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Biochemical, Immunological, and Cell Genetic Studies in Glycogenosis Type II

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INTRODUCTION

Glycogenosis type II (Pompe disease) 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 (E.C.3.2.1.20) [3]. Several clinical forms of glycogenosis II which differ in age of onset, organ involvement, and progression of the disease have been recognized [4–6]. In the infantile form (generalized glycogenosis II), nearly all tissues are affected, and symptoms become apparent shortly after birth. Hepatosplenomegaly and muscular weakness are present, and cardiac failure caused by the extensive accumulation of glycogen usually results in death within the first year of life. In the juvenile form, there is absent or minimal cardiac involvement; difficulty walking is generally the first symptom, and progressive weakness of the skeletal muscles is the main clinical feature. In adults, symptoms do not appear until the second or third decade of life, and the myopathy, usually beginning in the lower limbs, progresses at different rates. Patients over 60 years have been described [6–8].

During the past decade several investigators have tried to explain how these different clinical forms result from the same enzyme deficiency, but no clear answer has been given. It seems that glycogen accumulation in skeletal muscles of patients with the adult form is less pronounced than in cases of the infantile type [6, 8, 9]. Recently Mehler and Di Mauro [10] found residual acid α-glucosidase activity in muscles from patients with the adult or juvenile form of glycogenosis II but not in the infantile form.

The present paper presents a study of residual acid α-glucosidase activity in cultured fibroblasts from patients with the adult, juvenile, or infantile forms of glycogenosis II. The kinetic, electrophoretic, and immunological properties of the residual α-glucosidase in adult and juvenile cases were studied. Somatic cell hybridization

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experiments were carried out to investigate whether different gene mutations were responsible for the clinical heterogeneity in glycogenosis II.

MATERIALS AND METHODS

Cell Cultivation and Hybridization

Seven patients with the infantile form of glycogenosis II (code 6–12) and five patients with the juvenile or adult form (code 1–5) were studied (table 1). Subcultures of fibroblast strains of about the same passage from controls and patients were grown in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. Ten days after the cultures became confluent, they were trypsinized, and 4×10^6 cells of each strain were equally divided over four Falcon flasks (25 cm^2). One flask a week was harvested, and cell homogenates were prepared by sonication in distilled water for measurements of the α-glucosidase activity and the glycogen content. During cultivation, the medium was changed three times a week, including the day before harvesting. Separate cultures were kept confluent for 15 days and were then used for enzyme kinetic, electrophoretic, and immunological studies.

Cell hybridization was carried out by fusing cells from each parental strain using inactivated Sendai virus [11]. After 2–8 days of subsequent cultivation, cell homogenates were prepared, and the acid α-glucosidase activity of the mixed population of nonfused mononuclear cells and multikaryons was measured.

Enzyme Assays

Acid α-glucosidase assays with 2.2 mM 4-methylumbelliferyl-α-D-glucopyranoside (MUGlu, Koch-Light Laboratories, Colnbrook, U.K.) were carried out as described previously by incubating 10 μl cell homogenate with 20 μl substrate for 1 hr at 37°C [13]. Assays on single binuclear cultured cells were performed by incubating the cells in 0.08 μl of MUGlu substrate. Subsequent measurement of the concentration of methylumbelliferone was performed by microscope fluorometry [12]. The acid α-glucosidase activity in 10 μl cell homogenate was also measured with maltose (50 μl of 10 mg/ml, Baker, Phillipsburg, N.J.) or glycogen (50 μl of 50 mg/ml Gibco, Grand Island, N.Y.) as substrate; both were dissolved in potassium phosphate (0.2 M) -citrate (0.1 M) buffer, pH 4.4. After incubation and heating for 2 min at 100°C, the amount of liberated glucose was determined by adding 200 μl glucose reagent according to Koster et al. [14]. The pH profiles were made by mixing 10 μl cell homogenate, 50 μl substrate, dissolved in water, and 10 μl of phosphate-citrate buffer of proper pH. Electrophoresis was carried out at 4°C with cellulose-acetate gels (Cellogel, Chemetron, Italy) in 50 mM potassium phosphate (pH 6.8) for 2 hr (200 V).

