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Defective Sialic Acid Egress from Isolated Fibroblast Lysosomes of Patients with Salla Disease

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Normal fibroblasts exposed to *N*-acetylmannosamine yielded lysosome-rich granular fractions loaded with free (unbound) sialic acid, whose velocity of egress increased with increasing initial loading. Fibroblast granular fractions of patients with Salla disease exhibited negligible egress of sialic acid, whether endogenous or derived from *N*-acetylmannosamine exposure. Salla disease represents the first disorder demonstrated to be caused by defective transport of a monosaccharide out of cellular lysosomes.

LYSOSOMAL STORAGE DISORDERS result from several different biochemical abnormalities. Lysosomal enzymes may be defective, enzyme activator proteins may be deficient, or newly synthesized enzymes may lack recognition markers essential for targeting them to the lysosome (*1*). Recently, another group of lysosomal storage disorders was described, characterized by defective transport of small molecules across the lysosomal membrane. The first disorder of this class was nephropathic cystinosis, a fatal disease in which the disulfide cystine fails to traverse the lysosomal membrane at a normal rate and, consequently, accumulates within lysosomes (*2*). Later, a disorder of vitamin B_{12} storage was ascribed to defective lysosomal transport of free vitamin B_{12} out of lysosomes (*3*). We now report that impaired lysosomal transport of a carbohydrate, *N*-acetylneuraminic acid (sialic acid; NANA), results in Salla disease, a disorder characterized by free sialic acid storage within lysosomes.

Patients with Salla disease, which is inherited in an autosomal recessive fashion, have moderate to severe psychomotor retardation, spasticity, and ataxia with an early onset and slow progression (*4*). The majority of the patients in whom the disease has been detected are in Finland. They excrete large amounts of free (unbound) NANA and store 10 to 30 times the normal amounts of this compound within several tissues and cultured fibroblasts (*5*). Electron microscopy of tissues reveals characteristic vacuolated cytoplasmic inclusions, that are apparently lysosomes swollen with high concentrations of free NANA (*4*). The amounts and distributions of NANA-containing membrane glycoproteins and gangliosides are normal in cells from patients with Salla disease ("Salla disease cells"), and a normal amount of acid neuraminidase activity cleaves NANA from glycoconjugates in Salla disease lysosomes (*5*). The fate of free NANA produced has not been rigorously determined, but *N*-acetylneuraminate pyruvate-lyase (E.C. 4.1.3.3.), a cytoplasmic enzyme, does cleave the neuraminic acid ring structure as the first step in NANA catabolism. In Salla disease fibroblasts this enzyme has normal activity (*5*), which led us to investigate whether free NANA transport out of lysosomes and into the cytoplasm is the defective process in the disorder.

Previous studies demonstrated that [^{3}H]NANA bound to low-density lipoprotein (LDL) was taken up normally by the lysosomes of Salla disease fibroblasts but was cleared at a much slower rate than in normal cells (*6*). In these studies the specific radioactivity of the labeled free NANA was diluted by large amounts of nonradioactive NANA present in the Salla cells but not in the normal cells. This made direct kinetic measurements of NANA egress in normal and Salla disease cells difficult to compare. In the present study we directly measured NANA egress by using lysosome-rich granular fractions and assays of nonradioactive NANA. Normal granular fractions were loaded with NANA to levels observed in Salla disease by exposure of intact fibroblasts to high concentrations of the NANA precursor, *N*-acetylmannosamine (ManNAc) (*7*).

