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IMMUNOLOGICAL AND ISOELECTRIC FOCUSING STUDY OF β-GLUCOCEREBROSIDASE FROM NORMAL AND GAUCHER DISEASE

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SUMMARY : Comparison of normal and Gaucher disease β-glucocerebrosidase by agarose isoelectric focusing (IEF) demonstrated additional bands at the pI-6 area seen within the mutated enzyme, while both normal placenta and spleen enzyme preparations manifest only major activity at pI-5. Antiglucocerebrosidase antibodies precipitated both normal and pathological enzymes, however, more antibodies were needed to reach an equivalence with the normal enzymes than with the Gaucher's. Cross reactivity of the IEF isozymes were detected by direct immunodifusion on the prefocused gel.

The Gaucher disease presents multiple clinical forms that have in common an accumulation of glucocerebroside within cells of the reticuloendothelial system and a deficiency of the lysosomal enzyme β-glucocerebrosidase. Gaucher type I is the most common form, among Ashkenazi Jews itself comprizing clinical subtypes that vary between malignant childhood and benign adult forms. Since the deficiency in glucocerebrosidase is a common denominator to all types and variants in Gaucher disease, efforts were made to determine the relationship between the disease and the molecular expression of enzyme deficiency. Several β-glucosidase isozymes were found in different tissues and cells ; in normal leukocytes two isozymes, separated by IEF on granulated gel,with pI values of 5.2 and 6.4 were described along with an acidic one at pI 3.2-3.9 (1,2). In fibroblasts of normal and Gaucher donors major isozyme separated on sucrose IEF, of pH 4.8 was described along with two minor ones at pI 4.55 and 4.67. It was suggested that the Gaucher's fibroblasts are deficient in the pI 4.55 isozyme (3). Two forms of β-glucosidases from the normal human spleen were found to differ in pH optimal and temperature sensitivity, one of these being the major form which is deficient in Gaucher disease (4,5). Spleen enzymes, studied using granulated IEF, revealed a non-specific β-glucosidase and two β-glucocerebrosidases with pI value of 5.2

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and 6.4. The latter were both present as membrane bound and membrane free forms and were deficient in Gaucher spleen, while the non-specific form was present in the same amounts as in normal spleen (6,7).

Determination of molecular forms of the enzymes was done by polyacryl-amide electrophoresis in which the residual glucocerebrosidase activity in adult Gaucher fibroblasts co-migrated with the activity of normal cells(8). However, changes in molecular forms of the enzyme originated from neurological and non-neurological human brain tissues and fibroblasts were reported (9). Glucocerebrosidase from leukocytes and fibroblasts were also studied using cellogel electrophoresis in which fibroblasts extracts of Gaucher's disease did not migrate (10). We used agarose IEF to analyze β-glucocerebrosidase preparations of both normal and adult forms of Gaucher disease which enabled to test directly the sliced gel for enzyme activity and the use of combined immunoprecipitation on the prefocused gels.

MATERIALS AND METHODS

Enzyme preparations of normal placentae, normal spleens and Gaucher spleens were processed according to published procedures (11,12). Enzyme activity was measured by using either the natural substrate C^{14} glucocerebroside (13) or the artificial 4-methyl-umbelliferyl-β-D-glucopyranoside (4mUβglc) (14).

Antiglucocerebrosidase antibodies were obtained by immunization of four rabbits with purified enzyme (Table I) using 0.5 mg protein in complete Freund's adjuvant in four weekly intervals. The rabbits were boosted three weeks later and bled after another week. γ-globulins were separated from serum using two consecutive 40% ammonium sulfate precipitations. To determine antibody samples of purified placental glucocerebrosidase were mixed in serial dilutions of γ-globulins obtained from the immunized rabbits and incubated for 1 h at 37°C and overnight at 4°C. Supernatants obtained after centrifugation 20 min. at 10.000 g (Microfuge B, Beckman) were tested for remaining activity. Similar assay was done with spleen enzyme of normal and Gaucher preparation. The resulting precipitate were washed 4 times with phosphate-buffered saline to remove non-precipitated material and the amount of protein was determined by Lowry (15).