Glycogen Determination

Glycogen levels were determined by a modified procedure of Huijing [15]. The cell homogenates were heated for 2 min at 100°C. After cooling, a 50 μl aliquot was incubated with a 50 μl mixture of α-amylase (Diastase Hog Pancreas, Sigma, St. Louis, Mo., 50 μg/ml) and α-glucosidase (15018 EGBC, Boehringer, Indianapolis, Ind., 1.25 μg/ml) in sodium acetate (pH 6.0) for 1 hr. The samples were then heated for 2 min at 100°C, and the amount of liberated glucose was determined as described above.

Immunological Methods

An antiserum against human liver acid α-glucosidase was prepared as described previously [16]. The IgG fraction was obtained by ammonium sulphate precipitation and used for the present studies. Crude cell homogenates were diluted with phosphate buffered (10 mM, pH 6.8) saline (0.9% NaCl) to an α-glucosidase activity of 20-50 nmol MU per/hr/ml, and 15 μl aliquots were then incubated overnight at 4°C with 15 μl of serially diluted antiserum. The immune

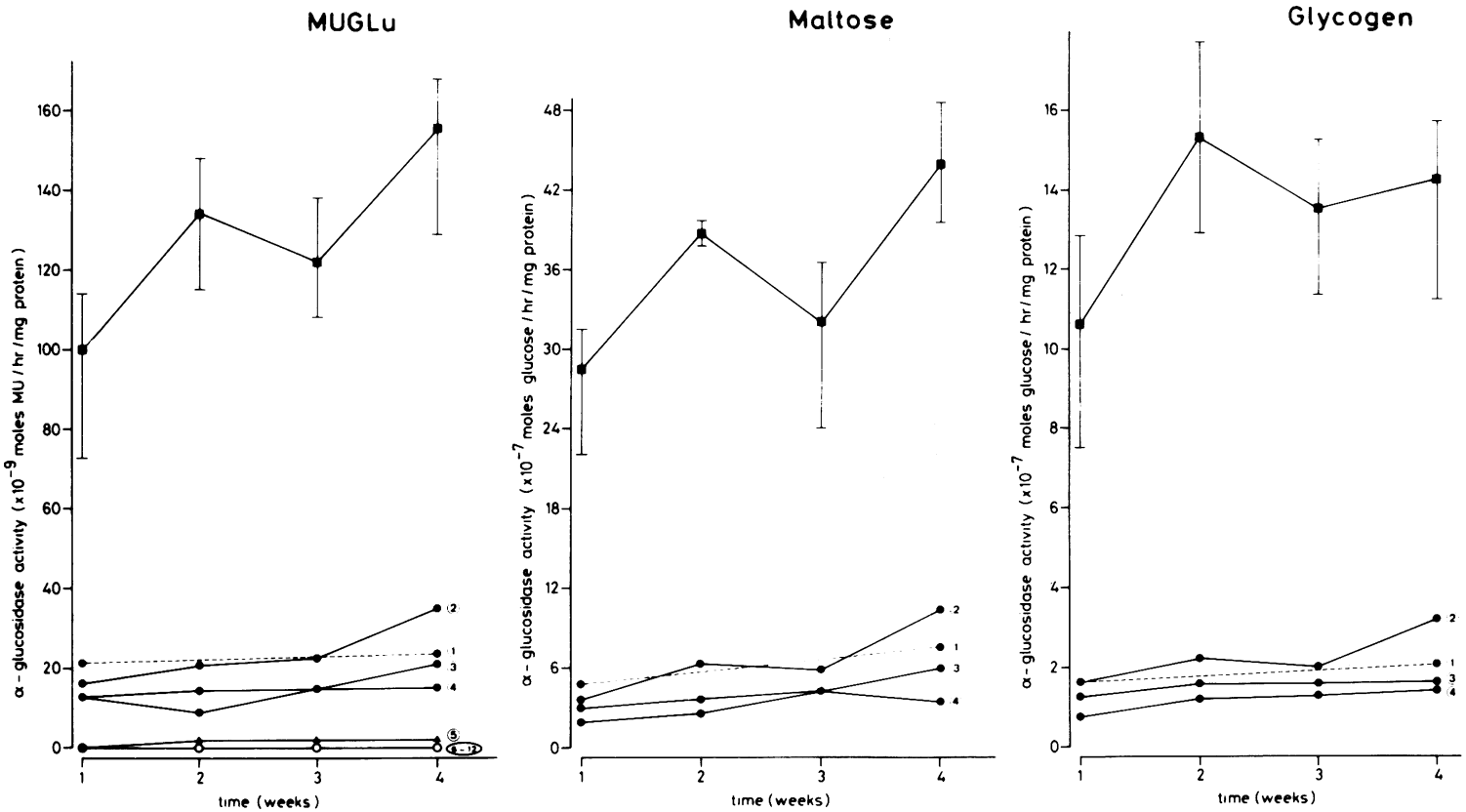
MUGLu
Maltose
Glycogen
α - glucosidase activity (x10⁻⁹ moles MU / hr / mg protein)
α - glucosidase activity (x10⁻⁷ moles glucose / hr / mg protein)
α - glucosidase activity (x10⁻⁷ moles glucose / hr / mg protein)
time (weeks)
time (weeks)
time (weeks)
1
2
3
4
5
6 - 12