Normal or Salla disease fibroblasts, grown to confluence in Eagle's minimum essential medium containing 10% fetal calf serum, 2 m*M* glutamine, streptomycin (100 μg/ml), and penicillin (100 U/ml), were exposed to 0 to 100 m*M* nonradioactive ManNAc for 3 to 14 days. The cells were harvested by trypsinization, washed once with cold phosphate-buffered saline, *p*H 7.4, and once in cold 0.25*M* sucrose, and disrupted by nitrogen cavitation [30 psi (2.0 atm), 10 minutes]. After centrifugation at 1200*g* for 5 minutes, the supernatant was centrifuged for 10 minutes at 17,000*g* to prepare a granular fraction enriched approximately threefold with respect to hexosaminidase

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activity (*2*). Normal fibroblasts exposed to ManNAc yielded granular fractions with up to 88 pmol of NANA per unit of hexosaminidase (no exposure: 2 pmol per unit of hexosaminidase); Salla disease cells exposed to 30 m*M* ManNAc produced granular fractions that had acquired up to 496 pmol of NANA per unit of hexosaminidase more than the endogenous level of free NANA in the granular fractions (see Table 1). Since lysosomes accumulate NANA in Salla disease (*5, 6*), this suggested that the lysosomes within the granular fractions were being loaded by the ManNAc exposure. Substantial amounts of free NANA were found in other compartments of the normal cells but were removed during isolation of the lysosome-rich fractions.

The NANA-loaded granular fractions were suspended at 37°C in a solution of 0.4*M* sucrose in 16 m*M* Hepes (*p*H 7.0) that also contained 1% bovine serum albumin, and 1 m*M* disodium EDTA; aliquots were removed at various times and centrifuged for 10 minutes at 17,000*g*. We analyzed pellets and supernatants for unbound sialic acid by high-performance liquid chromatography (HPLC) (*8*), with commercially prepared NANA (Sigma, St. Louis, Missouri) as standard and 2 to 10 nmol per sample of ManNAc or *N*-acetylglucosamine as internal standards. Samples were purified on 0.3 × 1 cm Dowex 2X8 (100 to 200 mesh) ion-exchange columns (acetate form) before HPLC analysis (*4*). Two hundred picomoles of NANA per sample was reliably measured by this method. Lysosomal rupture was assayed from the measurements of supernatant, pellet, and total hexosaminidase activity in each aliquot in the presence of 0.1% Triton X-100 (*2*). One unit of hexosaminidase hydrolyzed 1 nmol of substrate per minute at 37°C.

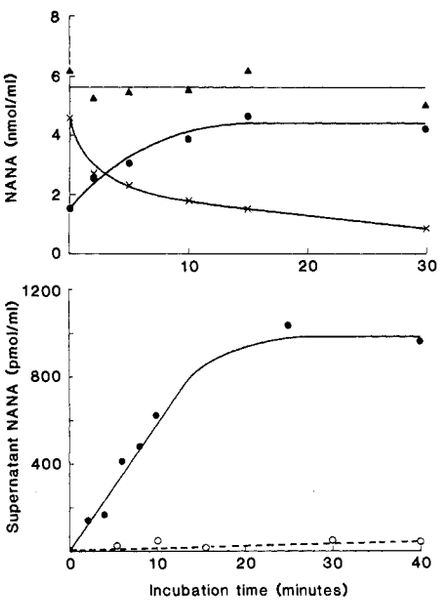

Fig. 1 (top). Recovery of free NANA from granular fraction supernatant and pellet after incubation for 0 to 30 minutes. Normal fibroblasts were exposed to 50 m*M* *N*-acetylmannosamine for 6 days, and the granular fractions were incubated at 37°C. Portions were removed at various times and centrifuged at 17,000*g* for 10 minutes; the NANA in each pellet (x) and supernatant (●) was determined, as well as total NANA recovered (▲). NANA loading in the granular fraction at zero time was 47.1 pmol per unit of hexosaminidase. Fig. 2 (bottom). Time course of appearance of free NANA outside normal loaded granular fractions (●) and Salla disease granular fractions (○) not loaded by exposure to ManNAc. Normal fibroblasts were exposed to 40 m*M* ManNAc for 10 days. Cells were harvested, and a granular fraction was incubated at 37°C. Portions (1 ml) were removed at various times and centrifuged at 17,000*g* for 10 minutes; unbound NANA was measured in the supernatant and pellet. Supernatant NANA was corrected for lysosomal rupture as gauged by soluble hexosaminidase. Normal and mutant granular fraction loadings were 57 and 66 pmol of NANA per unit of hexosaminidase at zero time of incubation.

