Enhanced Enzyme Activity after Incubation with Zinc Can Be Used to Distinguish Heterozygotes of Pompe's Disease

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ABSTRACT. In an attempt to identify heterozygotes for Pompe's disease, the acid α -D-glucosidase activity at pH 4 in phytohemagglutinin-stimulated lymphocytes was measured. In the standard assay an overlap in the enzyme activity between normal controls and obligate heterozygotes was demonstrated. When the assay was modified by adding zinc chloride into the incubation mixture, the enzyme activity of the lymphocyte homogenates did not change. However, the enzyme activity increased dramatically after intact lymphocytes were preincubated with zinc chloride. There was no more overlap in the acid α -D-glucosidase activity between normal controls and obligate heterozygotes when lymphocytes were incubated 1 h with 10^{-4} mol/liter zinc chloride or 2 h with 10^{-3} or 10^{-4} mol/liter zinc chloride. This modified enzyme activity should facilitate improved heterozygote detection. (*Pediatr Res* 23: 283-287, 1988)

Abbreviations

PHA, phytohemagglutinin
FCS, fetal calf serum
4-MU- α -glc, 4-methylumbelliferyl- α -D-glucopyranoside

Glycogen storage disease type II (Pompe's disease) (McKusick no. 23230) is an autosomal recessive disorder characterized by the lysosomal accumulation of glycogen (1, 2). The impaired glycogen degradation is due to a deficiency of acid α -glucosidase (EC 3.2.1.20) (1-6). Several clinical forms of Pompe's disease that differ in age of onset, organ involvement, and progression of the disease have been recognized (1-7). In Taiwan Pompe's disease is the most common type of glycogen storage disease (7-9). Most cases have had the infantile form (generalized glycogenosis II) with symptoms appearing shortly after birth. Cardiomegaly, hepatomegaly, and muscular weakness are usually present. Patients typically die within the 1st yr of life due to cardiac failure or respiratory tract complications. Therefore, the identification of both homozygous and heterozygous patients is important for genetic counseling and prenatal diagnosis. In our previous reports we established the normal reference range of acid α -D-glucosidase levels in different age groups in this population which is useful not only for clinical cases but also for prenatal diagnosis (8). Often heterozygote detection is difficult. For heterozygotes identification studies measuring enzyme activ-

ity from PHA-stimulated lymphocytes (10, 11), leukocytes (12, 13), cultivated fibroblasts (14), or skeletal muscles (15) have been reported. However, each method has its limitation. In an attempt to determine a more accurate method for the recognition of heterozygotes, we established an enzyme assay method using zinc stimulated intact lymphocytes which is superior to the method using PHA-stimulated lymphocytes.

MATERIALS AND METHODS

Subjects. From April 1981 to June 1986 18 families had one or more infant deaths with Pompe's disease (infantile generalized form). The criteria for diagnosis of Pompe's disease have been described previously (7-9). These criteria are: 1) presence of clinical features of hypotonia, cardiomegaly, and hepatomegaly; 2) depressed ST segments, inverted T waves, and a shortened P-R interval on electrocardiogram; 3) light microscopic and electron microscopic demonstration of lysosomal glycogen deposition; and 4) markedly deficient α -glucosidase activity in lymphocytes, muscle cells, and/or cultured fibroblasts. Obligate heterozygotes from 14 of these families were studied; five families were consanguineous.

Lymphocyte separation. Lymphocytes were separated using a Ficoll-Hypaque density gradient as previously described (7, 8). The monocytes were removed with Petri dish adherence. The polymorphonuclear leukocytes were removed with 6% dextran.

PHA-stimulated lymphocytes. Lymphocytes were cultured in RPMI-1640 containing 10% heat-inactivated FCS with 5 μ g/ml PHA or without. Cultures were carried out for 72 h in a 5% CO₂ incubator at 37° C. At the end of the culture lymphocytes were washed three times with isotonic saline and then the activity of α -glucosidase was measured.

α -D-glucosidase activity. The activity of acid α -glucosidase was assayed with 4-MU- α -glc (Koch-Light Laboratories, Colnbrook, England) as substrate by the method of Salafsky and Nadler (16) with the following modifications. Twenty- μ l cell suspensions (varying from 5×10^4 to 5×10^5) were incubated with 50 μ l of 1 mM 4-MU- α -glc in citrate phosphate buffer pH 4 and 6 for 2 h at 37° C with constant agitation. The reaction was terminated by adding 1 ml of 400 mM glycine-NaOH buffer, pH 10.5. Fluorescence was determined in an Hitachi or an Aminco-Bowman spectrofluorometer with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The standard contained 0.05 mM (8.81 mg/liter) of 4-methylumbelliferone (Koch-Light) in 0.05 M glycine carbonate buffer. Protein was determined by the method of Lowry *et al.* (17).