Isoelectric focusing (IEF) of enzyme preparations was done using agarose IEF(Pharmacia) with ampholines of pH 3,5-9,5 or ampholines at the narrow 4-6,5 range. 1% agarose was dissolved in distilled water containing 10% sorbitol and the ampholines were added. The mixture was poured on Gelbond and 20-30 µl of enzyme were applied. After electrofocusing for 1.5-2 hours, the agarose gel was fixed and stained with 0.2% Coomassie Blue . In parallel experiments where enzyme activity was studied, the agarose strip was removed after focusing, sliced and each fraction was incubated with the substrate to determine percent of activity in each fraction. For detection of antibody cross reactive material on the isoelectrically focused gel, the agarose in the lanes between samples was removed and new agarose in phosphate buffered saline at pH 8.0 was poured in. Longitudinal troughs were cut in the middle of the newly poured agarose and filled with 100 µl of 30 mg/ml γ-globulins. After 2 hours at 37°C or overnight

at 4°C precipitation lines were observed. The plate was washed extensively with saline to remove excess antisera and stained.

RESULTS

Table 1 demonstrates the different steps of purification of placental glucocerebrosidase measured by using the natural substrate. The antibodies obtained from the immunized rabbits were enzyme specific since they precipitated the enzyme as measured by remaining enzyme activity using both the natural and artificial substrates (Table 2). Enzyme preparations from normal and Gaucher spleens (Table 3) were used to determine the amount of cross reactive protein by incubating with increasing amounts of antiglucocerebrosidase antibodies. The protein of the complexed enzyme-antibody precipitates was measured and plotted against the antibody/enzyme ratios. Results illustrated in Fig.1 show that similar amount of protein was precipitated by the antibodies following reaction with both normal and two pathological enzymes. However, more antibodies were needed to reach an equivalence with the normal enzyme than with either of the Gaucher proteins.

Agarose IEF was used to compare the enzyme preparations of normal and pathological tissues . Fig. 2 demonstrates IEF at pH 4-6.5 range. The placental purified enzyme focused at the pI 5 area as well as the major band of normal spleen enzyme. The Gaucher enzymes presented a much reduced bands at this pI value and showed few additional protein bands at the pI 6 area. Repeated

Table 1

PURIFICATION OF β-GLUCOCEREBROSIDASE FROM NORMAL HUMAN PLACENTA

	Volume (ml)	Protein (mg/ml)	Specific Activity Units/mg	Total Units Activity	% Yield
Cholate Extraction	550	8.85	134.6	655165	100
Ammonium Sulfate Precipitation	560	3.27	257	470618	71.8
Butanol Extraction	280	0.53	1347	199894	30.5
Dialysis and Centrifugation	310	0.38	1237	145718	22.2
Octyl sepharose column and Ethanol precipitation	1.5	2	24363	73092	11.7

TABLE 2

PURIFIED PLACENTAL GLUCOCEREBROSIDASE INTERACTION WITH ANTIBODIES

Amount of added antibodies to 20 µl of 1.6 µg purified enzyme	Protein precipitate µg/ml	Supernatant activity	
		C^{14} glucocerebroside nMole/ml	4 mU-β-glc nMole/ml
None	0	365	228
80 µl	73	44.2 (12%)	21.2 (9.2%)
40 µl	65	95.6 (26%)	45 (19.7%)
20 µl	51	263.4 (72%)	182.2 (79%)
10 µl	38	294.7 (80%)	224 (98%)
2.5 µl	10	356. (97%)	227 (100%)

experiments in which the agarose gel was sliced immediately after focusing to fourteen equal samples of each lane, and tested for enzyme activity using 4mUβglc as substrate were undertaken. As can be seen in Fig. 3 the major percentage of activity of normal enzyme preparations concentrated in the pI 5 slices while the fragments of the focused Gaucher gel had additional activities at the pI 6-6.3 area. That these additional isozymes were also cross reacting material to the antiglucocerebrosidase antibodies is demonstrated in Fig. 4. In this figure isoelectrically focused samples of the different enzymes were incubated immediately after focusing, with the antibodies applied to troughs along the

Table 3

β-GLUCOCEREBROSIDASE PREPARATIONS

	Normal Spleen	Gaucher Spleen (G.R.)		Gaucher Spleen (C.Z.)
Cholate Extraction	36.8*	2.19	4.86	3.15
Ammonium Sulfate Precipitation	52.2	3.96	7.12	5.79
Butanol Wash & Dialysis	1020	34.9	45.3	75.4
Ethanol Precipitation	1728	210	140	136

*Specific activity as determined by using C^{14}-glucocerebroside in nmoles/mg protein per 1 hour.

