Comparative study on glucocerebrosidase in spleens from patients with Gaucher disease

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In Gaucher disease (glucosylceramide lipidosis), deficiency of glucocerebrosidase causes pathological storage of glucosylceramide, particularly in the spleen. A comparative biochemical and immunological analysis has therefore been made of glucocerebrosidase in spleens from normal subjects ($n = 4$) and from Gaucher disease patients with non-neuronopathic ($n = 5$) and neuronopathic ($n = 5$) phenotypes. The spleens from all Gaucher disease patients showed markedly decreased glucocerebrosidase activity. Discrimination of different phenotypes of Gaucher disease was not possible on the basis of the level of residual enzyme activity, or by measurements, using the immunopurified enzyme, of kinetic constants, pI or molecular mass forms. A severe decrease was found in the specific activity of glucocerebrosidase purified to homogeneity from the spleen of a patient with the non-neuronopathic phenotype of Gaucher disease, as compared with that of the enzyme purified from the spleen of a normal subject. This finding was confirmed by an immunological method developed for accurate assessment of the relative enzyme activity per molecule of glucocerebrosidase protein. The method revealed that the residual enzyme in the spleens of all investigated patients with a non-neuronopathic course of Gaucher disease had a more than 7-fold decreased activity of glucocerebrosidase (measured in the presence of taurocholate) per molecule of enzyme, and that the concentration of glucocerebrosidase molecules in the spleens of these patients was near normal. Observations made with immunoblotting experiments were consistent with these findings. In contrast, in the spleens of patients with neuronopathic phenotypes of Gaucher disease, the concentration of glucocerebrosidase molecules was severely decreased.

INTRODUCTION

Gaucher disease is a recessively inherited lysosomal storage disorder that is characterized by accumulation of glucocerebroside (glucosylceramide) due to a deficiency of glucocerebrosidase activity (Barranger & Ginns, 1989). The disorder is heterogeneous in clinical presentation. Three variants of the disease are recognized on the basis of clinical manifestations: type 1, the non-neuronopathic form; type 2, the acute neuronopathic form; and type 3, the subacute neuronopathic form (Barranger & Ginns, 1989). So far there has been no documentation of a correlation between the clinical manifestation of Gaucher disease and residual glucocerebrosidase activity measured in cells and tissues from patients (Wenger & Olsen, 1981; Barranger *et al.*, 1984; Barranger & Ginns, 1989).

Studies on the molecular properties of glucocerebrosidase in relation to Gaucher disease have largely used skin fibroblasts. Differences have been noted in the molecular mass forms (Ginns *et al.*, 1982) and in the maturation and stability (Jonsson *et al.*, 1987) of glucocerebrosidase in cells from patients with neuronopathic phenotypes of Gaucher disease, as compared with non-neuronopathic phenotypes. However, since fibroblasts are not involved in the pathology of Gaucher disease, additional studies concerning the properties of glucocerebrosidase in tissues actually affected in Gaucher disease are of importance. Because the spleen is an organ that is involved in the pathology of all phenotypes of Gaucher disease, we have carried out a comparative study of the properties of the residual glucocerebrosidase

in the spleens of normal subjects and of patients with different phenotypes of Gaucher disease.

MATERIALS AND METHODS

Materials

All spleens were obtained either as surgical specimens during therapeutic splenectomy or at autopsy. The phenotype of the patients was established by clinical examination. The ethnic background of the patients with the non-neuronopathic phenotype of Gaucher disease could not be established with certainty.

Glucocerebroside was labelled in the glucose moiety with ^{14}C as described by Ginns *et al.* (1982). Conduiritol β -epoxide was kindly supplied by Dr. N. Radin, Dr. A. Gal and Dr. G. Legler. Sodium taurocholate (Grade A) was from Calbiochem (San Diego, CA, U.S.A.), 4-methylumbelliferyl substrates from Koch-Light (Colnbrook, Bucks., U.K.) and Sigma (St. Louis, MO, U.S.A.), CNBr-activated Sepharose 4B from Pharmacia (Uppsala, Sweden) and rabbit anti-(mouse IgG) from Nordic (Tilburg, The Netherlands). All other reagents were of the purest grade available. Antiserum against purified placental glucocerebrosidase was raised in a rabbit, as described by Ginns *et al.* (1982). Monoclonal antibodies against glucocerebrosidase were obtained from mouse ascites or were purified from the culture medium of hybridoma cells, as described by Barneveld *et al.* (1983). The mixture of exoglycosidases was prepared from the culture medium of *Streptococcus pneumoniae* by differential

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ammonium sulphate precipitation according to Glasgow *et al.* (1977).

Preparation of splenic extracts

Spleens were homogenized in 2 vol. of 50 mM-potassium phosphate buffer (pH 6.5) containing 0.25% (v/v) Triton X-100, using an Ultra-Turrax and sonication. The homogenate was centrifuged for 1 h at 100 000 *g* and the supernatant (called the splenic extract) was collected.

Purification of glucocerebrosidase

Glucocerebrosidase was purified from crude tissue extracts using a 1:1 mixture of the anti-glucocerebrosidase monoclonal antibodies 8E4 and 2C7 immobilized on CNBr-activated Sepharose 4B beads, as described by Aerts *et al.* (1986a). On a small scale, glucocerebrosidase was isolated by incubation of tissue extracts with immunosorbent overnight at 4 °C with rotation. Unbound material was removed by centrifugation and repeated washing in potassium phosphate buffer (pH 6.5) containing 0.25% (v/v) Triton X-100. Next, the beads were washed successively with 1% (v/v) Triton X-100, 30% (v/v) ethylene glycol and 50% (v/v) ethylene glycol. Enzyme was eluted from the immunosorbent using 90% (v/v) ethylene glycol or by suspension in SDS/PAGE sample buffer and boiling of the suspension for 5 min. On a larger scale, enzyme was purified by immunoaffinity chromatography using the same immunosorbent and 90% (v/v) ethylene glycol as eluting agent, exactly as described by Aerts *et al.* (1986a).