Acid α -D-glucosidase activity from lymphocytes after incubation with zinc. To elucidate the effect of zinc, the acid α -D-glucosidase activity in intact lymphocytes from four homozygotes, 28 heterozygotes, and 20 healthy controls were measured after being incubated with final concentration of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} mol/liter zinc chloride and incubated for 1, 2, and 16 h.

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Acid α -D-glucosidase activity from lymphocyte homogenates after incubation with zinc. For the study of the effect of zinc in pH 4 citrate buffer on the α -D-glucosidase in lymphocyte homogenates, homogenates from four homozygotes, 20 heterozygotes, and 20 healthy controls were incubated with final concentrations of 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} mol/liter zinc chloride with or without EDTA for 15 and 30 min, then the acid α -D-glucosidase activity was immediately determined.

Controls. The lymphocytes used as controls were separated with Ficoll-Hypaque density gradient from 20 age- and sex-matched healthy persons.

Statistical analysis. The differences between groups were assessed by the approximate *t* test, or Student's *t* test, depending

on the equality of the variances of the two groups being compared.

RESULTS

Stimulated versus unstimulated. The lymphocyte acid α -D-glucosidase activity increased after PHA stimulation in both heterozygotes or affected homozygotes but decreased in normal healthy controls (Fig. 1). Comparison of acid α -D-glucosidase activity in PHA-stimulated lymphocytes both in normal healthy controls and heterozygotes showed no statistical difference.

Modified enzyme activity with the addition of zinc. The acid α -D-glucosidase activity from lymphocyte homogenate after

Fig. 1. Comparison of acid α -D-glucosidase activity in PHA-stimulated lymphocytes among homozygous, heterozygous, and normal controls. The α -D-glucosidase in heterozygotes can be divided into two groups, group a (*Gr. a*) (which is parent a in Tables 1 to 5) is slightly low; but group b (*Gr. b*) (which is parent b in Tables 1 to 5) is very low. $\bar{s}$, without; $\bar{p}$, post.

Table 1. Activity of the acid α -D-glucosidase* (pH 4) from lymphocyte homogenate after incubation with $ZnCl_2$ for 15 min [mean $\pm$ SD (range)]

Lymphocyte homogenate	Homogenate incubation with Zn^{++} (mol/liter) for 15 min							With EDTA	Nothing
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Normal (<i>n</i> = 20)	25.07 ± 4.62 (20.52–30.06)	26.89 ± 5.48 (20.46–32.82)	25.20 ± 6.24 (18.82–32.41)	24.40 ± 7.66 (26.48–32.52)	27.35 ± 5.56 (21.42–32.62)	22.41 ± 5.42 (16.74–28.52)	21.11 ± 6.12 (14.84–28.32)	23.29 ± 10.96 (12.88–34.42)	26.67 ± 5.34 (18.42–32.18)
Heterozygotes†									
Patient a (<i>n</i> = 14)	9.70 ± 2.34 (6.42–13.81)	10.63 ± 3.61 (6.32–14.70)	11.20 ± 3.84 (6.82–15.24)	11.01 ± 4.21 (6.38–15.28)	10.24 ± 3.76 (6.42–14.74)	10.51 ± 4.04 (6.34–15.12)	10.16 ± 3.68 (6.14–15.80)	11.17 ± 1.06 (9.72–14.40)	12.04 ± 2.06 (10.42–14.80)
Patient b (<i>n</i> = 14)	3.92 ± 0.42 (2.12–4.86)	4.11 ± 0.48 (2.84–4.78)	4.68 ± 0.52 (2.92–5.02)	4.11 ± 0.36 (2.88–4.74)	4.07 ± 0.40 (2.56–4.82)	3.69 ± 0.32 (2.34–4.41)	3.67 ± 0.48 (2.16–4.32)	3.42 ± 0.50 (1.06–5.43)	3.48 ± 0.48 (1.08–5.62)
Homozygotes (<i>n</i> = 4)	0.20 ± 0.24 (0.01–0.80)	0.21 ± 0.32 (0.01–0.90)	0.24 ± 0.28 (0.01–0.84)	0.22 ± 0.30 (0.01–0.88)	0.18 ± 0.12 (0.01–0.72)	0.18 ± 0.18 (0.01–0.74)	0.17 ± 0.14 (0.01–0.70)	0.38 ± 0.12 (0.10–1.24)	0.36 ± 0.14 (0.10–1.26)

* α -D-Glucosidase was measured in n mol/mg protein/h.