When normal granular fractions were loaded by exposure of whole cells to 50 m*M* ManNAc, the free NANA produced rapidly left the granular fraction pellet and appeared within the supernatant (Fig. 1). The total amount of NANA recovered remained constant as the amount inside the granular fraction decreased and the amount outside increased. The positive identification of NANA in the supernatant verified that NANA itself was transported, rather than a derivative formed before or during passage through the lysosomal membrane. Supernatant NANA corrected for rupture of lysosomes (Fig. 2) increased linearly with time of incubation up to approximately 15 minutes. Negligible egress of NANA was observed from granular fractions of patients with Salla disease (Fig. 2). In subsequent experiments, free NANA egress was calculated on the basis of the appearance of free NANA outside granular fractions after 12 minutes of incubation, with correction for lysosomal rupture.

Velocities of free NANA egress from normal and Salla disease lysosome-rich granular fractions were determined at several levels of initial NANA loading (Fig. 3). After exposure of normal fibroblasts to as much as 100 m*M* ManNAc, the granular fractions contained between 2 and 88 pmol of free NANA per unit of hexosaminidase at zero time. The velocity of NANA egress increased approximately linearly throughout this range of loading with no apparent plateau of velocity at high loading. Four strains of Salla disease fibroblasts, not exposed to ManNAc, yielded granular fractions containing endogenous free NANA levels of between 18 and 90 pmol per unit of hexosaminidase. At every level of endogenous loading, the velocity of egress of free NANA was negligible (Fig. 3).

Mass spectrometric analysis of the material stored in Salla disease lysosomes (*5*) and the material derived from loading of normal fibroblasts with ManNAc (*7*) has been performed. In both cases, the fragmentation pattern was identical to that of commercially prepared NANA rather than to that of some related compound such as an *O*-acetylated derivative. In addition, HPLC methods such as those used in our studies readily distinguish NANA from its *O*-acetylated derivatives (*8*). These findings offered assurance that we were measuring the egress of free NANA out of both normal and mutant granular fractions, and rendered extremely unlikely the possibility that in Salla disease some *O*-acetylated derivative of NANA accumulated because of failure of its enzymatic conversion to free NANA.

Free, endogenous NANA in the mutant granular fractions might have been stored in a lysosomal compartment not normally displaying egress, while the free NANA derived from ManNAc entered a different granular fraction compartment characterized by rapid NANA egress. We examined this possibility by determining the fate of ManNAc-derived NANA in Salla disease granular fractions. Mutant fibroblasts were exposed to 30 m*M* ManNAc for 5 days, increasing the free NANA content in the granular fractions by 8- to 22-fold over

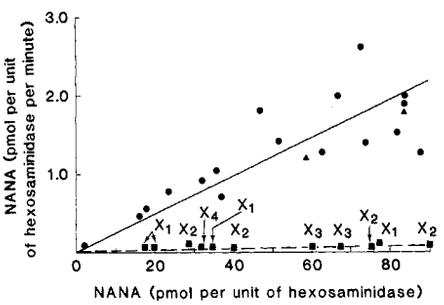

Fig. 3. Velocity of egress of free NANA from granular fractions isolated from normal and Salla disease fibroblasts. The abscissa shows the loading of free NANA achieved. Two normal fibroblast strains (GM 3349 and 494) were exposed to ManNAc (0 to 100 m*M*) for 3 to 10 days, and granular fractions were prepared (●, ▲). Granular fractions were also prepared from four strains of Salla disease fibroblasts (■, x_1 to x_4) not exposed to ManNAc. Variations in endogenous sialic acid content of the same mutant cell strain may reflect differences in phase of cell growth or of granular fraction recovery. The ordinate depicts initial velocity expressed as picomoles of free NANA leaving the lysosome and appearing in the supernatant over the first 12 minutes per unit of initial hexosaminidase. The values were corrected for lysosomal rupture as judged by hexosaminidase release.