Enzyme-binding assay

An identical amount of rabbit anti-(mouse IgG) was coated on to each well of a microtitre plate by incubation for 1 h at 37 °C with a solution containing 3 µg of the rabbit immunoglobulins/ml (150 µl/well). Next, an identical amount of anti-glucocerebrosidase monoclonal antibody 8E4 was coated on each well by incubation for 16 h at 4 °C with a solution containing 0.5 µg of the antibody/ml (125 µl/well). Unbound antibody was removed by washing in phosphate-buffered saline (1.38 mM-NaCl, 6.5 mM-Na₂HPO₄, 0.5 mM-KH₂PO₄ and 3 mM-KCl, pH 7.2) containing 0.05% (v/v) Tween-20. Next, the wells were incubated overnight at 4 °C and for 2 h at room temperature with a dilution range of splenic extracts prepared in 50 mM-potassium phosphate buffer, pH 6.5, containing 0.1% (v/v) Triton X-100 and 10 mg of ovalbumin/ml. Next, the wells were washed with the same buffer to remove unbound material. Bound glucocerebrosidase was detected by incubating the wells for 2 h at room temperature with β -glucosidase assay mixture, consisting of 100/200 mM-citrate/phosphate buffer (pH 5.2), 3.7 mM-4-methylumbelliferyl β -glucoside, 0.1% (v/v) Triton X-100 and 0.2% (w/v) sodium taurocholate. The reaction was stopped by addition of excess glycine/NaOH (pH 10.6) and the 4-methylumbelliferone formed was measured fluorometrically.

Isoelectric focusing, SDS/PAGE and immunoblotting

Preparative isoelectric focusing in granulated Ultradex gels, SDS/PAGE and immunoblotting were performed exactly as described by Aerts *et al.* (1985).

Enzyme assays

Glucocerebrosidase activity was measured with glucocerebroside (labelled with 1-¹⁴C in the glucose moiety) as substrate, as described by Barneveld *et al.* (1983), or as the β -glucosidase activity with 4-methylumbelliferyl β -glucoside as substrate that could be inhibited by conduritol β -epoxide (Aerts *et al.*, 1985). The activities of β -hexosaminidase, β -glucuronidase, α -glucos-

idase, α -fucosidase and α -galactosidase were measured with the corresponding 4-methylumbelliferyl substrates.

Proportions of glucocerebrosidase in the form I and the form II states

Form I glucocerebrosidase is the conduritol- β -epoxide-inhibitable β -glucosidase activity which is precipitated by immobilized anti-glucocerebrosidase antibodies, whereas form II glucocerebrosidase is the conduritol- β -epoxide-sensitive activity which is not immunoprecipitated (Aerts *et al.*, 1987). The proportions of form I and form II glucocerebrosidase were measured in extracts by immunotitration, exactly as described by Aerts *et al.* (1985). Freshly prepared extracts were used, since form II, the aggregated form, is unstable and dissociates with time to give the monomeric form I enzyme (Aerts *et al.*, 1985).

Estimation of activator protein content

The activator protein known as sphingolipid activator protein 2 [SAP-2; saposin C; see Glew *et al.* (1988) for a review] was partially purified from extracts of spleen according to Peters *et al.* (1977) as follows. The extracts were boiled for 5 min and then centrifuged for 20 min at 50 000 *g*. The supernatant was adjusted to pH 4.7 with concentrated acetic acid and centrifuged for 20 min at 50 000 *g*. The supernatant was tested for its capacity to stimulate the enzymic activity of placental glucocerebrosidase purified according to Aerts *et al.* (1986a). For the test, placental enzyme (3–5 ng of protein) was assayed in the presence of 25 µl of activator protein preparation in 50 mM-acetate buffer (pH 4.5) containing 0.1% (v/v) Triton X-100, 3.7 mM-4-methylumbelliferyl β -glucoside and 10 mg of bovine serum albumin/ml. One unit of activator protein is defined as the amount which stimulates the enzyme activity by 100% in this assay.

RESULTS

Analysis of splenic extracts

Spleen was extracted in 4 vol. of extraction buffer as described in the Materials and methods section. Table 1 summarizes the results obtained for spleens from normal subjects and from patients with different phenotypes of Gaucher disease.

Level of glucocerebrosidase. In all Gaucher disease spleens investigated, a clear decrease in glucocerebrosidase activity assayed with 4-methylumbelliferyl β -glucoside as substrate was detected, the residual level always being less than 15% of the mean control value. Similar findings were made when glucocerebrosidase was assayed with radioactively labelled glucosylceramide (results not shown). The level of residual glucocerebrosidase was on average somewhat lower in extracts of spleens from patients with neuronopathic variants of Gaucher disease (types 2 and 3) compared with that observed in extracts of spleens from type 1 patients. However, an absolute correlation between phenotype and residual level of enzyme activity was not noted.

Level of activating protein. It has been known for some years that the enzymic activity of glucocerebrosidase *in vitro* is increased by interaction of the enzyme with another lysosomal protein variously referred to as factor P (Ho & O'Brien, 1971) coglucosidase (Radin & Berent, 1982) sphingolipid-activating protein 2 (Fujibayashi & Wenger, 1985) or, more recently, as saposin C (O'Brien *et al.*, 1988).

