, B. Braun, Melsungen).

Leucocytes from venous blood were collected as quoted earlier
(5). Leucocytes from 5 ml blood each were suspended in 0.5 ml
50 mM sodium acetate buffer, pH 4.2 (for neuraminidase assay),
and in 0.5 ml water (for the GalNAc-6-S sulfatase assay),
respectively, and homogenized by ultrasonication for 3 x 5 sec.

Glycoprotein neuraminidase was assayed with $\alpha 2 \rightarrow 3$ sialyl[1-³H]
lactitol (II 'NeuAc'-H-Lac) and with 4-methylumbelliferyl-N-
acetylneuraminic acid (4-MU-NeuAc) (23) as substrates. The prepara-
tion of II 'NeuAc'-H-Lac has been described (4). The specific
radioactivity was 99 Ci/mol. The standard incubation mixture for
the neuraminidase assay contained 0.55 μmol substrate, 40-140 μg
(fibroblasts) or 280-480 μg (leucocytes)/cell protein, 12 μmol
sodium acetate buffer, pH 4.2, and 0.06 μmol sodium azide in a
final volume of 140 μl . After incubation for 1 h (fibroblasts) or
2 h (leucocytes) at 37° C the reaction was stopped by the addition
of 460 μl 21 mM NaOH. The incubation mixture was transferred to a
2 ml column of Dowex 1x2, 200-400 mesh, equilibrated with 10 mM
Tris/HCl, pH 7.0. The column was washed with 0.5 ml of this
buffer, and the reaction product ([³H]lactitol) was eluted with
4 ml of the same buffer. Radioactivity was determined after
addition of 8 ml Instagel (Packard).

The determination of GalNAc-6-S sulfatase activity towards
6-sulfo-N-acetylgalactosamine-glucuronic acid-6-sulfo-N-acetyl-
[1-³H]galactosaminitol (5) has been described previously (11).

Other lysosomal enzymes (11) and protein (9) were quantitated
as described. Sialic acid content in fibroblasts was determined
by the method of Warren (24), and in the urine by that of
Skoza and Mohos (22).

RESULTS

Measurement of GalNAc-6-S sulfatase activity in fibroblast
homogenates from the patient M.Q. confirmed the diagnosis of
Morquio disease type A. The residual enzyme activity was lower
than 1 % of normal activity (table I) with a pH-optimum of 3.0 as
expected for the severe form of Morquio disease (6). The affinity
of the mutant enzyme to the trisaccharide substrate was not
altered: the K_m -value of 15 $\mu\text{mol}/\text{l}$ (data not shown) was the same
as that found for the normal and the residual GalNAc-6-S
sulfatase of Morquio A fibroblasts (6). The sulfatase activities
of the fibroblasts of the patient's parents and sister ranged
from 28 to 49 % of normal (table I), as expected for
heterozygous individuals.

Glycoprotein neuraminidase activity in fibroblast homogenates
from the patient was about 5 % of mean normal activity using

either II $^3\text{NeuAc-}^3\text{H-Lac}$ (table I) or 4-MU-NeuAc (table II) as substrates. This value was confirmed in two different laboratories using fibroblasts from two different skin biopsies. In fibroblasts from the parents and the sister, the neuraminidase activity was within the normal range (table I). The k_m -value of the residual neuraminidase with sialyllactitol as substrate (0.61 mmol/l) did not differ significantly from the k_m -value of normal neuraminidase (0.66 mmol/l; data not shown). The same was true for the thermal stability: upon preincubation at 50°C for various lengths of time, the neuraminidase of mutant and normal cells lost half of its activity after 8 and 10 minutes, respectively. Upon mixing of cell homogenates from the patient and a normal individual, neuraminidase activity corresponding to the calculated average was found (table III). This argues against the presence of a reversibly acting inhibitor or the absence of an activator for the neuraminidase in the patient's cells.

In contrast to the findings in fibroblasts, neuraminidase activity of the patient's peripheral leucocytes was within the normal range, whereas the activity of GalNAc-6-S sulfatase was reduced to 3 % of mean normal activity (table IV).

