fluids or control solutions, and vortex-shaken. The solution was centrifuged at 2000*g* for 20 minutes, and the liposome pellet was resuspended in HBSS to the final injection volume.
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Niemann-Pick Disease: A Genetic Model in Siamese Cats

Abstract. *Three Siamese cats were found to have a progressive neurological disease that became obvious when they were 4 to 5 months of age. Their brains contained an excess of G_{M2} and G_{M3} gangliosides, and their livers a nine- to tenfold excess of sphingomyelin and cholesterol. A total deficiency of lysosomal (pH 5.0) sphingomyelinase was found in the leukocytes, liver, and brain of the cats, although the activity of the microsomal (pH 7.4, magnesium-dependent) sphingomyelinase was normal in brain. These cats appear to have a genetic disease identical to Niemann-Pick disease type A.*

Niemann-Pick disease (NPD) constitutes a group of recessively inherited syndromes in which sphingomyelin and, secondarily, other lipids, are stored in various organs of the patients. In NPD type A, hepatomegaly and slowing of motor and mental progress become evident early in the first year of life (*1*). Deterioration continues until the patients reach a vegetative state, and death usually occurs before 3 years of age. The liver and spleen contain foam cells filled with sphingomyelin and cholesterol. In the brains of these children there is an increase in G_{M2} and G_{M3} gangliosides (*2*). There is a pronounced deficiency of lysosomal acid (*p*H 5.0) sphingomyelinase (E.C. 3.2.4.12) activity, but the brain contains normal levels of a nonlysosomal magnesium-dependent neutral (*p*H 7.4) sphingomyelinase activity (*3*). Patients and carriers can be identified by examination of leukocytes and cultured skin fibroblasts. Although prenatal diagnosis is available for couples at risk, no treatment is available for children affected with this fatal autosomal recessive disease. We now report a genetic disease in Siamese cats that is identical in pathological and biochemical findings to human NPD type A.

Tissues from three affected cats from three unrelated litters were examined. The tissues from the first two cats had already been fixed in Formalin. These cats were 4 to 5 months old when the owners noted tremors, ataxia, hind-leg weakness, lack of appetite, and lack of interest in their surroundings. Treatment with antibiotics and corticosteroids provided only transitory improvement, and the cats were humanely killed at about 6 months of age. Examination of the tissues by light microscopy revealed the cytoplasmic vacuolization of neurons, hepatocytes, and cells of the reticuloendothelial system suggestive of a lipid storage disease. Because G_{M1} and G_{M2} gangliosides have been reported in Siamese cats, the brain ganglioside pattern was examined, and an excess of G_{M2} and G_{M3} gangliosides was found. The Formalin-fixed liver of one of these cats was available, and qualitative analysis on thin-layer chromatography showed the great excess of sphingomyelin and cholesterol consistent with NPD.

When a third cat was brought to the veterinarian with similar symptoms some months later, blood samples were obtained and leukocytes were prepared for assays of lysosomal enzyme activities as described previously (*4*). The leukocytes showed no measurable sphingomyelinase activity when this was measured at *p*H 5.0 (11 controls averaged 4.97 nmole of substrate hydrolyzed per milligram of protein per hour with a range of 2.99 to 7.20 nmole/mg-hour); an unaffected littermate had a value of 2.63 nmole/mg-hour and is considered a heterozygote for this defect. The parents of these two cats are not available for study.

β-Galactosidase activities were normal in the third affected cat and in its littermate, with values of 42.1 and 40.8 nmole of substrate hydrolyzed per milligram of protein per hour, respectively (14 controls averaged 30.1 nmole/mg-hour with a range of 21.4 to 64.3 nmole/mg-hour). The activities of β-*N*-acetylglucosaminidase (and the percentage activity of hexosaminidase A) were also normal in the affected cat and the unaffected littermate with values of 1332 nmole/mg-hour (57 percent) and 965 nmole/mg-hour (38 percent), respectively [14 controls averaged 864 nmole of substrate hydrolyzed per milligram of protein per hour (67 percent) with a range of 423 to 1928 (50 to 83 percent hexosaminidase A)]. The cat continued to deteriorate clinically until it was 13 months old when it was unable to stand or to feed itself. It was humanely killed with an injection of sodium barbital and the tissues were immediately removed for light and electron microscopy, as well as for measurement of the lipid