We have measured the level of activating protein in spleen extracts as described in the Materials and methods section. None of the 'activator fractions' prepared from control spleens showed a significant capacity to stimulate the activity of purified

Table 1. Characterization of spleen proteins from control subjects and patients with different phenotypes of Gaucher disease

Extracts of spleen were prepared exactly as described in the Materials and methods section. Protein and glucocerebrosidase activity with 4-methylumbelliferyl β -glucoside as substrate were determined as described by Aerts *et al.* (1987). The proportions of form I and form II glucocerebrosidase were determined as described by Aerts *et al.* (1987). The activator protein content was determined as described in the Materials and methods section. The concentrations of two platelet-specific antigens [platelet factor 4 (PF-4) and β -thromboglobulin (β -TG)] were measured by enzyme-linked immunoassays. Activities of the acid hydrolases were measured as described in the Materials and methods section. Abbreviations: α -Glu, α -glucosidase; α -Gal, α -galactosidase; β -Gluc, β -glucuronidase; β -Hex, β -hexosaminidase; n.d., not determined.

Spleen from:	Protein (mg/ml of extract)	Glucocerebrosidase (nmol/h per mg)	Form II (%)	Activator protein* (relative amount)	Platelet-specific antigen (ng/ml)			Lysosomal hydrolase (nmol/h per mg)		
					β -TG	PF-4	β -Hex	β -Gluc	α -Glu	α -Gal
Control subjects	27	20.8	12	0.18	7185	6019	388	358	43	42
	44	10.8	8	0.05	4049	3426	700	293	70	35
	10	16.0	19	0.30	6179	2647	134	170	12	15
	28	12.8	5	0.03	601	237	371	286	47	58
Type I patients	23	2.8	25	2.41	3010	1939	2230	1224	75	48
	30	2.1	31	3.12	5735	3945	2162	1926	70	68
	20	1.9	20	1.80	267	161	568	1328	103	65
	20	1.5	15	0.45	4414	3106	1150	640	35	63
	7	1.4	n.d.	n.d.	n.d.	n.d.	544	192	25	13
	11	1.8	n.d.	n.d.	n.d.	n.d.	848	448	28	16
Type II patients	20	2.6	19	1.40	2034	1465	1350	2800	54	98
	24	1.3	28	2.80	7007	5589	1864	1332	66	36
Type III patients	21	3.2	12	1.00	1125	1390	1150	1490	79	87
	29	0.8	29	2.15	1199	535	2288	2740	104	88
	28	2.1	24	2.00	4480	4168	1568	1780	38	102

* Defined as the amount of activator protein which stimulates purified glucocerebrosidase by 100% in the standard assays with 25 μ l of purified activator preparation.

glucocerebrosidase in the stimulation assay described in the Materials and methods section (Table 1). In contrast, comparable fractions prepared from spleens from most Gaucher disease patients showed a considerable capacity to stimulate glucocerebrosidase (Table 1). It should be noted that large differences exist in this parameter between spleens from individual Gaucher disease patients. In agreement with previous observations (Peters *et al.*, 1977), the extent of the increase in the amount of activator protein in Gaucher disease spleen did not correlate with the clinical phenotype.

Proportions of form I and form II glucocerebrosidase. We have shown previously that glucocerebrosidase may be solubilized from the spleen in two forms that differ in enzymic, physicochemical and immunological properties (Aerts *et al.*, 1985). A proportion of the enzyme is extracted in an immunoprecipitable monomeric form (form I) and the rest is present in an activated state as a component of a non-immunoprecipitable aggregate (form II) that also includes sphingolipid activator protein 2 (Aerts *et al.*, 1987; Sa Miranda *et al.*, 1988). The contribution of form II glucocerebrosidase to the total glucocerebrosidase activity in freshly prepared extracts of spleen was found to be higher on average in splenic extracts from Gaucher patients compared with that from control subjects (Table 1). However, no correlation was observed between the clinical phenotype of Gaucher disease and the percentage of glucocerebrosidase present as form II.

Some correlation exists between the proportion of form II glucocerebrosidase in splenic extracts and the level of activating protein in fractions prepared from the same splenic extracts (Table 1). A high percentage of form II enzyme in extracts is accompanied by a high capacity of the corresponding activator protein preparation to stimulate glucocerebrosidase activity.

Estimation of platelet concentration of spleens. We have observed that platelets are particularly rich in protein(s) able to

activate glucocerebrosidase *in vitro* (J. M. F. G. Aerts & S. Brul, unpublished work). An increase in the platelet content of spleen could thus be responsible for the relative increases in activating protein and form II glucocerebrosidase. Indeed, it has been known for some time that the absolute number of platelets is increased in spleens of Gaucher disease patients compared with the normal situation (Aster, 1966). However, it is not yet clear whether the platelet concentration (i.e. number of platelets per volume of spleen) is also significantly increased. We therefore determined the concentrations of two platelet-specific antigens in extracts of the various spleens. β -Thromboglobulin and platelet factor 4 (PF-4) were determined with respectively a β -thromboglobulin radioimmunoassay (Amersham International, Amersham, Bucks., U.K.) and a PF-4 radioimmunoassay (Abbot Laboratories, North Chicago, IL, U.S.A.) (Kaplan *et al.*, 1978). No correlation was observed between platelet-specific antigen concentration on the one hand and, on the other hand, Gaucher disease status, activating protein content or percentage of form II glucocerebrosidase (Table 1).

Level of lysosomal hydrolases. Table 1 shows that the levels of a number of lysosomal enzymes were considerably increased in spleens from most patients with Gaucher disease. A clear increase was also noted for β -galactosidase, α -fucosidase and α -mannosidase, but not for sphingomyelinase and ceramidase (results not shown).