FIG. 1

complexes were spun down at 40,000 *g* for 30 min, and the activity of unprecipitated α-glucosidase was measured in the supernatant using MUGlu substrate.

RESULTS

Cell cultivation procedures were standardized for comparison of the acid α-glucosidase activity in fibroblasts from controls and from patients with the different clinical forms of glycogenosis II. Figure 1 illustrates that the specific activity of acid α-glucosidase for each of the substrates increased in fibroblasts from controls and patients with the late onset form of glycogenosis II during the 4 week cultivation. During this period, the amount of protein per flask doubled. The lowest acid α-glucosidase activity in fibroblasts from adult patients (cases 1–4) was 7% of the mean control value (case 4); the highest activity was 22% (case 2) irrespective of the substrate used (fig. 1 and table 1). The activity in the juvenile form (case 5) was below the level of detection in the assays with maltose or glycogen, but 1%–2% residual activity was found with MUGlu as substrate. In cells from all seven patients with the infantile form of glycogenosis II (cases 6–12), the activity was less than 1% for all three substrates. The residual activity of acid α-glucosidase at all periods of cultivation is higher in fibroblasts from patients who survived to a more advanced age, and the specific activities for maltose, glycogen, and MUGlu were always reduced to the same extent.

The mean glycogen content was 0.5 mg/mg protein in fibroblasts from patients with glycogenosis II, over the whole period of cultivation, and 0.25 mg/mg protein in normal fibroblasts. No significant differences in glycogen content were observed among cells from different types of patients. The accumulation of glycogen in skeletal muscle, however, was less in patients with a late onset of symptoms than in patients with the infantile type of glycogenosis II (table 1).

A pH profile was made of the α-glucosidase activity in the various cell strains, using three different substrates. Figure 2 shows that the activity of the neutral enzyme is highest towards MUGIu substrate; with maltose the activity is much less and with glycogen its activity is not detectable. The pH profiles further demonstrate that the genetic defect in glycogenosis II only involves acid α-glucosidase. The activity of neutral α-glucosidase was similar in fibroblasts from the different types of patients and from controls (table 1). By comparing the different pH profiles, we concluded that the activity measured at pH 4.4 in fibroblasts from adult patients must be due to residual activity of acid α-glucosidase.

The K_m values of acid α-glucosidase did not vary among the adult patients: the mean K_m for MUGlu was 1.1 mM, for maltose, 11.9 mM, and for glycogen, 29.7 mM. These values are comparable with those of acid α-glucosidase in control cells.