† In our experience the α -D-glucosidase in heterozygotes can be divided into two groups, one (parent a) is slightly low (>10 nmol/mg protein/h) and the other (parent b) is very low (<10 nmol/mg protein/h).

Table 2. Activity of the acid α -D-glucosidase* (pH 4) from lymphocyte homogenate after incubation with ZnCl_2 for 30 min [mean $\pm$ SD (range)]

Lymphocyte homogenate	Homogenate incubation with Zn^{++} (mol/liter) for 30 min							With EDTA	Nothing
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Normal (n = 20)	23.51 ± 3.24 (19.42–27.34)	25.97 ± 4.36 (19.52–30.84)	26.59 ± 4.21 (19.36–31.32)	28.68 ± 4.71 (21.84–33.72)	31.78 ± 5.40 (22.52–37.56)	26.31 ± 6.38 (19.14–34.36)	24.81 ± 4.98 (19.02–30.16)	23.29 ± 10.96 (12.92–36.82)	26.67 ± 5.34 (16.32–35.80)
Heterozygotes†									
Patient a (n = 14)	6.70 ± 2.32 (3.12–9.18)	7.17 ± 3.28 (3.10–10.82)	7.56 ± 3.42 (3.02–11.54)	8.68 ± 4.53 (3.21–14.32)	9.83 ± 4.27 (4.12–15.38)	8.60 ± 3.83 (3.42–12.82)	8.88 ± 2.87 (3.04–11.52)	11.17 ± 1.06 (10.14–14.21)	12.05 ± 2.06 (10.02–15.32)
Patient b (n = 14)	2.06 ± 0.14 (1.04–3.14)	2.24 ± 0.30 (1.08–3.16)	2.38 ± 0.26 (1.06–3.04)	2.82 ± 0.30 (1.04–3.86)	3.38 ± 0.14 (1.12–5.14)	2.62 ± 0.32 (1.10–4.08)	2.22 ± 0.18 (1.11–3.44)	4.49 ± 1.48 (1.01–5.46)	4.83 ± 1.28 (1.06–6.02)
Homozygotes (n = 4)	0.06 ± 0.02 (0.01–0.52)	0.08 ± 0.04 (0.01–0.64)	0.11 ± 0.08 (0.01–0.72)	0.16 ± 0.14 (0.01–0.82)	0.17 ± 0.20 (0.01–0.90)	0.10 ± 0.21 (0.01–0.91)	0.08 ± 0.14 (0.01–0.80)	0.22 ± 0.10 (0.01–1.02)	0.32 ± 0.12 (0.01–1.22)

* α -D-glucosidase was measured in nmol/mg protein/h.† In our experience the α -D-glucosidase in heterozygotes can be divided into two groups, one (patient a) is slightly low (>10 nmol/mg protein/h) and the other (patient b) is very low (<10 nmol/mg protein/h).Table 3. Activity of the acid α -D-glucosidase* (pH 4) from lymphocytes after incubation with ZnCl_2 for 1 h [mean $\pm$ SD (range)]

Lymphocytes	Whole cells incubation with Zn^{++} (mol/liter) for 1 h				Without Zn^{++}	With EDTA
	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
Normal (n = 20)	22.45† ^a ± 8.26 (18.28–32.52)	26.63 ^b ± 8.82 (14.24–36.72)	37.98 ^{c,f} ± 8.74 (27.52–48.62)	46.83 ^{d,g} ± 10.95 (24.12–58.42)	29.09 ^e ± 16.82 (12.36–50.31)	27.84 ^h ± 15.68 (13.52–48.86)
Heterozygotes‡						
Patient a (n = 14)	14.17 ± 7.61 (6.36–24.72)	19.76 ± 6.12 (11.42–27.56)	24.04 ^f ± 6.71 (14.62–34.82)	17.61 ^g ± 8.44 (9.42–23.64)	17.74 ± 4.65 (10.26–22.62)	16.82 ± 5.27 (10.12–23.54)
Patient b (n = 14)	3.99 ^a ± 4.13 (0.08–9.14)	5.48 ^b ± 5.39 (0.08–12.42)	10.57 ^c ± 10.34 (0.09–21.52)	5.58 ^d ± 4.80 (1.12–12.14)	3.41 ^e ± 2.24 (1.02–7.42)	3.04 ^h ± 2.82 (1.06–8.42)
Homozygotes (n = 4)	0.34 ± 0.12 (0.01–0.90)	0.36 ± 0.14 (0.01–0.92)	0.38 ± 0.16 (0.01–0.94)	0.32 ± 0.10 (0.01–0.94)	0.38 ± 0.12 (0.01–1.06)	0.31 ± 0.16 (0.01–1.08)