However, discrimination between different phenotypes of Gaucher disease is not possible on the basis of these parameters. It should be noted that the general increase in lysosomal enzyme levels is highest in those spleens that also show a high level of activating protein.

Relative specific activity and concentration of glucocerebrosidase

An immunological enzyme-binding assay carried out with monoclonal antibody 8E4 was used to establish the relative

Fig. 1. Comparison of specific activities of glucocerebrosidase by immunobinding of enzyme activity

The amount of glucocerebrosidase activity bound by a fixed amount of anti-glucocerebrosidase monoclonal antibody 8E4 upon incubation with increasing dilutions of a splenic extract was determined by an enzymic assay (for experimental details, see the Materials and methods section). Bound glucocerebrosidase activity is plotted against dilution range of extracts of spleens. (a) Extracts of spleen of control subjects (●) and type 1 Gaucher disease patients (○); (b) extracts of spleen of Gaucher type 2 (□) and type 3 patients (◇); (c) extracts of a control subject (●), of a type 1 patient (○), and of a 1:1 mixture of the extracts of the control subject and type 1 patient (■).

specific activity per unit of glucocerebrosidase antigen in extracts of various spleens. The principle of the method is as follows. The wells of a microtitre plate are coated with a fixed, limiting amount of antibody, a titration is carried out with different amounts of glucocerebrosidase, and the enzyme activity bound in each well is measured. As shown in Fig. 1(a), at the highest enzyme concentrations, near-equal amounts of glucocerebrosidase activity are bound per well, confirming that the amount of immobilized monoclonal antibody is limiting. We established that the affinities of normal and type 1 glucocerebrosidase for immobilized monoclonal antibody 8E4 are similar; when extracts of spleens from type 1 patients and controls were mixed in a 1:1 ratio, an enzyme-binding curve was obtained which was almost exactly intermediate (Fig. 1c). This can only occur if the normal and mutant enzymes compete in a comparable manner for binding to immobilized antibody. Thus, since the affinities of mutant and normal glucocerebrosidases for immobilized monoclonal antibody 8E4 are equal, it is possible to compare the catalytic activities of normal and mutant enzymes per unit of antigen by comparing the amounts of enzyme activity that are maximally bound by a limiting amount of immobilized antibody.

It can be seen in Fig. 1(a) that the extracts prepared from different control spleens show very similar enzyme-binding curves. The maximal amount of enzyme activity bound, i.e. the amount of activity at the highest concentrations of enzyme added, was very similar for different control spleens, indicating that the catalytic activity per molecule of antigen is similar. Fig. 1(a) further shows that extracts of spleens from the non-neuronopathic (type 1) patients investigated all gave similarly shaped enzyme-binding curves. The maximal activity bound was, however, about 7–20-fold less than that in extracts from control spleens, indicating that in all the investigated cases, type 1 glucocerebrosidase was catalytically about 7–20-fold less efficient than the normal enzyme.

Finally, Fig. 1(b) reveals that the enzyme-binding curves for extracts of spleens from the neuronopathic Gaucher disease patients (types 2 and 3) examined were essentially different from

those observed for type 1 Gaucher disease cases. Even at the highest concentrations of glucocerebrosidase added, the wells were not saturated with antigen. In principle, this could be explained in two ways. Firstly, the number of glucocerebrosidase molecules in type 2 and 3 spleens could be far lower than that in type 1 and control spleens. Alternatively, the binding affinity of type 2 and 3 glucocerebrosidases could be far lower than that of the type 1 or control enzyme. We prefer the first explanation, at least for type 3 Gaucher disease, because the shapes of the type 3 enzyme-binding curves are exactly as would be predicted for an enzyme of normal affinity which was present at very low concentration. The activity of glucocerebrosidase in the type 2 spleens was too low to allow meaningful conclusions to be drawn.

Combining the results of Fig. 1 with the data on glucocerebrosidase activity in the extract used in the experiment allows calculations to be made of the concentration of glucocerebrosidase antigen in the various splenic extracts. These results are shown in Table 2. The mean of the maximal glucocerebrosidase activity bound in control spleen extracts was defined as the 100% value for the relative specific activity of normal glucocerebrosidase. The mean amount of glucocerebrosidase activity per g wet weight of control spleen was $1.20 \pm 0.08 \mu\text{mol/h}$. By combining the value for the relative specific activity of glucocerebrosidase, as determined in Fig. 1, and the amount of glucocerebrosidase activity per g wet weight of spleen, the relative concentration of antigen in the spleen could be calculated. As shown in Table 2, the antigen concentrations in individual control spleens ranged from 78 to 130%.

In the case of spleens from patients with type 1 Gaucher disease, a correction must be made for the fact that the relative specific activity per antigen molecule is only 5–15% of the control value. In other words, although the type 1 spleens contain only 5–15% of the glucocerebrosidase activity found in

Table 2. Comparison of concentrations and relative specific activities of glucocerebrosidase molecules in spleens from control subjects and patients with different phenotypes of Gaucher disease

The mean value for the maximal amount of glucocerebrosidase activity bound by a fixed amount of monoclonal anti-glucocerebrosidase antibody 8E4 is defined as 100%; this is a measure of the turnover per molecule of control glucocerebrosidase antigen. This value, in combination with the mean glucocerebrosidase activity per mg wet weight of control spleen, is used to calculate the mean concentration of glucocerebrosidase molecules in control spleen, which is defined as 100%. For calculation of the relative concentrations of glucocerebrosidase antigen in individual spleens, see the text. For control subjects, means $\pm$ S.E.M. are given, with ranges in parentheses.