Also, heat inactivation studies did not reveal a different behavior of the normal and

FIG. 1.—Acid α-glucosidase activity in fibroblasts after different periods of cultivation with the following substrates: MUGlu (*left*), maltose (*middle*), and glycogen (*right*). ■—■ = mean activity of four control strains with indication of extreme values; ●—● = adult patients (cases 1–4); ▲—▲ = juvenile patient (case 5); and O—O = infantile patients (cases 6–12).

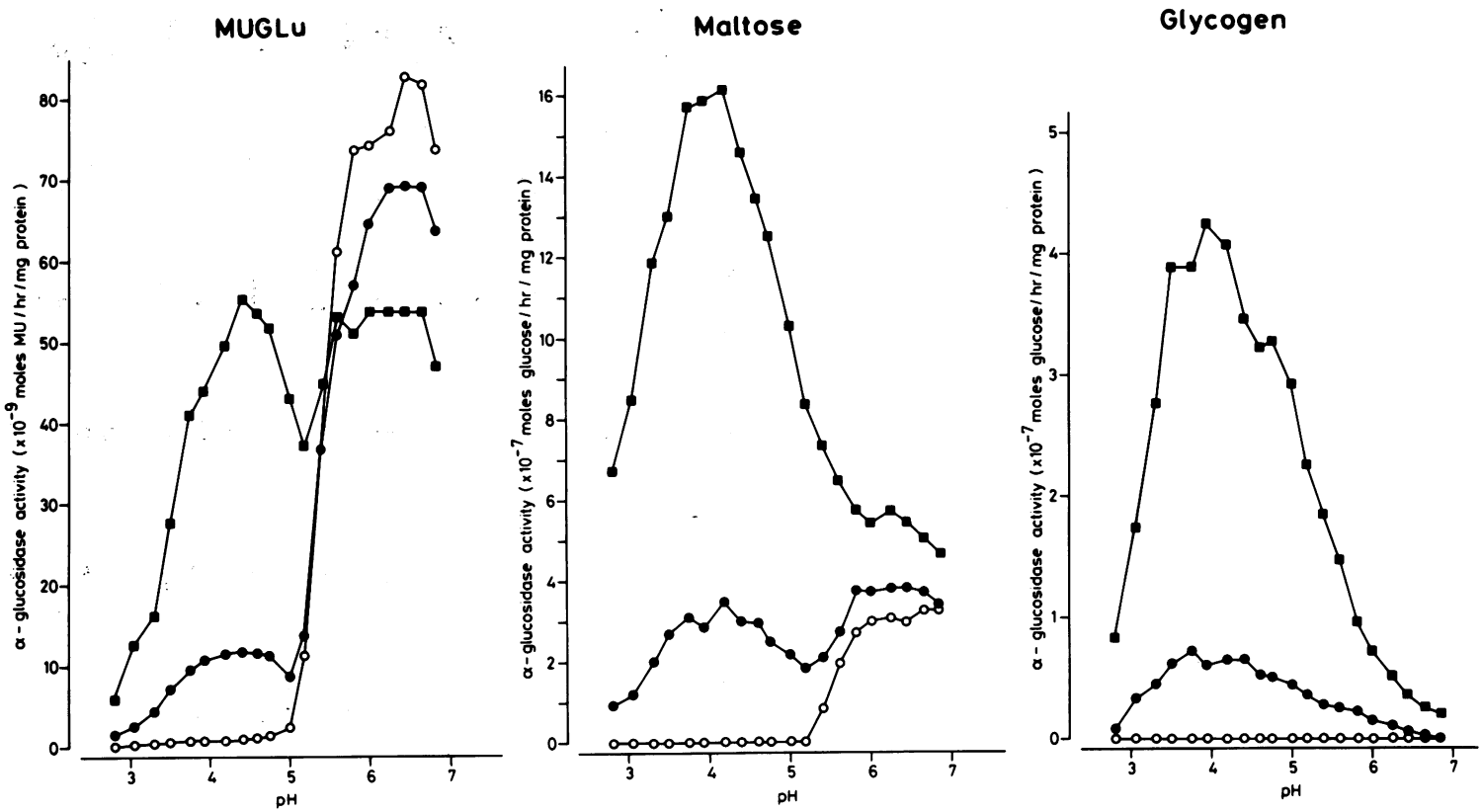
MUGLu
Maltose
Glycogen
α - glucosidase activity (x10⁻⁹ moles MU / hr / mg protein)
α - glucosidase activity (x10⁻⁷ moles glucose / hr / mg protein)
α - glucosidase activity (x10⁻⁷ moles glucose / hr / mg protein)
pH
pH
pH