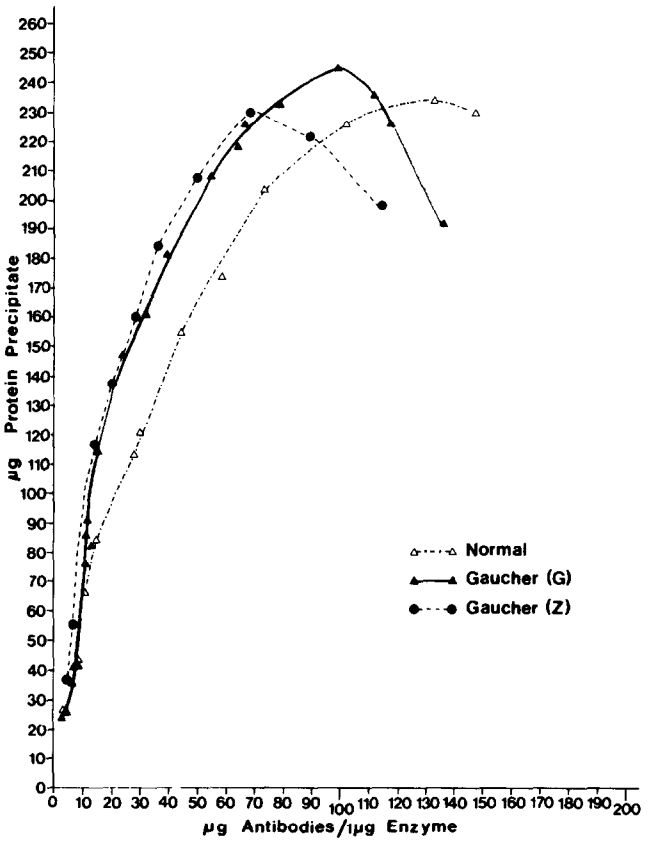

Fig. 1: Immunoprecipitation of human spleen glucocerebrosidase from normal and Gaucher disease with rabbit γ-globulin antibodies.

samples. The precipitin lines appearing for all samples in the acidic area, and at the lower, more basic, part of the gels only for the pathological enzymes.

DISCUSSION

Comparing normal and pathological glucocerebrosidase preparations by isoelectric focusing in agarose, antibody precipitation and combination of the two methods revealed differences between the enzyme of normal placenta and spleen, and those of the Gaucher disease. The findings that antibody precipitates enzymes of normal and Gaucher's tissues similarly, are in agreement with the study in which the percent of remaining activity of both normal and Gaucher enzymes were tested following precipitation with antibodies (12). However, almost double antibody concentration needed to reach maximum precipitin with the normal enzyme suggest that the latter contains more antigenic determinants per

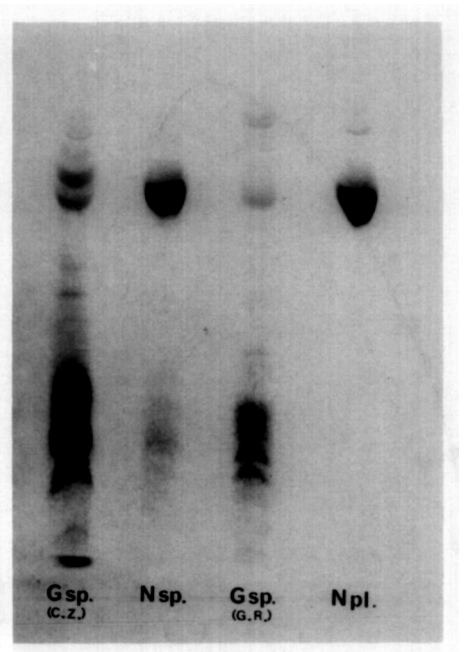

Fig. 2: Agarose-IEF of enzyme preparation using ampholins pH range 4-6.5, stained with Coomassie Blue.
Gsp - Gaucher spleen (C.Z.);
Nsp - Enzyme of normal spleen;
Gsp - Gaucher spleen (G.R.);
Npl - Enzyme of normal placenta.