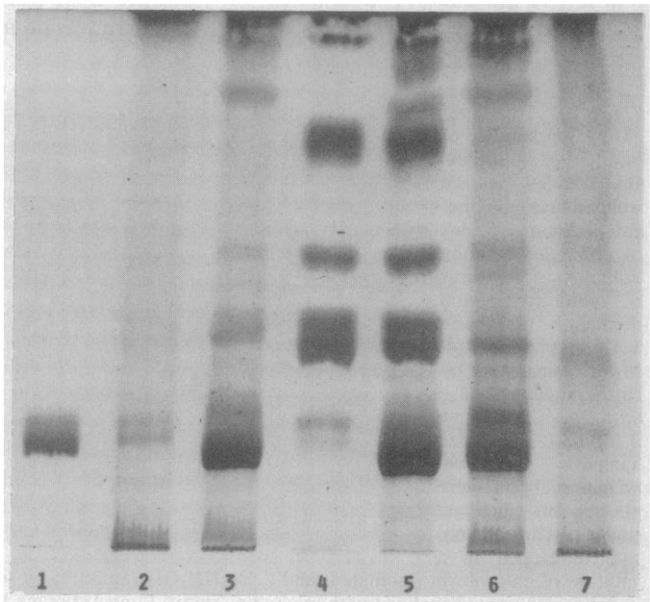

Fig. 1. Thin-layer chromatography of liver phospholipids. Liver samples were homogenized in ten volumes of a mixture of chloroform and methanol (2:1, by volume) and the extract was washed with one-fifth volume of 0.1*M* KCl; the lower phase was washed again with the Folch upper phase. The lower phase was dried with nitrogen, and the residue was dissolved in one volume of the chloroform-methanol mixture. Portions of equal size (on the basis of the wet weight of liver) were spotted on silica gel plates (Brinkmann) and developed in a mixture of chloroform, methanol, acetic acid, and water (75:45:12:6, by volume). The plates were dried and the lipids were visualized by spraying with 50 percent sulfuric acid and heating at 100°C for 20 minutes. Lane 1, sphingomyelin standard; lane 2, Formalin-fixed control cat liver; lane 3, Formalin-fixed liver from affected cat; lane 4, fresh frozen control cat liver; lane 5, fresh frozen affected cat liver; lane 6, fresh frozen liver from human with NPD type A; and lane 7, fresh frozen control human liver.

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Table 1. Sphingomyelinase activities in cat brain extracts. The results are expressed as nanomoles of substrate hydrolyzed per milligram of protein per hour. Samples were prepared as described by Rao and Spence (*7*). Brains from four control cats were used in these studies. All results are the mean of duplicate experiments that agree within 5 percent.

Subject	*p*H 5.0		*p*H 7.4	
	No additions	With EDTA	$+Mg^{2+}$	+EDTA
		Total homogenate		
Affected cat		0	128	0.8
Control cats				
Mean	24.5	24.0	142	4.1
Range	21.6 to 27.3	21.6 to 26.5	123 to 160	3.7 to 5.0
		Supernatant fraction		
Affected cat	0	0	0.6	0.7
Control cats				
Mean	75.4	75.6	19.4	33.8
Range	56.6 to 97.6	56.4 to 97.1	15.7 to 25.6	25.8 to 44.7
		Pellet extract		
Affected cat	0	0	150	0.8
Control cats				
Mean	15.8	15.5	127	1.4
Range	14.9 to 17.0	14.9 to 16.3	116 to 135	1.0 to 1.6

content and of the enzymatic activities.

Detailed microscopic examination revealed pathology identical to that observed in the first two animals and in tissues from children with NPD type A (*5*). Samples of liver tissue from this cat and from controls were homogenized in 0.1 percent Triton X-100 containing 0.05*M* NaCl and assayed for a number of lysosomal enzyme activities. Using the 4-methylumbelliferyl (4MU) derivatives we assayed the total homogenate for acid β-galactosidase, α-L-fucosidase, β-glucuronidase, acid phosphatase, and β-*N*-acetylglucosaminidase according to published procedures (*4*). Sphingomyelinase activity was measured at *p*H 5.0 in liver and at *p*H 5.0 and 7.4 in brain as described previously (*6*). The liver sample from the affected cat had no detectable sphingomyelinase activity. Liver samples from 12 control cats averaged 6.30 nmole of sphingomyelin hydrolyzed per milligram of protein per hour (range 3.20 to 13.8 nmole/mg-hour). All other lysosomal enzymes analyzed were either normal or increased.

Brain samples from this cat and control cats were fractionated as described by Rao and Spence (*7*). This method effectively separates the lysosomal sphingomyelinase activity from the magnesium-dependent microsomal sphingomyelinase activity (*p*H 7.4). Three samples were examined: the total homogenate, the supernatant fraction, and the pellet extract. In all fractions from the affected cat there was no measurable sphingomyelinase activity when it was assayed at *p*H 5.0, but the activities of acid β-galactosidase, α-L-fucosidase, β-glucuronidase, acid phosphatase, and β-*N*-acetylglucosaminidase were normal. Table 1 shows that compared to four control cats the affected cat had a normal level of magnesium-dependent sphingomyelinase activity at *p*H 7.4, whereas the activity at *p*H 5.0 could not be measured. This finding is identical to that found in brains of patients with NPD type A (*3*).