* α -D-Glucosidase was measured in nmol/mg protein/h.† Mean values in the same column followed by a common letter are significantly different ($p < 0.001$) by Student's or approximate t test.‡ In our experience the α -D-glucosidase in heterozygotes can be divided into two groups, one (parent a) is slightly low (>10 nmol/mg protein/h) and the other (parent b) is very low (<10 nmol/mg protein/h).

being incubated with different concentrations of zinc chloride for 15 and 30 min is shown in Tables 1 and 2. Comparison of acid α -D-glucosidase activity among each group incubated with and without zinc chloride revealed no statistical difference.

The acid α -D-glucosidase activity from whole intact lymphocytes increased after incubation with zinc chloride for 1 (Table 3) and 2 (Fig. 2; Table 4) h but not after 16 h. When the incubation time was set at 1 h, the maximal increased enzyme activity was at the zinc chloride concentration of 10^{-4} mol/liter. With zinc preincubation the group of heterozygotes with enzyme activity above and below 10 nmol/mg protein/h from the normal

controls could be distinguished clearly (Table 3). When the incubation time increased to 2 h, the maximal enzyme activity was still at the zinc chloride concentration of 10^{-4} mol/liter. There was also a statistical difference between heterozygotes with enzyme activity above 10 nmol/mg protein/h group and normal controls at 10^{-3} and 10^{-4} mol/liter zinc chloride (Table 4). After the incubation time increased to 16 h, the zinc chloride was unable to distinguish between heterozygotes and normal controls (Table 5).

DISCUSSION

Pompe's disease, type II glycogenosis, is the most common type of glycogenosis in Taiwan (7-9). The disease involves mainly Southern Chinese and is rare in Northern Chinese (8) and Japanese (18). In our estimation the frequency of heterozygotes in Taiwan is about 0.5 to 1% which is similar to the Netherlands and Belgium (3). This is a high frequency hereditary disease. Therefore, genetic counseling and heterozygotes detection are very important. Hirschhorn *et al.* (10) and Taniguchi *et al.* (11) have reported that the assay of acid α -D-glucosidase activity in PHA-stimulated lymphocytes is useful in the detection of heterozygotes of this disease. However, we found that there was overlap in the acid α -D-glucosidase activity of PHA-stimulated lymphocytes between normal controls and obligate heterozygotes. Therefore, we tried to improve the sensitivity of the method for a better differentiation of the heterozygotes using metallic ions including Ag^+ , Hg^{++} , Zn^{++} , Cu^{++} , and Cd^{++} . No remarkable improvement in the enzyme activity of lymphocyte homogenate was found by Zn^{++} , Cu^{++} , Cd^{++} , and EDTA. Instead, a strong inhibition by Ag^+ and Hg^{++} was noted. It is surprising that α -D-glucosidase activity from intact lymphocytes increased markedly after incubation with zinc. Furthermore, the acid α -D-glucosidase activity of lymphocytes incubated 1 h with 10^{-4} mol/liter zinc chloride or 2 h with 10^{-3} or 10^{-4} mol/liter zinc chloride did not overlap between the normal controls and obligate heterozygotes. This result suggests that the zinc-enhanced enzyme activity may be useful for heterozygote detection.

Fig. 2. The activity of the acid α -D-glucosidase (pH 4) from lymphocytes after incubation with ZnCl_2 for 2 h.