Subject	Glucocerebrosidase		
	Specific activity (nmol/h per mg wet weight of spleen)	Relative activity per molecule of antigen (%)	Relative concentration of antigen (%)
Controls	1.20 ± 0.08 (0.98–1.45)	100 ± 5 (90–105)	100 ± 12 (78–130)
Type 1 Gaucher	0.09 0.09 0.12 0.15 0.17	14.2 11.2 10.0 13.1 12.0	53 67 100 95 118
Type 3 Gaucher	0.06 0.07 0.09	> 42.8 > 36.0 > 33.1	< 12 < 16 < 22

control spleens, the concentration of antigen in type 1 spleens is 53–118 % of normal.

In the case of type 3 Gaucher disease, only an estimation of maximum antigen concentration can be made. Fig. 1 suggests that the relative specific activity per type 3 antigen is at least 40 % of that of the normal enzyme. It can thus be calculated that, in all three types 3 Gaucher disease splenic extracts, the antigen concentration was less than 18 % of that in comparable control spleen extracts. The actual value may be much lower, since the binding curve suggests that the catalytic activity per type 3 antigen may be much higher than 40 % of the control value. In a separate experiment, using a more concentrated type 3 enzyme preparation, we observed that the relative specific activity of the enzyme in this particular spleen was at least 80 % of the control value (results not shown).

Reliable estimation of the relative specific activity of type 2 glucocerebrosidase is not feasible, owing to the low amounts of enzyme (see Fig. 1). Consequently, a meaningful estimation of antigen concentrations in type 2 Gaucher disease spleens is not possible.

Studies on purified glucocerebrosidase

Specific activity of glucocerebrosidases purified from control and type 1 spleens by immunoaffinity chromatography. Glucocerebrosidase was purified from the spleens of a normal subject and of a type 1 Gaucher disease patient by immunoaffinity chromatography. The enzyme was purified to apparent homogeneity as indicated by SDS/PAGE and silver staining of the final enzyme preparations (results not shown). The results of the isolations are summarized in Table 3. The recovery of glucocerebrosidase was similar for control and Gaucher disease spleens, indicating that the isolation procedure does not differentiate between the control and mutant enzymes. The specific activity of the type 1 glucocerebrosidase studied was about 8-fold less than that of the control enzyme.

Kinetic parameters of immunopurified glucocerebrosidases from normal and Gaucher spleens. Glucocerebrosidase was purified on a small scale from the various spleens investigated by incubation

overnight at 4 °C of tissue extract (10 ml) with 8E4/2C7–Sephrose 4B beads. The incubation of extracts with immunosorbent overnight resulted in all cases in binding of 70–90 % of the enzyme activity to the beads.

It was noted previously for homogeneous placental glucocerebrosidase that a number of kinetic parameters, such as K_m values for artificial substrates and stimulation by taurocholate, were identical for the enzyme in solution and for the enzyme immobilized on immunobeads (Aerts *et al.*, 1986a). The kinetic properties of glucocerebrosidase in various splenic extracts were therefore established using enzyme purified by binding to immunobeads. K_m values with respect to 4-methylumbelliferyl β -glucoside, determined under the conditions described previously (Aerts *et al.*, 1985) were found to be between 1.7 and 2.1 mM for all enzyme preparations. In all cases the enzyme activity was stimulated by about 18-fold by 0.2 % (w/v) sodium taurocholate if assayed in the presence of 0.1 % (v/v) Triton X-100 at pH 5.2.

Molecular mass species of immunopurified glucocerebrosidase from normal and Gaucher spleens. Glucocerebrosidase from various splenic extracts was purified as described in the Materials and methods section. After SDS/PAGE the various forms of the enzyme were revealed by immunoblotting using a rabbit anti-(placental glucocerebrosidase) antiserum.

Fig. 2 shows that extracts of spleen from normal subjects contain species of glucocerebrosidase ranging in molecular mass from 58 to 66 kDa; these forms are similar to those previously observed in cultured skin fibroblasts (Ginns *et al.*, 1982; Jonsson *et al.*, 1987). Differences were observed in the relative intensity of the individual bands in spleen extracts from different normal individuals or even in those prepared from different samples of the same biopsies. In extracts of spleens from type 1 Gaucher disease patients, near-normal or slightly reduced amounts of antigen with comparable heterogeneity in apparent molecular mass were detected (Fig. 2). In sharp contrast, cross-reactive material was hardly detectable, if at all, in similar extracts of spleens from types 2 and 3 Gaucher disease patients (Fig. 2). In all cases 60–90 % of the glucocerebrosidase activity present in the original extract was recovered on the immunobeads before electrophoresis. The severe deficiency of glucocerebrosidase revealed by immunoblotting with a polyclonal antiserum in spleens from types 2 and 3 Gaucher disease, but not in the case of type 1 Gaucher disease spleens, reflects the actual situation in

Table 3. Isolation of glucocerebrosidase from control and mutant spleens by immunoaffinity chromatography

Extracts were prepared from spleens as described in the Materials and methods section. The preparations were subjected to immunoaffinity chromatography using immobilized anti-glucocerebrosidase monoclonal antibodies as immunosorbent and 90 % (v/v) ethylene glycol as eluting agent, exactly as described by Aerts *et al.* (1986a)

Parameter	Spleen from	
	Control subject	Type 1 patient
Wet weight of spleen sample (g)	150	500
Activity in extract (μ mol/h)	185	105
Activity after first immunoaffinity step (μ mol/h)	117	77
Activity after second immunoaffinity step (μ mol/h)	113	66
Recovery (%)	61	63
Specific activity of final preparation (nmol/h per mg of protein)	3.1	0.4
Purification (-fold)	220 000	280 000

Fig. 2. Molecular mass species of glucocerebrosidase purified from spleen

Molecular mass species of immunopurified glucocerebrosidase were revealed by SDS/PAGE and immunoblotting. The glucocerebrosidase present in an extract of 0.15 g wet weight of spleen was immunoprecipitated with anti-glucocerebrosidase monoclonal antibodies 8E4 and 2C7 immobilized by coupling to CNBr-activated Sepharose. Enzyme activity bound to the immunosorbent was measured as described by Aerts *et al.* (1986b). Glucocerebrosidase was eluted from the immunosorbent by boiling in sample buffer and was then subjected to SDS/PAGE. Protein was transferred electrophoretically to nitrocellulose filters, and glucocerebrosidase was revealed after incubation with rabbit anti-glucocerebrosidase using the peroxidase–second antibody conjugate technique. The glucocerebrosidase activity (nmol/h) applied to each lane was as follows: lane a, 180; b, 140; c, 125; d, 140; e, 15; f, 14; g, 6; h, 11; i, 16; j, 12.