To elucidate the genetic background of this coexistent deficiencies of GalNAc-6-S sulfatase and neuraminidase in the fibroblasts of the patient, complementation studies were performed with fibroblasts from patients with isolated deficiencies of the respective enzymes. The results are shown in table V. Fusion of fibroblasts of the propositus with Morquio A fibroblasts did not

produce any increase in GalNAc-6-S sulfatase activity. Fusion of the patients fibroblasts with sialidosis fibroblasts, however, resulted in a significant elevation of neuraminidase activity.

In fibroblasts of the patient, the content of bound, water extractable sialic acid (2.86 nmol/mg protein) was not increased (normal controls 6.04 and 6.12 nmol/mg protein, respectively). In the urine of the patient, the content of bound sialic acid, 0.357 $\mu\text{mol/mg creatinine}$, was somewhat increased (normal controls: 0.074 and 0.084 $\mu\text{mol/mg}$, respectively), but amounted for only about 10 % of the sialic acid content in the urine of a sialidosis patient (3.51 $\mu\text{mol/mg creatinine}$).

The results on patient M.Q. prompted us to reinvestigate other Morquio A cell lines. Neuraminidase activity was measured in 10 different Morquio A cell strains. As shown in table I, two cell lines were within the 1s-, six cell lines within the 2s-, and two cell lines below the 2s-ranges of mean normal neuraminidase activity.

DISCUSSION

This report describes a patient with coexistent deficiencies of GalNAc-6-S sulfatase and of glycoprotein neuraminidase in fibroblast homogenates. The GalNAc-6-S sulfatase deficiency appears to have caused the development of the clinical symptoms typical of Morquio disease. The neuraminidase deficiency, on the other hand, has been without apparent clinical consequences up to now, perhaps because of the relatively high residual activity as compared to that found in sialidosis patients. Neuraminidase deficiency, however, could not be observed in peripheral leucocytes (26). It seems therefore possible that the primary metabolic lesion is a genetic defect of GalNAc-6-S sulfatase, and that the neuraminidase deficiency is a secondary event. Support for this assumption comes from the finding of a gene dosage effect for the sulfatase, but not the sialidase, as only the former activity was reduced in obligate heterozygotes.

Investigation of further Morquio A cell lines revealed the reduced neuraminidase activity not being unique for this Morquio patient. In two Morquio A patients, both with the severe form of the disease, neuraminidase activity was almost as low as in patient M.Q., whereas the neuraminidase activity of six patients was in the low normal range. Interestingly, both patients with the highest neuraminidase activity exhibit the mild form of Morquio disease type A.

What is the cause of the reduced sialidase activity? The fusion experiments suggest that two different gene mutations are involved in the neuraminidase deficiencies of our patient and a patient with sialidosis (mucopolipidosis I). In contrast, GalNAc-6-S sulfatase deficiency was not corrected after hybridization with Morquio A fibroblasts, indicating that the mutation should be within this particular gene. Though the localization of the genes coding for neuraminidase and GalNAc-6-S sulfatase is as yet unknown, a linkage either between the structural genes or between the structural gene for the sulfatase and another gene involved in the expression of the neuraminidase seems possible.

Another likely explanation for the neuraminidase deficiency could be inhibition by storage material. It had been shown by Mian et al. (15) that *C. perfringens* neuraminidase can be inhibited by sulfated glycopeptides. However, Morquio fibroblasts store only minute amounts of chondroitin sulfate and do not produce keratan sulfate (14). Mixing experiments gave no indication of the presence of a reversibly acting inhibitor in the patient's fibroblasts (table III). On the other hand, fibroblasts from other mucopolysaccharidosis patients with excessive storage of sulfated glycosaminoglycans exhibited normal or only marginally reduced neuraminidase activity (table I). Thus, the reason for the neuraminidase deficiency in fibroblasts of some patients with Morquio A disease remains speculative at present.

Our observations are of obvious relevance for the biochemical diagnosis of the neuraminidase deficiency diseases, particularly, as cultured fibroblasts are the preferred material for testing. Thus, if neuraminidase deficiency should be detected, it would be necessary to determine additional enzyme activities in order to decide if one is dealing with a sialidosis with an "isolated" neuraminidase deficiency, a combined β -galactosidase/neuraminidase defect, a mucopolipidosis II or III, or, as in the present case, a mucopolysaccharidosis, type Morquio A. This is of particular importance with respect to the prognosis affixed to a certain diagnosis, as the clinical courses of the different diseases vary greatly.