FIG. 2

TABLE 1

CLINICAL AND LABORATORY DATA OF PATIENTS WITH VARIOUS FORMS OF GLYCOGENOSIS TYPE II

CODE	TYPE	AGE OF ONSET	AGE	ACTIVITY IN FIBROBLASTS*		MUSCLE† GLYCOGEN
				Acid α-Glucosidase	Neutral α-Glucosidase	
1	adult	± 35 yr	56 yr	2.1	74	...
2	adult	± 35 yr	48 yr	3.2	62	...
3	adult	± 14 yr	32 yr‡	1.6	77	107
4	adult	± 11 yr	26 yr	1.4	59	622
5	juvenile	unknown	18 yr‡	< 0.2	61	...
6–12	infantile	3 months	< 2 yr‡	< 0.2	66 (no. = 7)	1,300 (no. = 7)
Controls	...	...	...	14	69 (no. = 4)	32–79 (no. =13)

* Mean activities after 4 weeks cultivation: acid α-glucosidase measured with glycogen as substrate at pH 4.4, expressed as x 10^{-7} mol glucose/hr/mg protein; neutral α-glucosidase measured with MUGlu as substrate at pH 6.5, expressed as x 10^{-9} mol MU/hr/mg protein.

† Amount expressed in μg glycogen/mg protein.

‡ Deceased.

the "mutant" enzyme (fig. 3). The electrophoretic pattern of the mutant enzyme in fibroblasts from the adult patients cannot be distinguished from normal acid α-glucosidase (fig. 4). No band of activity was detected in a cell homogenate from the infantile type.

The nature of the residual acid α-glucosidase activity in adult patients with glycogenosis II was further investigated immunologically with an antiserum raised against acid α-glucosidase from human liver. This antiserum does not crossreact with the neutral enzyme. Homogenates of control fibroblasts and fibroblasts from patients with the adult and juvenile forms of glycogenosis type II were diluted to obtain equal acid α-glucosidase activity per ml. Increasing amounts of antiserum were subsequently added to fixed amounts of cell homogenates. Figure 5 shows that the mutant enzyme precipitates equally well as the normal enzyme using these conditions. The normal enzyme precipitates slightly better than the mutant enzyme when the protein concentration of the control homogenate is increased, while the activity is kept constant by adding a homogenate of cells from a patient with the infantile form of the disease. The precipitation curve of the normal enzyme (open squares) then parallels the curve obtained when the control homogenate is used in the same dilution as those from the patients (not shown). From the latter, we conclude that little cross reactive material is present in fibroblasts from patients with the infantile form and that the decrease in enzyme activity in the late onset forms is predominantly due to a reduction of the number of enzyme molecules rather than to a reduced catalytic activity per molecule.

To investigate whether different gene mutations are involved in the various clinical forms of glycogenosis II, complementation studies were performed after somatic cell hybridization. Fibroblasts from patients of the adult, juvenile, and infantile types were

FIG. 2.—The pH profile of α-glucosidase activity with different substrates: MUGlu (*left*), maltose (*middle*), and glycogen (*right*). ■—■ = control; ●—● = adult patient; and ○—○ = infantile patient.