Fig. 3. Isoelectric point of glucocerebrosidase purified from spleen: effect of exoglycosidases

Glucocerebrosidase purified by immunoaffinity chromatography was subjected to native isoelectric focusing in Ultrodex flat-bed gels as described by Aerts *et al.* (1986a). The gel was fractionated and glucocerebrosidase activity was measured in the individual fractions. Enzyme preparations were incubated overnight at 37 °C with a mixture of exoglycosidases consisting of neuraminidase, β -galactosidase and β -hexosaminidase. The recovery of glucocerebrosidase activity was always more than 70%. The effect of exoglycosidase treatment on the apparent isoelectric point was determined using isoelectric focusing in flat-bed gels exactly as described above. The isoelectric point profiles of glucocerebrosidase activity of enzyme purified from the spleen of a normal subject (a), of a type 1 Gaucher disease patient (b) and of a type 3 Gaucher disease patient (c) before (○) and after (●) exoglycosidase treatment are shown.

the spleens, and confirms the results obtained using the enzyme-binding assay (Fig. 1).

Isoelectric point species of glucocerebrosidase immunopurified from control and Gaucher spleens. As shown in Fig. 3, immunopurified enzyme from type 1 Gaucher spleen is similar to that from the control spleen with respect to the isoelectric point species seen. A different profile was observed for the type 3 immunopurified enzyme, with peaks at pI 5, pI 6.0–6.4 and pI 7.0–7.2.

Effect of deglycosylation on glucocerebrosidases. Upon incubation of purified glucocerebrosidase from control and types 1 and 3 Gaucher disease spleens with a mixture of exoglycosidases (neuraminidase, β -galactosidase and β -hexosaminidase), a single major isoelectric point form (pI 7.0–7.2) was formed (Fig. 3). This indicates that in the spleen, as in other tissues (Aerts *et al.*, 1986b), the heterogeneity in apparent isoelectric points of control and Gaucher glucocerebrosidases is caused by heterogeneous oligosaccharide chain composition.

DISCUSSION

Discrimination between different phenotypes of Gaucher disease has not proved feasible on the basis of the extent of residual glucocerebrosidase activity or the level of protein(s) able to activate the enzyme in extracts from spleens from Gaucher disease patients (cf. Barranger & Ginns, 1989). Several groups have investigated additional biochemical parameters. These include the relative specific activity of mutant glucocerebrosidase as measured in the presence of taurocholate and the residual concentration of glucocerebrosidase antigen. With respect to the relative specific activity of the enzyme in extracts of spleen, mean values of 24% of control in non-neuronopathic cases and of 4% in neuronopathic cases have been reported (Pentchev *et al.*, 1983). Other studies suggest identical values for this parameter in fibroblasts of all variants, the values ranging from 30–60% (Beutler *et al.*, 1984) to 4–8% (Grabowski *et al.*, 1985). With respect to the residual concentration of glucocerebrosidase antigen, a 40–60% decrease has been reported in spleens from all Gaucher disease variants (Pentchev *et al.*, 1983), whereas in fibroblasts less than 13% of the antigen was noted for neuronopathic cases and 15–60% for non-neuronopathic cases, compared with controls (Beutler *et al.*, 1984).

Another parameter that has been studied is the stability of newly synthesized enzyme in cultured fibroblasts. Metabolic labelling experiments indicated that the type 1 Gaucher disease fibroblasts investigated contained near-normal amounts of glucocerebrosidase antigen (Jonsson *et al.*, 1987). In contrast, greatly decreased amounts of enzyme protein were observed in fibroblasts from neuronopathic cases due to instability of the enzyme (Jonsson *et al.*, 1987). This instability was particularly marked in fibroblasts from type 2 patients. In the light of these findings an alternative explanation is possible for the earlier conclusion of Ginns *et al.* (1983) that in type 2 fibroblasts, there is an absence of material cross-reacting with monoclonal antibody 8E4; the amount of antigen may have been too low in these cells for detection by the immunoblotting procedure employed. More recent studies have revealed that there are exceptions with respect to apparent normal stability of glucocerebrosidase in cultured fibroblasts from type 1 Gaucher disease patients (Bergmann & Grabowski, 1989; Van Weely *et al.*, 1989).

In this study, the relative specific activity of glucocerebrosidase and the relative concentration of glucocerebrosidase protein were studied in spleen. Using two independent methods it was observed that in the spleens of the five type 1 Gaucher disease patients investigated, glucocerebrosidase was markedly defective catalytically when measured in the presence of taurocholate, whereas the concentration of antigen was not greatly altered (see also Willemsen *et al.*, 1988). Direct determination of the specific activity of purified type 1 glucocerebrosidase substantiated these findings. In the spleens of three type 3 Gaucher disease patients, the concentration of antigen was found to be markedly decreased whereas the relative specific activity per molecule of antigen did not appear to be greatly altered. These findings are consistent with our previous observations made with respect to differences in the stability of glucocerebrosidase in cultured fibroblasts from the majority of patients with non-neuronopathic phenotype on the one hand and from patients with neuronopathic phenotypes on the other hand (Jonsson *et al.*, 1987). However, in the light of the occurrence of some cases of type 1 Gaucher disease showing abnormal lability of the enzyme in cultured fibroblasts, it seems likely that exceptions will be found in this parameter in spleens from type 1 Gaucher disease patients also.