The enlarged yellowish liver was examined for its lipid content after extraction with chloroform-methanol (2:1, by volume). Compared to the livers of five control cats, the liver of the affected cat showed about a ninefold increase in its cholesterol content (31.4 mg/g wet weight, compared to a mean of 3.62 mg/g for controls); total phospholipids in the affected cat showed a more than fourfold increase (110 mg/g wet weight, compared to a mean of 23.9 mg/g for controls). As a percentage of phospholipid, sphingomyelin showed about a tenfold increase (64.4 percent compared to 6.6 percent for controls). This lipid storage is comparable to that observed in livers from patients with NPD type A (Fig. 1).

The brain gangliosides were examined after extraction with ten volumes each of chloroform and methanol (2:1 and 1:1, by volume) and partitioning into the upper phase with one-fifth volume of 0.1*M* KCl. As shown in Fig. 2, there was a significant increase in G_{M2} and G_{M3} gangliosides compared to control cat brain, and this compares to that seen in the brain of a child who died at 26 months with NPD type A.

Several enzymatically confirmed lysosomal storage disorders have been reported in Siamese cats, including G_{M1} gangliosidosis, G_{M2} gangliosidosis, and Maroteaux-Lamy syndrome (*8*). The pathological, enzymatic, and chemical studies reported here indicate that the Siamese cats we examined had a genetic disease identical to NPD type A. Among the littermates of the third cat we examined there were two, a male and a female, that had similar symptoms and were humanely killed at 6 months of age with no studies performed. The healthy male littermate that we suspect is a carrier of the disease has sired two litters with a total of eight kittens. Five of these kittens, two females and three males, appear to have half-normal sphingomyelinase activity and may be carriers of this autosomal recessive disease. The affected cats reported here are clinically and pathologically similar to two previously reported cats who were thought to have a sphingomyelin lipidosis (*9*).

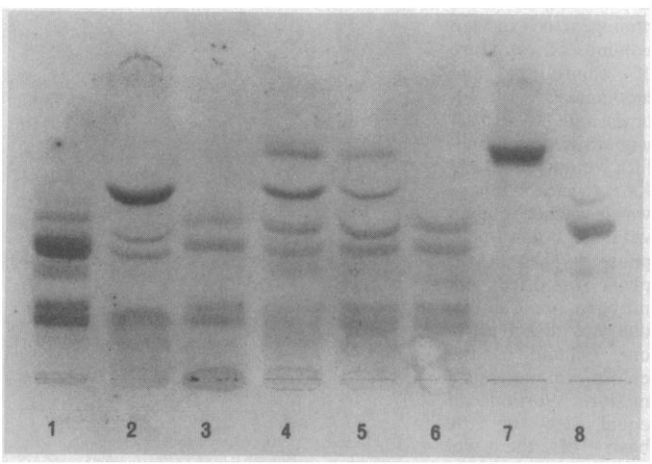

Fig. 2. Thin-layer chromatography of brain gangliosides. Gangliosides were isolated from bovine, cat, and human brains by extraction with ten volumes of mixtures of chloroform and methanol (2:1 and 1:1, by volume) followed by partition into the aqueous phase with one-fifth volume 0.1*M* KCl. Equal size samples per wet weight of brain were spotted on silica gel plates (Brinkmann) and developed in a mixture of chloroform, methanol, and 2.5*N* NH_4OH (60:35:8, by volume). After drying, the gangliosides were visualized with resorcinol spray. Lane 1, bovine brain gangliosides; lane 2, gangliosides from the brain of a 3-year-old child who died with Tay-Sachs disease; lane 3, gangliosides from the brain of a 2-year-old-child who died with no evidence of neurological disease; lane 4, gangliosides from the brain of a 26-month-old child who died with NPD type A; lane 5, gangliosides from the brain of the affected cat described herein; lane 6, gangliosides from the brain of a 1-year-old control cat; lane 7, G_{M3} ganglioside isolated from a spleen of a Gaucher's disease patient; and lane 8, partially purified G_{M1} ganglioside from bovine brain.

However, enzymatic studies were not reported for those cats since only Formalin-fixed tissues were obtained. Very recently a poodle with NPD was reported (*10*).

