Immunological studies on lysosomal sphingomyelinase:
Identification of a 28 000-Da component deficient in urine from patients with Niemann-Pick disease types A and B

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The immunoblotting technique was used to identify sphingomyelinase protein in samples of tissue and urine after subjection to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. In a sphingomyelinase preparation purified from control urine a prominent band was seen with an M_r of 28 000 Da. Glycoprotein fractions from urine and placenta, a membrane extract from spleen, and a partially purified sphingomyelinase preparation from placenta contained the 28 000-Da band plus additional, higher-Mr bands. The 28 000-Da band was detectable in urine from a patient with Niemann-Pick disease type C, but not in urine from patients with Niemann-Pick disease types A and B. It is concluded that sphingomyelinase is composed of at least one polypeptide with an M_r of 28 000 Da and that this polypeptide is deficient in the urine of patients with Niemann-Pick disease types A and B.

Acid sphingomyelinase is a lysosomal enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Although detergents must be employed in order to obtain maximal extraction of the enzyme from tissues, a considerable proportion of the activity (40% in brain, 55% in liver, and 50% in fibroblasts) can be obtained in a soluble form by extracting tissues or cells with aqueous buffers in the absence of detergents (De Rooij et al., 1975). A soluble form of sphingomyelinase is also present in urine (Seidel et al., 1978; Schram et al., 1984) and in the medium of fibroblasts cultured in the presence of a weak base (Weitz et al., 1984).
The enzyme activity is deficient in the lysosomal storage disorders known as Niemann-Pick disease types A and B (for a review, see Harzer et al., 1978; Reich et al., 1979; Brady, 1983). Type A is the severe phenotype with massive hepatosplenomegaly and storage of sphingomyelin, neurological involvement, and an almost total deficiency of sphingomyelinase. Type B is a milder form without neurological involvement; there is moderate storage of sphingomyelin and the residual sphingomyelinase activity is higher than in type-A patients. In the neurological disorder known as Niemann-Pick disease type C, only a small amount of sphingomyelin accumulates and the activity of sphingomyelinase is normal or only slightly decreased (see Harzer et al., 1978).

Considerable confusion exists with regard to the minimum molecular mass of purified sphingomyelinase; the values reported in the literature range from 28 300 to 89 100 Da (Jones et al., 1981; Pentchev et al., 1977; Sakuragawa, 1982; Yamanaka and Suzuki, 1982).

We have recently obtained an antiserum against sphingomyelinase using a partially purified preparation of urinary sphingomyelinase as antigen (Weitz et al., 1984). The antiserum is able to precipitate about 70% of the sphingomyelinase activity present in urine or in an aqueous extract of placenta (Weitz et al., 1984). In this paper we describe a study in which the immunoblotting technique was used to identify proteins in samples of tissue and urine after subjection to polyacrylamide-gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS). The results show that at least one component in sphingomyelinase has an Mr of about 28 000 and that this component is not detectable in urine from patients with Niemann-Pick disease types A and B.

Materials and Methods

Protein determination

Protein was determined according to the method of Lowry et al. (1951).

Polyacrylamide-gel electrophoresis and immunoblotting

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed on 10% slab gels according to Laemmli (1970). The protein samples were denatured by boiling in the presence of SDS and mercaptoethanol. After electrophoresis the protein bands were transferred to nitrocellulose filters (Schleicher and Schüll, Dassel, Federal Republic of Germany; filter BA 85, pore size 0.45 μM) using the electrophoretic procedure (blotting) of Towbin et al. (1979). The filters were washed three times with Tris-buffered saline (pH 7.0) containing 0.05% Tween 20 and then incubated at 37°C for 1 h with a 1:500 dilution of anti-(sphingomyelinase) antiserum in Tris-buffered saline/0.05% Tween 20. After washing the filters five times, they were incubated for 1 h with goat anti-(rabbit IgG) antiserum conjugated with horseradish peroxidase (Bio-Rad) at room temperature. After washing the filters five times they were stained for peroxidase using the procedure described by Bio-Rad.
Fig. 1. Reaction of anti-(urinary sphingomyelinase) antibodies with proteins in a urinary glycoprotein fraction, in partially or highly purified urinary sphingomyelinase, and in partially purified placental sphingomyelinase. Samples were analysed by PAGE in the presence of 1.0% SDS followed by immunoblotting as described in Materials and Methods. A and B were two separate runs. A1, urinary glycoproteins (10 μg of protein); A2, the partially purified urinary sphingomyelinase preparation used as antigen (10 μg of protein); B1, urinary sphingomyelinase purified 5.10⁵-fold (1 μg of protein); B2, partially purified placental sphingomyelinase from Sigma (15 μg of protein).

Antibodies and enzyme preparations

These were obtained as described previously (Weitz et al., 1984).

Diagnosis of Niemann-Pick disease

The disease was diagnosed by J.A. Barranger and N. Barton and was classified according to the criteria summarized by Brady (1983). Urine was collected, concentrated 50-fold by Amicon filtration, and stored at 4°C.