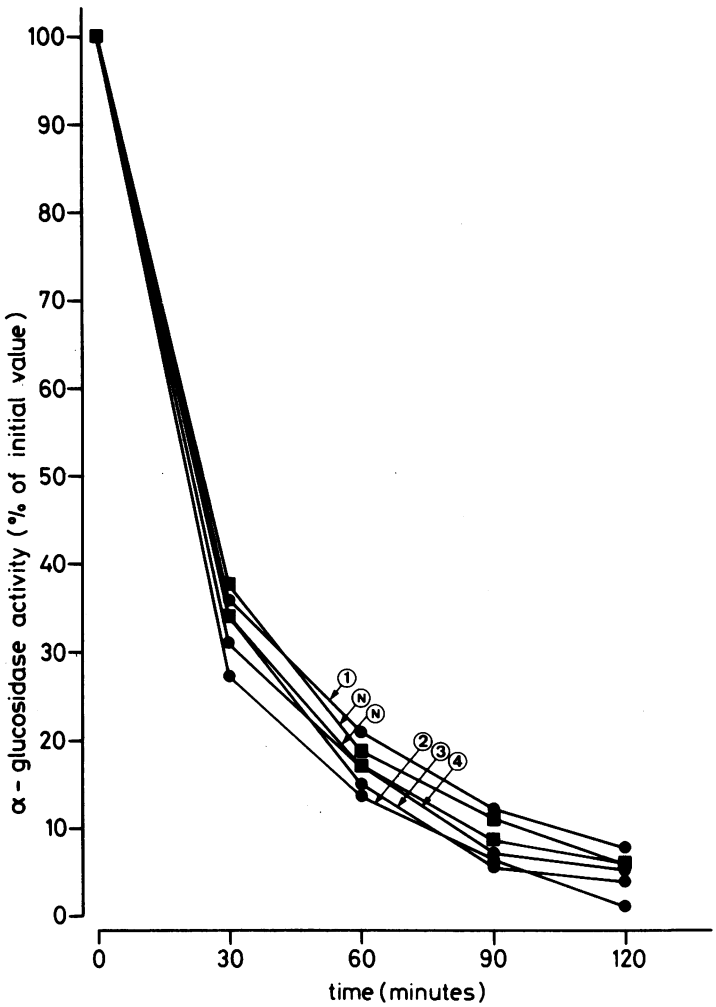

FIG. 3.—Heat inactivation of acid α-glucosidase. Experimental conditions: 56°C; 0.2 M sodium acetate (pH 4.4); protein concentration in each of the samples 1 mg/ml. ■—■ = controls; ●—● = adult patients; and nos. = cases listed in table 1.

fused in different combinations using inactivated Sendai virus. The activity of acid α-glucosidase was measured 2–8 days after fusion and subsequent cultivation. More than 20 such combinations were tested and the activity in each case was measured with MUGlu, maltose, and glycogen as substrate. No increase of acid α-glucosidase activity was observed in any of these complementation experiments.

DISCUSSION

The present study shows that there is a significant difference in the level of residual acid α-glucosidase activity in cultured fibroblasts from patients with the adult form and

those with the infantile form of glycogenosis II. In cells from the infantile form, the activity always remained below 1% of the control values despite prolonged cultivation. In contrast, the activity in fibroblasts from patients with the adult form varied between 7% and 22%. The activity of acid α-glucosidase in the juvenile form (case 5) was very close to that of the infantile cases, but no overlap with this latter category was found. This inverse correlation between the level of residual activity and the severity of the disease appears to hold within the group of adult patients. The activity measured at pH 4.4 is not due to the neutral enzyme as was demonstrated by the pH profile as well as by the fact that the activity at this pH was removed by the antiserum raised against purified acid α-glucosidase. Normal activities of neutral α-glucosidase were found in fibroblasts from all types of patients. It therefore seems unlikely that the neutral enzyme plays a role in the clinical expression of glycogenosis II as suggested by some investigators [6, 17, 18].

No differences could be detected between the physicochemical properties of the enzyme in fibroblasts from adult patients with glycogenosis II and controls. Electrophoresis of the mutant enzyme showed one band with the same mobility as normal acid α-glucosidase. The activity of the mutant enzyme for MUGlu, maltose, and glycogen was reduced to the same extent. These combined data indicate that the mutation in the late onset forms does not directly interfere with the conformation of the different catalytic centers of the enzyme [19, 20, 23]. Our immunological studies support this view because the amount of cross reacting material decreased proportionally with the enzyme activity, and the hydrolytic activity per molecule did not differ significantly. These data suggest that the acid α-glucosidase deficiency is caused by a decreased rate of synthesis of the enzyme or by a structural alteration resulting in an increased rate of degradation. The infantile form of glycogenosis II might represent an extreme example of this situation since virtually no enzymatic activity could be detected in the patients' fibroblasts, and the amount of cross reacting material was estimated to be very low. This has also been reported by others [21–23].