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Abstract No. 38) that more than 80 % of the neuraminidase activity of granulocytes was not produced by the enzyme which is deficient in sialidosis. Measurement of total leucocyte neuraminidase is therefore unreliable to detect the deficiency of a distinct neuraminidase isoenzyme.

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Table I

Activities of neuraminidase, GalNAc-6-S sulfatase and β -galactosidase in fibroblast homogenates

Genotype	Neuraminidase ¹	GalNAc-6-S sulfatase ²	β -Galactosidase ³
Normal	18.4 [±] 6.8 ⁴ (n=7)	31 [±] 11 ⁴ (n=15)	45 [±] 14 ⁴ (n=15)
Sialidosis, severe form (mucopolipidosis I)	0.13	12.3	54
Sialidosis, β -Gal ⁻ /Neur ⁻ type	0.67	n.d.	3.9
Mucopolysaccharidosis III A, Sanfilippo A	18.2	n.d.	111
Mucopolysaccharidosis III D; Sanfilippo D	14.2	n.d.	67
Mucopolysaccharidosis VI, Maroteaux-Lamy	7.5	n.d.	24
Mucopolysaccharidosis IV A, Morquio A			
Patient 1	8.5	0.10	49
2	9.8	0.15	45
3	9.2	0.55	49
4	7.9	0.35	65
5	26.1	0.63	43
6	15.4	0.68	42
7	6.4	0.35	59
8	7.5	0.06	41
9	1.8	0.40	53
10	2.6	0.21	56
Family Q.:			
Patient M.Q.	1.2	0.06	23
Mother	28.2	13.2	36
Father	13.1	8.6	36
Sister	23.1	15.1	34

¹ pmole/min/mg of protein,

² nmole/hr/mg of protein,

³ mU/mg of protein,

⁴ mean $\pm$ S.D.;

n.d. = not determined.

Table II

Neuraminidase activity in fibroblast homogenates using 4-MU-NeuAc as substrate

Cell line	Neuraminidase activity
Normal controls: Cell line 1	5.85
Cell line 2	3.97
Family Q: Patient M.Q.	0.32
Mother	4.99
Father	2.48
Sister	4.10

Results are expressed as nmol/hr/mg of protein

Table III

Mixing experiment with fibroblast homogenates from patient M.Q. and normal controls

Homogenate	Neuraminidase found	activity ⁺ calculated
Normal control (N)	22.1	-
Patient (M.Q.)	1.3	-
Mixture N + M.Q.	12.2	11.7

⁺ Results are expressed as pmole/min/mg of protein (substrate: II³NeuAc-³H-Lac).

Table IV

Activities of neuraminidase and GalNAc-6-S sulfatase in peripheral leucocytes

Enzyme source	Neuraminidase ¹	GalNAc-6-S sulfatase ²
Family Q.		
Patient	0.273	0.32
Mother	0.298	4.15
Father	0.297	4.57
Grandmother	0.307	10.30
Grandfather	0.358	8.24
Normal individuals (n = 7)	0.373 $\pm$ 0.06	9.8 $\pm$ 2.5

¹ pmole/min/mg of protein (substrate II³NeuAc-³H-Lac)

² nmole/hr/mg of protein

Table V

Cell hybridization and co-cultivation of fibroblasts from patient M.Q. with GalNAc-6-S sulfatase deficient (MPS IV A) and neuraminidase deficient (ML I) fibroblasts

Combination of cell strains		GalNAc-6-S sulfatase activity ¹		Neuraminidase activity ²	
		Hybridization ³	Co-cultivation	Hybridization ³	Co-cultivation
M.Q.	x MPS IV A	0.09	0.09	-	-
MPS IV A	x MPS IV A	0.10	0.11	-	-
M.Q.	x M.Q.	0.06	0.05	1.3	0.9
M.Q.	x ML I	-	-	3.5	1.1
ML I	x ML I	-	-	0.1	0.1

¹nmole/hr/mg of protein

²pmole/min/mg of protein (substrate: II ³NeuAc-³H-Lac)

³The portion of di- and multikaryons was between 40 and 45 percent of total cell number.

The values represent the mean of two parallel fusion experiments.