Table 1. Granular fraction loading and egress velocities of endogenous and *N*-acetylmannosamine–derived free NANA in Salla disease fibroblasts. Confluent Salla disease fibroblasts were exposed to 0 or 30 m*M* *N*-acetylmannosamine (ManNAc) for 5 days. Cells were harvested and a lysosome-rich granular fraction prepared. Free NANA egress was determined as described in the text, after measurement of NANA loading at 0 time of incubation. Mutant strains include several depicted in Fig. 3.

Salla disease fibroblast strain	NANA loading (pmol hexosaminidase unit^{-1})			Velocity of egress (pmol NANA hexosaminidase unit^{-1} min^{-1})		
	Endogenous	After ManNAc exposure	Attributable to ManNAc	Endogenous	After ManNAc exposure	Attributable to ManNAc
x_2	28	276	248	0.1	0.1	0.0
x_6	27	380	353	0.0	0.3	0.3
x_1	19	422	403	0.0	0.6	0.6
x_5	69	535	466	0.2	0.7	0.5
x_3	60	556	496	0.1	0.2	0.1
Normal fibroblast	2	88	86	0.1	2.0	1.9

endogenous levels (Table 1). The amount of free NANA in the granular fraction directly attributable to ManNAc exposure varied from 248 to 496 pmol per unit of hexosaminidase, or three to six times the maximum NANA levels achieved in normal granular fractions by any regimen of ManNAc exposure (Fig. 3). This enormous concentration of free NANA in Salla disease granular fractions indicated that the target compartment (the granular fraction compartment in which NANA accumulated in the mutant cells and from which NANA exited in normal cells) was being loaded during ManNAc exposure.

Furthermore, the egress of ManNAc-derived NANA from Salla disease granular fractions was minimal, averaging 0.3 pmol per unit of hexosaminidase per minute at a mean loading of 393 pmol per unit of hexosaminidase compared with an egress of 1.9 pmol per unit of hexosaminidase per minute in normals loaded to only 86 pmol per unit of hexosaminidase (Table 1). ManNAc-derived NANA was apparently handled in a manner analogous to endogenous free NANA, and the exposure to ManNAc per se had not weakened or disrupted lysosomal membranes permitting leakage of free NANA out of the mutant or normal granular fraction vesicles.

We examined the possibility that Salla disease represents a generalized defect in lysosomal membrane transport by measuring the velocity of cystine egress from normal and Salla disease granular fractions. Fibroblasts were exposed to 0.5 m*M* cystine dimethyl ester for 30 minutes, and cystine-loaded granular fractions were prepared (*2*). The zero-time cystine loading of the Salla disease granular fraction was 0.37 nmol per unit of hexosaminidase, with an egress velocity of 8.4 pmol half-cystine per unit of hexosaminidase per minute; normal control loading was 0.27 nmol per unit of hexosaminidase with an egress velocity of 6.8 pmol half-cystine per unit of hexosaminidase per minute. This normalcy of cystine egress in Salla disease indicates that the impairment of free NANA transport was a specific rather than a generalized lysosomal membrane defect.

The velocity of NANA egress was greater than zero in highly loaded Salla disease granular fractions (Table 1). This may have occurred because corrections for lysosomal rupture involved small differences between large numbers, a complication not encountered at lower levels of NANA loading (Fig. 3). Alternatively, several or all of the Salla disease cell strains may represent mutants with residual amounts of a functional transport system for free NANA. This interpretation is supported by the existence of clinically and biochemically more severe forms of generalized lysosomal NANA storage disease (*9*), for which evidence of defective lysosomal transport of NANA has also been found (*10*).