Discrimination between variants of Gaucher disease on the basis of isoelectric point and molecular mass composition of residual glucocerebrosidase in spleen is not feasible. A considerable heterogeneity in the pattern of molecular species was

noticed between extracts of different pieces of spleen from the same individual. Moreover, the residual amounts of antigen in the spleens of neuronopathic Gaucher disease cases was too low for reliable analysis of molecular species composition. A similar conclusion using fibroblasts was reached by others (Fabbro *et al.*, 1987) and by us (Van Weely *et al.*, 1989).

In the light of the findings of Grabowski *et al.* (1985) that the glucocerebrosidase of American Ashkenazi Jewish type 1 Gaucher disease patients shows unique enzymic properties, it should be mentioned that our study did not include material from patients of clear Ashkenazi Jewish origin. We have subsequently had the opportunity of examining the enzyme in the spleen of one such patient. As observed for the other type 1 patients examined, the relative specific activity of the glucocerebrosidase in this patient was about 5% of the control value, and the amount of cross-reactive material was near normal.

At present, considerable attention is being paid to analysis of the nucleotide sequence of the structural gene coding for glucocerebrosidase (see Barranger & Ginns, 1989, for a review). Independent studies have shown that distinct mutations exist in the structural gene of glucocerebrosidase in relation to Gaucher disease (Tsuji *et al.*, 1987, 1988; Graves *et al.*, 1988; Wigderson *et al.*, 1989). So far, at least four distinct point mutations have been identified in Gaucher disease. Heteroallelism, i.e. the occurrence of different combinations of mutant alleles, has also been observed in Gaucher disease patients (see Barranger & Ginns, 1989, for a review). At present a clear correlation between glucocerebrosidase genotype and Gaucher phenotype is still lacking. Individuals homoallelic for the mutation resulting in the substitution of Pro-444 for Leu-444 seem to develop severe neurological complications. Individuals with at least one allele containing the mutation resulting in substitution of Ser-370 for Asn-370 in glucocerebrosidase have so far been found to be free of neurological complications. The latter mutation is rather frequent (although in heteroallelic form) in Ashkenazi and non-Ashkenazi type 1 Gaucher disease patients in the United States (Tsuji *et al.*, 1988). Our findings of near-normal amounts of glucocerebrosidase antigen in spleens from type 1 Gaucher disease patients (53–118% of control concentrations) suggests that all of these individuals have at least one glucocerebrosidase allele coding for a mutant enzyme with normal stability. The investigated neuronopathic Gaucher disease cases do not, however, have such alleles. It will be of interest to establish the nature of the mutations in glucocerebrosidase alleles in the patients investigated in this study.

The catalytic properties of the immunopurified mutant enzyme have been studied under a variety of conditions. Our preliminary findings indicate that the relative specific activity of glucocerebrosidase from type 1 Gaucher disease patients is highly dependent on the assay conditions, ranging from severely curtailed in the presence of taurocholate to near normal in the presence of appropriate amounts of activator protein and phosphatidylserine. The extent of interaction of glucocerebrosidase with activator protein *in situ* may therefore be of particular importance in determining the extent to which glucosylceramide catabolism is deficient in cases of Gaucher disease. The physiological significance of sphingolipid activator protein 2 in glucosylceramide degradation has been substantiated by the observation of a case of Gaucher disease caused by a deficiency in activator protein (see Kleinschmidt *et al.*, 1987).

In conclusion, the results of our study show that both (1) an apparently decreased catalytic activity of mutant glucocerebrosidase in combination with a near normal concentration of mutant enzyme protein, and (2) apparently near-normal catalytic activity of mutant glucocerebrosidase in combination with severely decreased enzyme protein concentration, lead to

pathological manifestations in spleen, an organ consistently involved in the pathology of Gaucher disease.

We are most grateful to the following people for supplying some of the obduction material used in this study: Dr. J. Ploem (Sint Lucas Ziekenhuis, Amsterdam), Professor R. Goudsmit (Department of Haematology, Academic Medical Centre of the University of Amsterdam), Dr. P. Maaswinkel (Department of Pediatrics, University of Leiden), Dr. B. Poorthuis (Stichting Klinisch Genetisch Centrum, Leiden), and Dr. G. Besley (Royal Hospital for Sick Children, Edinburgh). We thank Dr. K. Nieuwenhuis and Professor J. Sixma (Department of Haematology, University of Utrecht) for carrying out the determination of platelet antigens, and Ms. Wendy van Noppen and Mr. Marcel Dutrieux for their help in the preparation of this manuscript.