Table 4. Activity of the acid α -D-glucosidase* (pH 4) from lymphocytes after incubation with ZnCl_2 for 2 h [mean $\pm$ SD (range)]

Lymphocytes	Whole cells incubation with Zn^{++} (mol/liter) for 2 h				Without Zn^{++}	With EDTA
	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
Normal (n = 20)	16.43 ^{†a} ± 6.68 (6.52– 28.82)	18.81 ^b ± 9.52 (8.12– 30.56)	41.96 ^{c,f} ± 11.32 (28.24– 58.92)	57.54 ^{d,g} ± 10.28 (42.42– 70.18)	24.65 ^e ± 10.82 (12.42– 36.92)	23.42 ^h ± 9.63 (12.14– 36.56)
Heterozygotes [‡]						
Parent a (n = 14)	10.36 ± 2.27 (7.24– 14.32)	16.14 ± 5.07 (10.41– 22.72)	20.59 ^f ± 9.84 (10.82– 31.26)	21.06 ^g ± 6.59 (12.92– 28.32)	14.74 ± 4.65 (9.82– 20.48)	13.67 ± 5.32 (8.12– 20.72)
Parent b (n =)	2.85 ^a ± 4.18 (0.92– 0.48)	7.25 ^b ± 5.11 (0.92– 14.54)	16.61 ^c ± 10.42 (4.82– 30.12)	16.96 ^d ± 9.88 (6.52– 28.42)	3.41 ^e ± 2.55 (0.92– 7.42)	3.08 ^h ± 2.47 (0.94– 7.62)
Homozygotes (n = 4)	0.22 ± 0.10 (0.01– 0.90)	0.26 ± 0.14 (0.01– 0.92)	0.30 ± 0.10 (0.01– 0.92)	0.30 ± 0.20 (0.01– 0.94)	0.36 ± 0.10 (0.01– 1.02)	0.29 ± 0.09 (0.01– 1.00)

* α -D-Glucosidase was measured in nmol/mg protein/h.

[†] Mean values in the same column followed by a common letter are significantly different ($p < 0.001$) by Student's or approximate t test.

[‡] In our experience the α -D-glucosidase in heterozygotes can be divided into two groups, one (parent a) is slightly low (>10 nmol/mg protein/h) and the other (parent b) is very low (<10 nmol/mg protein/h).

Table 5. Activity of the acid α -D-glucosidase* (pH 4) from lymphocytes after incubation with $ZnCl_2$ for 16 h [mean $\pm$ SD (range)]

Lymphocytes	Whole cells incubation with Zn^{++} (mol/liter) for 16 h				Without Zn^{++}	With EDTA
	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
Normal (n = 20)	2.06 ± 3.36 (0.10–5.82)	6.31 ^{†,a,b} ± 4.19 (1.02–11.72)	26.14 ^{c,d} ± 11.05 (12.41–48.52)	27.50 ^{e,g} ± 14.86 (12.62–45.82)	19.65 ^h ± 8.82 (10.42–30.52)	18.42 ⁱ ± 7.46 (10.64–28.42)
Heterozygotes [‡]						
Parent a (n = 14)	1.57 ± 1.92 (0.14–3.12)	5.93 ± 6.07 (0.94–12.73)	11.45 ^e ± 5.17 (5.72–19.42)	9.93 ± 6.13 (2.14–17.92)	13.41 ± 4.25 (8.42–19.48)	12.82 ± 4.94 (7.24–18.22)
Parent b (n = 14)	0.39 ± 0.11 (0.21–1.04)	2.47 ^b ± 1.74 (0.90–5.12)	6.35 ^d ± 2.61 (1.01–10.52)	6.55 ^g ± 5.18 (0.94–12.81)	4.74 ± 3.65 (0.92–9.02)	4.14 ± 3.21 (0.96–9.41)
Homozygotes (n = 4)	0.12 ± 0.08 (0.01–0.90)	0.16 ^a ± 0.10 (0.01–0.91)	0.26 ^{c,e} ± 0.12 (0.01–0.94)	0.26 ^f ± 0.10 (0.01–1.02)	0.28 ^h ± 0.06 (0.01–1.06)	0.20 ⁱ ± 0.08 (0.01–1.08)

* α -D-Glucosidase was measured in nmol/mg protein/h.† Mean values in the same column followed by a common letter are significantly different ($p < 0.001$) by Student's or approximate t test.‡ In our experience the α -D-glucosidase in heterozygotes can be divided into two groups, one (parent a) is slightly low (>10 nmol/mg protein/h) and the other (parent b) is very low (<10 nmol/mg protein/h).

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