The failure to demonstrate saturation, that is, a plateau of velocity of normal NANA egress with increasing loading (Fig. 3), is consistent with either a diffusional or facilitated transport system. Demonstration of saturation kinetics may require substantially higher free NANA loading. Indeed, we have been unable to demonstrate counter transport of free NANA in the normal fibroblast system used in these studies, most likely because such a demonstration also depends on some degree of saturation of the putative NANA carrier (*11*). Nevertheless, defective free NANA transport in an autosomal recessive metabolic disease, in the face of normal cystine transport and lack of accumulation of other small molecules, suggests that a single gene product, a specific carrier, mediates the NANA transport across the lysosomal membrane.

Data presented here establish the metabolic defect in Salla disease as an impairment of normal transport of free NANA out of cellular lysosomes. NANA represents the first monosaccharide for which there is direct kinetic evidence of egress out of lysosomes, and Salla disease is the first disorder demonstrated to be caused by defective lysosomal transport of a carbohydrate. We expect that certain carbohydrates other than sialic acid exit lysosomes by carrier-mediated transport, just as do certain amino acids other than cystine (*12*) and certain other small molecules such as vitamin B_{12} (*3*). A new class of lysosomal storage disorders can be expected to expand as specific transport systems are delineated. The elucidation of these metabolic defects will provide the groundwork for determining how functional integral membrane macromolecules are targeted to lysosomes, and for designing potential therapy directed at depleting small molecules stored within lysosomes because of defective lysosomal membrane transport (*13*).

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11. A demonstration of countertransport or transactivation would prove that the system is carrier mediated [W. Wilbrandt and T. Rosenberg, *Pharmacol. Rev.* **13**, 109 (1961)] but is not always possible to obtain. For example, while countertransport of cystine was readily apparent in normal (but not cystinotic) leucocyte granular fractions [W. A. Gahl *et al.*, *Biochem. J.* **216**, 393 (1983)], we were unable to demonstrate it in normal fibroblast granular fractions (F. Tietze and W. Gahl, unpublished observations), despite easily measurable egress of cystine in

that system (F. Tietze, L. H. Rome, J. DeB. Butler, G. S. Harper, W. A. Gahl, *Biochem. J.*, in press). Therefore, the demonstration of sialic acid countertransport may require a system other than normal fibroblast granular fractions loaded by exposure to ManNAc.

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Karyotype Analysis of *Leishmania* Species and Its Use in Classification and Clinical Diagnosis

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Chromosomes of four species of *Leishmania* represented by ten different geographic isolates were analyzed by pulsed field gradient gel electrophoresis (PFG) to assess chromosome stability in these parasitic protozoans. Among different geographic isolates of the same subspecies, more than two-thirds of chromosomes had similar sizes, ethidium bromide staining intensities, and locations of α,β-tubulin genes. However, among New World *Leishmania*, members of different species or subspecies have fewer than one-third of their chromosomes in common. Therefore, PFG karyotypes of *Leishmania* exhibit intraspecific variability similar to that reported for other parasitic protozoans. The greater similarities of the karyotypes of members of the same *Leishmania* subspecies may indicate that they represent valid taxa. These similarities also allowed the use of PFG in clinical diagnosis for rapid and accurate typing of patient isolates.

THE DNA SEQUENCE OF AN ORGANISM encodes species-specific information. In eukaryotes, this information is packaged into chromosomes in the nucleus. Chromosomes of parasitic protozoans cannot be resolved by light microscopy because they do not condense during the cell cycle. Thus, their genes cannot be mapped to specific chromosomes by cytogenetics, even in the rare instances when genetic markers are available.

Pulsed field gradient gel electrophoresis (PFG) has been used to separate protistan chromosome-sized DNA molecules up to about 2 megabase pairs or more, including those of some protozoan agents of human disease (*1–7*). The separated DNA molecules, which appear as discrete bands in agarose gels, have been shown to represent the DNA of the full-length chromosomes (*1, 8*).