The use of the drug AY-9944 to produce an animal model of NPD in rats has been reported (*11*). This drug produces pathological changes similar to those seen in NPD, and causes an increase in the sphingomyelin content of the liver and a reduction in sphingomyelinase activity, possibly because of impaired enzyme synthesis. Thus animal models of some human genetic diseases may be produced by specific chemicals, but genetic models are more useful for studying methods of treatment. The availability of Siamese cats with a severe neurovisceral lipidosis apparently identical to human NPD type A should facilitate studies of treatment for this fatal lipid storage disease of humans.

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Immunofluorescence of NADPH–Cytochrome *c* (P-450) Reductase in Rat and Minipig Tissues Injected with Phenobarbital

Abstract. *The enzyme NADPH–cytochrome* c *(P-450) reductase was identified by indirect immunofluorescence in hepatocytes, bronchioles, and proximal tubules of liver, lung, and kidney, respectively, of rats and minipigs that had been injected with phenobarbital or saline. The distribution of this component of the cytochrome P-450–mediated microsomal system may be relevant to sites of drug toxicity and carcinogenesis.*

The enzyme NADPH-cytochrome *c* (P-450) reductase (E.C. 1.6.2.4), as well as cytochrome P-450, cytochrome b_5, and NADH-cytochrome b_5 reductase are components of the known microsomal electron transport systems that participate in the metabolism of endogenous substrates such as steroids and fatty acids, and in the metabolism of many exogenous substrates, including various drugs and carcinogens (*1*, *2*). Selected components of these systems are increased by the administration of drugs such as phenobarbital or certain polycyclic aromatic hydrocarbons (*1*). Paradoxically, the microsomal drug-metabolizing system may participate in the activation of many potential carcinogens to their proximate metabolites (*3*). Phenobarbital has been shown both to promote and to inhibit tumor formation in experimental models (*4*). Thus, the question of the relation of cytochrome P-450–mediated enzyme systems to carcinogenesis is a complicated one. It is logical to ask which cell types contain the mixed function oxidase enzymes, and if these cell types correspond to those which are frequent sites of tumor formation.

NADPH-cytochrome *c* (P-450) reductase has been shown biochemically to be present in liver, lung, and kidney microsomes (*5*). However, since microsomes are a product of tissue homogenates, microsomal data provide no insight into the cell type containing the enzyme, unless the tissue is very homogeneous. Baron *et al.* (*6*) have demonstrated NADPH-cytochrome *c* reductase in livers of control and phenobarbital-treated rats by immunohistochemical techniques. We have investigated by immunofluorescence the extrahepatic, as well as the hepatic, distribution of NADPH-cytochrome *c* (P-450) reductase in rats and minipigs (small pigs bred by Bastrop, College Station, Texas).

Male Sprague-Dawley rats were injected intraperitoneally with phenobarbital (80 mg/kg) or saline daily for 4 days, fasted, and then killed. The minipigs were given phenobarbital (15 mg/kg) in a bolus of food once daily for 6 days and then killed. Portions of liver, lung, and kidney were removed and frozen immediately in liquid nitrogen for immunocytochemistry. Biochemical assays were performed on microsomes prepared by standard methods from the remainder of the tissue from each organ; induction of the phenobarbital-treated rats was confirmed by a two- to threefold increase in NADPH-cytochrome *c* (P-450) reductase activity, and a three- to fivefold increase in cytochrome P-450 content when liver microsomes from phenobarbital- and saline-treated rats were compared. No change in activities was seen in kidney microsomes and only a slight increase was seen in lung microsomes from the phenobarbital-treated rats.

Sections of tissue (5 to 6 μm) prepared in a cryostat were stained by the indirect fluorescent antibody technique (*7*). For this purpose we used rabbit antibody to pig NADPH-cytochrome *c* reductase (0.025 mg/ml), prepared against the protease-solubilized form of the reductase and purified by affinity chromatography (*8*). Although there are differences in the reductase purified by proteolytic as opposed to detergent solubilization, antibody prepared against the proteolytically isolated form is also specific for the detergent-solubilized form as well as the intact, membrane-bound enzyme (*9*). Goat antibody to rabbit immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC) was used as the secondary antibody. Controls included: unstained sections, sections stained with secondary antibody only, nonimmune IgG substituted for the primary antibody, and primary antibody that had been incubated with cytochrome P-450 reductase to block antibody binding sites. Although only the controls for liver are shown (Fig. 1), controls were performed on each tissue and consistently showed minimal or no fluorescence.

In the rat, specific fluorescence was seen throughout the liver (Fig. 2, a and d) in the hepatocyte cytoplasm but not in the nucleus. In both saline- and phenobarbital-treated rats, the fluorescence was more intense in centrilobular than in periportal zones; livers from phenobarbital-treated rats showed increased fluorescence compared to the livers from saline-treated animals. Fluorescence in the

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