REFERENCES

- Aerts, J. M. F. G., Donker-Koopman, W. E., Van der Vliet, M., Jonsson, L. M. V., Ginns, E. I., Murray, G. J., Barranger, J. A., Tager, J. M. & Schram, A. W. (1985) *Eur. J. Biochem.* **150**, 565–574
- Aerts, J. M. F. G., Donker-Koopman, W. E., Murray, G. J., Barranger, J. A., Tager, J. M. & Schram, A. W. (1986a) *Anal. Biochem.* **154**, 655–663
- Aerts, J. M. F. G., Donker-Koopman, W. E., Koot, M., Murray, G. J., Barranger, J. A., Tager, J. M. & Schram, A. W. (1986b) *Biochim. Biophys. Acta* **863**, 63–70
- Aerts, J. M. F. G., Donker-Koopman, W. E., van Laar, C., Brul, S., Murray, G. J., Wenger, D. A., Barranger, J. A., Tager, J. M. & Schram, A. W. (1987) *Eur. J. Biochem.* **163**, 583–584
- Aster, R. H. (1966) *J. Clin. Invest.* **45**, 645–647
- Barneveld, R., Tegelaers, F. P. W., Ginns, E. I., Visser, P., Laanen, E. A., Brady, R. O., Galjaard, H., Barranger, J. A., Reuser, A. J. J. & Tager, J. M. (1983) *Eur. J. Biochem.* **134**, 585–589
- Barranger, J. A. & Ginns, E. I. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds.), pp. 1677–1698, McGraw-Hill, New York
- Barranger, J. A., Murray, G. J. & Ginns, E. I. (1984) in *Molecular Basis of Lysosomal Storage Disorders* (Barranger, J. A. & Brady, R. O., eds.), pp. 311–323, Academic Press, New York
- Bergmann, J. E. & Grabowski, G. A. (1989) *Am. J. Hum. Genet.* **44**, 741–750
- Beutler, E., Kuhl, W. & Sorge, J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6506–6510
- Fabbro, D., Desnick, R. J. & Grabowski, G. A. (1987) *Am. J. Hum. Genet.* **40**, 15–31
- Fujibayashi, S. & Wenger, D. A. (1985) *Clin. Chim. Acta* **146**, 147–156
- Ginns, E. I., Brady, R. O., Pirruccello, S., Moore, C., Sorrell, S., Furbish, F. S., Murray, G. J., Tager, J. M. & Barranger, J. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5607–5610
- Ginns, E. I., Tegelaers, F. P. W., Barneveld, R., Galjaard, H., Reuser, A. J. J., Brady, R. O., Tager, J. M. & Barranger, J. A. (1983) *Clin. Chim. Acta* **131**, 283–287
- Glasgow, L. R., Paulson, J. C. & Hill, R. L. (1977) *J. Biol. Chem.* **252**, 8615–8623
- Glew, R. H., Basu, A., LaMarca, K. L. & Prence, E. M. (1988) *Lab. Invest.* **58**, 5–25
- Grabowski, G. A., Goldblatt, J., Dinur, T., Kruse, J., Svennerholm, L., Gatt, S. & Desnick, R. J. (1985) *Am. J. Med. Genet.* **21**, 529–549
- Graves, P. N., Grabowski, G. A., Eisner, R., Palese, R. & Smith, F. I. (1988) *DNA* **7**, 521–528
- Ho, M. W. & O'Brien, J. S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2810–2813
- Jonsson, L. M. V., Murray, G. J., Sorrell, S., Strijland, A., Aerts, J. M. F. G., Ginns, E. I., Barranger, J. A., Tager, J. M. & Schram, A. W. (1987) *Eur. J. Biochem.* **164**, 171–179
- Kaplan, K. L., Nossel, H. L., Drillings, M. & Lesznik, S. (1978) *Br. J. Haematol.* **39**, 129–146
- Kleinschmidt, T., Christomanou, H. & Brainitzer, G. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 1571–1578
- O'Brien, J. S., Kretz, K. A., Dewje, N., Wenger, D. A., Esch, F. & Fluharty, A. (1988) *Science* **241**, 1098–1101
- Pentchev, P. G., Neumeyer, P., Svennerholm, L., Groth, C. G. & Brady, R. O. (1983) *Am. J. Hum. Genet.* **35**, 621–628
- Peters, S. P., Coffee, C. J., Glew, R. H., Lee, R. E., Wenger, D. A., Li, S. C. & Li, Y. T. (1977) *Arch. Biochem. Biophys.* **183**, 290–297

- Radin, N. S. & Berent, S. L. (1982) *Methods Enzymol.* **53**, 569–603
- Sa Miranda, M. C., Aerts, J. M. F. G., Pinto, R. A. A., Magalhaes, J. A., Barranger, J. A., Tager, J. M. & Schram, A. W. (1988) *Biochim. Biophys. Acta* **965**, 163–168
- Tsuji, S., Choudary, P. V., Martin, B. M., Stubblefield, B. K., Mayor, J. A., Barranger, J. A. & Ginns, E. I. (1987) *N. Engl. J. Med.* **316**, 570–575
- Tsuji, S., Martin, B. M., Barranger, J. A., Stubblefield, B. K., Lamavea, M. E. & Ginns, E. I. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2349–2352
- Van Weely, S., Aerts, J. M. F. G., Van Leeuwen, M., Peterson, M. E., Goldblatt, J., Tager, J. M., Barranger, J. A. & Schram, A. W. (1989) in *Lysosomal Storage Disease. Biological and Medical Aspects* (Salvayre, R., Douste-Blazy, L. & Gatt, S. eds.), pp. 51–57, Plenum, New York
- Wenger, D. A. & Olsen, G. C. (1981) in *Lysosomes and Lysosomal Storage Diseases* (Callahan, J. W. & Lowden, J. A., eds.), pp. 151–171, Raven Press, New York
- Wigderson, M., Firon, N., Horowitz, Z., Wilder, S., Frishberg, Y., Reiner, O. & Horowitz, M. (1989) *Am. J. Hum. Genet.* **44**, 365–377
- Willemsen, R., Aerts, J. M., Van Dongen, J., Goudsmit, R., Schram, A. W., Tager, J. M. & Reuser, A. J. J. (1988) *J. Ultrastruct. Pathol.* **12**, 471–478

Received 2 October 1989/13 February 1990; accepted 7 March 1990