We have applied the extraordinary resolving power of PFG to analyze the genomic organization of *Leishmania*. These obligate intracellular protozoan parasites of macrophages, monocytes, and histiocytes cause severe public health problems worldwide, including a recently discovered autochthonous focus of cutaneous leishmaniasis in Texas (*9*). *Leishmania* cause diseases ranging from skin ulcers (Oriental sore) to systemic visceral leishmaniasis, or kala-azar. The species and subspecies of the parasite (*10*) and the immunogenetic background of the host (*11*) affect disease development. Because only certain of its members may cause a given clinical syndrome, it is vital to elucidate the genetics of *Leishmania* and to classify the causative organism for epidemiological studies.

We first explored the variability of PFG karyotypes of the two developmental stages of *Leishmania*—the nonmotile amastigote, found within cells of vertebrate hosts, and the flagellated promastigote, found extracellularly in the gut of the sandfly vector. Both stages can be cultivated in vitro.

We examined the PFG karyotypes of *L. major*, an etiologic agent of cutaneous leishmaniasis from Africa (*12*). Several PFG gels were run at different pulse frequencies for all experiments described to separate optimally chromosomes of different molecular weights. With a 60-second pulse frequency, at least 15 bands were distinguished, with sizes between about 340 kb and 800 kb (Fig. 1). At this pulse frequency, large chromosomes (more than 800 kb) are not separated and some DNA remains in the sample well. The staining intensity of several bands is nonstoichiometric, indicating that multiple chromosomes are present in a band of one size class; this is also the case for yeast (*1*), *Trypanosoma brucei* (*5*), and *Plasmodium falciparum* (*7*). Nonstoichiometrically staining bands may represent more than one chromosome coincidentally of the same size or multiple copies of a single chromosome (aneuploidy). By using several pulse frequencies for optimal separation of DNA molecules of different lengths, at least 20 bands are revealed in different *Leishmania* species.

We determined the size of each chromosome to within ±20 kb with annealed lambda phage as size markers. Reproducibility of size determinations from replicate samples and for gels run at different pulse frequencies was good, with individual chromosomes being measured with a 5% coefficient of variation.

The ethidium bromide–stained PFG karyotypes of promastigotes, amastigotes from skin lesions, and amastigotes from culture (*13*) were the same at all pulse

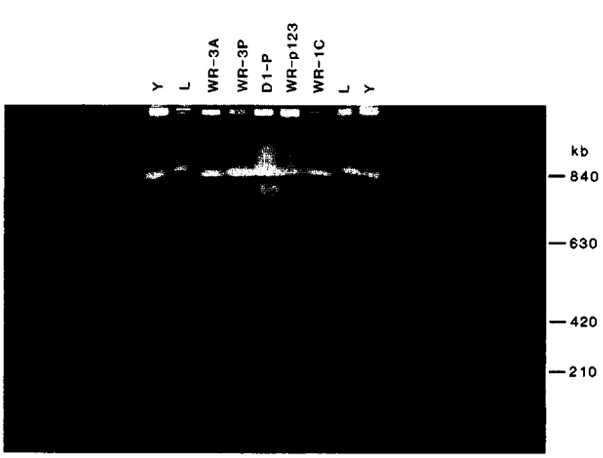

Fig. 1. Comparison of PFG karyotypes of *L. major*. See (*12*) for abbreviations. WR-3A, clone No. 3 amastigotes harvested from mouse skin lesions; WR-3P, clone No. 3 promastigotes; D1-P, promastigotes; WR-p123P, uncloned promastigotes from a 123rd subculture; WR-1C, clone No. 1 culture amastigotes. Annealed bacteriophage lambda DNA ladders (L) and yeast DNA (Y) are included as size standards. Samples were prepared (*2, 12, 13, 18*) and electrophoresed in a double inhomogeneous electrophoresis apparatus (20 by 20 cm) (15°C, 42 hours, 12 V/cm, pulse frequency of 60 seconds).

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