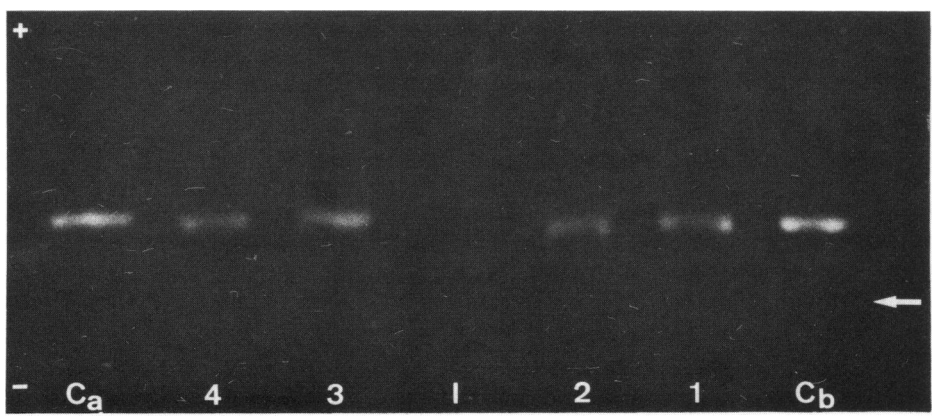

FIG. 4. — Cellulose acetate gel electrophoresis of acid α-glucosidase. Ca and Cb = control strains; I = infantile form of glycogenosis type II; and 1–4 = adult forms of glycogenosis type II.