Results and Discussion

The partially purified urinary sphingomyelinase preparation with which the rabbits were immunized contained several protein bands when subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. After immunoblotting with the anti-(urinary sphingomyelinase) antiserum, a prominent band at an Mr of about 28 000 was seen, together with a minor band at 32 000 (Fig. 1, lane A2). In a urinary sphingomyelinase preparation purified 5000-fold (G. Weitz, unpublished results) a prominent band at 28 000 Da was seen and a minor band at an Mr of about 73 000 Da (Fig. 1, lane B1). Glycoprotein fractions from urine (Fig. 1, lane A1) or placenta (Fig. 1, lane B2) contained the 28 000-Da component plus additional bands.
When commercially available placental sphingomyelinase was analysed by immunoblotting with anti-(urinary sphingomyelinase) antibodies, the most prominent band seen had an $M_r$ of about 28 000 (Fig. 2, lane 1). A band with the same $M_r$ was seen in a glycoprotein fraction from splenic membranes (Fig. 2, lane 2).

Fig. 2 also shows the results of an immunoblotting experiment with urinary glycoprotein fractions from a control person and patients with different types of Niemann-Pick disease. A prominent band at 28 000 Da was observed in control material and in material from the patient with Niemann-Pick disease type C (Fig. 2, lanes 3,4). It should be remembered that sphingomyelinase activity is not deficient in urine from patients with Niemann-Pick disease type C (Seidel et al., 1978;
Weitz et al., 1984). The 28 000-Da band was not detectable in a glycoprotein fraction from urine of patients with Niemann-Pick disease types B and A (Fig. 2, lanes 5,6). The amount of protein applied to the gel in lane 5 was about the same as that applied in lanes 3 and 4. However, the amount applied in lane 6 was considerably less, due to the limited volume of urine available. The absence of a detectable band at 28 000 in lanes 5 and 6 is in agreement with the fact that there is no detectable sphingomyelinase activity in urine of patients with Niemann-Pick disease types A and B (Weitz et al., 1984). When preimmune serum was used instead of the antiserum, no bands were seen.

It should be noted that besides the 28 000-Da band additional protein bands are deficient in the urine from patients with Niemann-Pick disease types A and B. These bands might represent components of sphingomyelinase. However, the presence of the 28 000-Da band in

![Image]

**Fig. 3.** Effect of the detergent Nonidet P-40 on the binding of native sphingomyelinase to anti-(urinary sphingomyelinase) antibodies. IgG molecules in the antiserum or control serum were immobilized by binding to Protein A-Sepharose 4B. The immobilized IgGs were incubated with urinary glycoproteins (100 μg of protein) in the absence or presence of Nonidet P-40 as described in the text. (Nonidet P-40, like Triton X-100, is an octyl phenol ethylene oxide condensate.) The immune complexes were eluted with glycine-HCl and subjected to SDS-PAGE. The proteins were transferred electrophoretically to nitrocellulose filters and analysed by the immunoblotting technique.
purified urinary sphingomyelinase (Fig. 1B) leads us to the hypothesis that at least the 28 000-Da polypeptide represents (part of) sphingomyelinase.

We have shown that detergents inhibit the formation of immune complexes between native sphingomyelinase and the antibodies present in the anti-(urinary sphingomyelinase) antiserum (to be published elsewhere). For instance, if a commercially available placental sphingomyelinase preparation is incubated with antibodies immobilized by adsorption to Protein A-Sepharose 4B, little immunoprecipitation of the sphingomyelinase activity occurs. In the experiment of Fig. 3 anti-(urinary sphingomyelinase) antibodies or control IgG were incubated with a glycoprotein fraction from urine in the absence or presence of the non-ionic detergent Nonidet P-40. Subsequently immune complexes were eluted with glycine-HCl, subjected to SDS-PAGE, and analysed by the immunoblotting procedure. Apart from the IgG bands, a strong band with an Mr of about 28 000 Da was seen in the lane corresponding to the incubation with anti-(urinary sphingomyelinase) antibodies in the absence of Nonidet P-40 (Fig. 2, lane 4).

In the presence of Nonidet P-40 the 28 000-Da band was less prominent (Fig. 3, lane 2). This observation is consistent with the fact that Nonidet P-40 at the concentration used in the experiment of Fig. 3 inhibits the formation of immune complexes between the antibodies and native sphingomyelinase by about 75% (to be published elsewhere). Using control serum (lanes 1 and 3 of Fig. 3), some 28 000-Da material is visualized. This is most probably due to nonspecific adsorption of sphingomyelinase to Sepharose beads. The bands of high molecular mass visualized in lanes 2 and 4 might also represent sphingomyelinase; this possibility cannot be excluded, particularly since bands of high molecular mass are also observed in the purified urinary enzyme (Fig. 1, B1) and the placental sphingomyelinase (Fig. 1, B2).

Conclusion

The results of the immunological studies described in this paper provide evidence that a polypeptide (or polypeptides) with an Mr of about 28 000 Da contributes to the structure of sphingomyelinase. The absence of detectable sphingomyelinase activity in the urine of patients with Niemann-Pick disease types A and B is associated with a marked deficiency or complete absence of the 28 000-Da polypeptide(s).

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