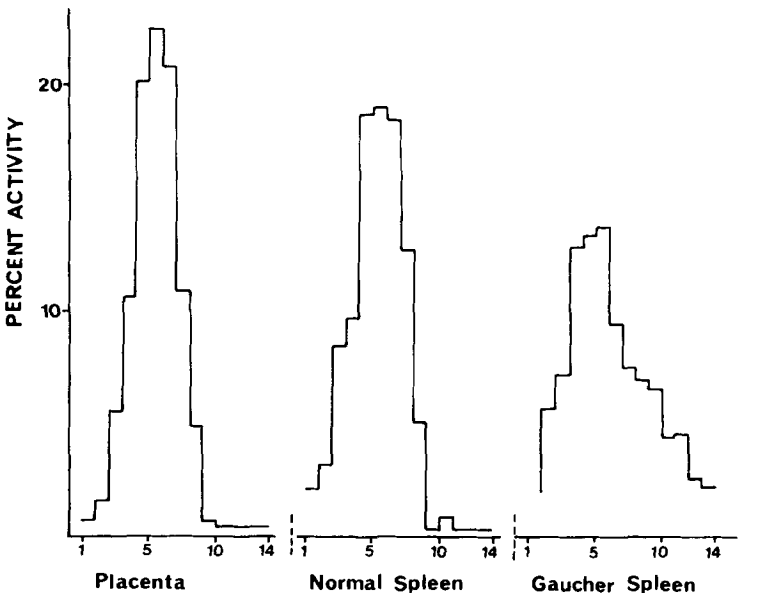

Fig. 3: Percent of activity determined in each slice of agarose gel immediately after isoelectric focusing, placenta, normal and Gaucher spleen enzymes, tested by 4mUβ-glc substrate.

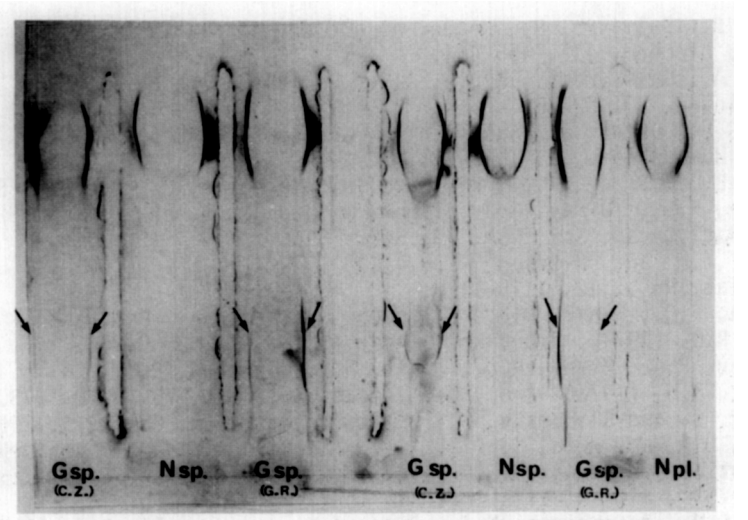

Fig. 4: Immunodiffusion of antiglucocerebrosidase antibodies on preisoelectrically focused enzyme preparations (pH range 4-6.5). Abbreviations as in Fig. 2.

protein molecule, or alternatively, possesses determinants which are missing from the pathological enzyme. It was recently demonstrated that glucocerebrosidase cross reactive material in Gaucher extracts was lower than that found in normal spleens (16). The aim of many studies of β-glucocerebrosidases and β-glucosidases were made to determine the specific isozyme deficient in the Gaucher tissues (1-10,12). Our finding by the use of agarose-IEF and antibody precipitation further suggests that a structurally different isozyme or isozymes appearance in the Gaucher spleen tissues which cross react with the antibodies to normal placental enzyme, might represent the altered protein enzyme that has reduced activity. Further studies in which mono-specific antibody to the Gaucher isozymes are under investigation.

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