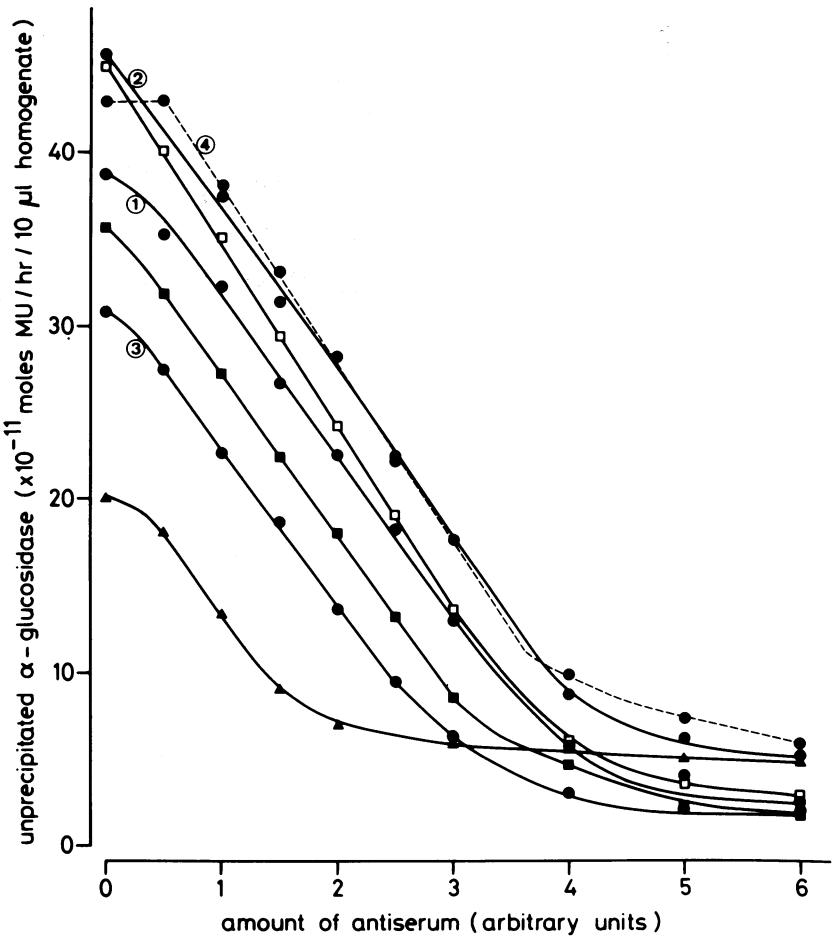

FIG. 5.—Immunoprecipitation of acid α-glucosidase. Increasing amounts of IgG were added to a fixed amount of crude cell homogenate. ■—■ = control fibroblasts (protein concentration 1.6 mg/ml); □—□ = mixture of control cells and cells from a patient with the infantile form of glycogenosis type II (protein concentration 6.1 mg/ml); ●—● = adult patients (protein concentration no. 1: 2.6 mg/ml, no. 2: 5.7 mg/ml, no. 3: 7.5 mg/ml, no. 4: 10.6 mg/ml); ▲—▲ = juvenile patient (protein concentration 15 mg/ml); and nos. = cases listed in table 1.

The different levels of residual activity in fibroblasts from patients with the infantile vs. the late onset forms of glycogenosis II might be due to allelic mutations. Such genetic heterogeneity could not be demonstrated in our somatic cell hybridization studies. No increase in acid α-glucosidase activity was found (1) after fusion of cells from different clinical variants followed by analyses of cell homogenates containing nonfused mononuclear cells and heterokaryons or (2) in microchemical assays on single binuclear [12]. In other lysosomal storage diseases, complementation analyses are useful in demonstrating different gene mutations [11, 24, 25].

If the finding of residual α-glucosidase activity in fibroblasts from all patients with

the adult type of glycogenosis II tested might be extrapolated to the activity in skeletal muscle, this would explain why accumulation of glycogen in this tissue is less severe in the adult than in the infantile form (table 1) [6, 8–10]. More investigations on the α-glucosidase activity in muscle biopsies from patients with late onset forms are required before definite conclusions can be made. Cardiac involvement seems to be a quantitative rather than a qualitative difference between the adult and infantile form since patients have been described where the heart was "slightly" involved or where cardiac symptoms gradually developed with increasing age [7, 26, 27]. In fibroblasts, the glycogen accumulation was found to be similar in various types of glycogenosis II despite the different levels of acid α-glucosidase activity. However, the extent of glycogen storage in various tissues in vivo will not only depend on acid α-glucosidase activity but also on carbohydrate supply, turnover of extra lysosomal glycogen, hormonal control, and rate of cell renewal [28]. In this respect, the cultured fibroblasts do not seem to be a suitable model for studying glycogen storage, but they provide a proper tool for the biochemical and genetic analysis of the normal and mutant enzymes.

SUMMARY

Fibroblasts from patients with the adult, juvenile, and infantile form of glycogenosis type II (Pompe disease) were cultured under standardized conditions, and the activity of acid α-glucosidase (E.C.3.2.1.20) towards glycogen, maltose, and 4-methylumbelliferyl-α-D-glucopyranoside was measured. Glycogen levels in muscle biopsies and in cultured fibroblasts from patients were determined. Residual enzyme activities varying from 7%–22% were detected in fibroblasts from patients with the adult form but not from patients with the infantile form of glycogenosis II. An inverse correlation was found between the severity of the clinical manifestation and the degree of residual enzyme activity in the fibroblasts. The kinetic and electrophoretic properties of acid α-glucosidase in fibroblasts from the adult patients and from control individuals were similar. Immunological studies suggested that the decrease of acid α-glucosidase activity is caused by a mutation that affects the production or degradation of the enzyme rather than its catalytic activity. Complementation studies were carried out by fusing fibroblasts from patients with the adult, juvenile, and infantile form of glycogenosis II, but neither conventional assays on multikaryons nor enzyme assays on single binuclear heterokaryons gave any evidence for genetic heterogeneity among these forms.

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