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Cross-reacting material in Gaucher disease fibroblasts

(enzyme/antigen/monoclonal antibody/glucocerebrosidase)

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ABSTRACT **Glucocerebrosidase is the enzyme that is deficient in Gaucher diseases. Four monoclonal antibodies reacting with at least two different epitopes of this enzyme have been produced. The amounts of glucocerebrosidase in fibroblasts of patients with all three types of Gaucher disease were investigated by radioiodinating two of the antibodies and measuring their binding to fibroblast extracts immobilized on nitrocellulose filters. The amount of glucocerebrosidase antigen was decreased in all cases of Gaucher disease, particularly in the fibroblasts of patients with the more severe neuronopathic forms of the disorder, types II and III. The catalytic activity was reduced to a greater extent than the amount of antigen in all cases, so that the specific activity of the residual enzyme was found to be diminished. Although measurements in individual cases were quite reproducible and the amount of antigen detected by monoclonal antibodies reacting with different epitopes was quite similar, there was considerable variation between patients. This finding is consistent with the apparent within-type genetic heterogeneity of Gaucher disease, even within the Ashkenazi Jewish population in which it is most prevalent.**

Gaucher disease is one of the more common glycolipid storage disorders. Characterized by a deficiency of glucocerebrosidase activity, the disease occurs in three forms. Type I or the "adult" type of disease is by far the most common. Here storage of glucocerebroside in liver, spleen, and bone is prominent and there are no central nervous system manifestations. Types II and III are characterized by central nervous system as well as visceral involvement. All three forms of the disease appear to be caused by a mutation of the same gene, since complementation cannot be demonstrated (1, 2); the cause of the difference between these types of Gaucher disease remains obscure.

We have now produced a panel of monoclonal antibodies against at least two distinct epitopes of glucocerebrosidase. Using these antibodies, we have been able to quantitate the amount of cross-reacting material (CRM) in fibroblasts from patients with the various forms of Gaucher disease.

MATERIALS AND METHODS

Production of Monoclonal Antibodies. BALB/c/BYJ strain mice (bred at our institution) were immunized with three injections totaling 30–100 μg of purified glucocerebrosidase (3). By using minor modifications of standard techniques (4), spleen cells were fused with X63-Ag 8.6.5.3 myeloma cells, the mixture was selected with hypoxanthine/aminopterin/thymidine (HAT) medium, and the fused cells were plated first at a density of approximately five cells per well and then, after subcloning, at two and then one cell per well. Purified glucocerebrosidase was labeled with ^{125}I by using lactoperoxidase (5).

Supernatant fluids from 1000 wells were screened for anti-glucocerebrosidase activity. For each, 200 μl of fluid was absorbed onto nitrocellulose paper on a micro-filtration sample manifold. Unreacted sites were blocked with 3% bovine serum albumin in phosphate-buffered saline (9 parts 0.154 M NaCl, 1 part 0.1 M K_2HPO_4/KH_2PO_4, pH 7.4). The nitrocellulose was then treated with ^{125}I-labeled glucocerebrosidase (^{125}I-glucocerebrosidase) (5–15 μg/20 ml of 3% bovine serum albumin in phosphate-buffered saline) at 4°C for 16 hr, followed by washing and autoradiography. Positive clones were subcultured.

Four hybridoma cell lines, each of which generated a monoclonal antibody against glucocerebrosidase, were produced. These clones were injected intraperitoneally into mice, ascites fluid was harvested after several days, and the antibody was purified by precipitation with ammonium sulfate and chromatography on DEAE-cellulose. It was then labeled with ^{125}I by using the lactoperoxidase technique (5).

Fibroblast Cultures. Cultured human skin fibroblasts from five unrelated patients with type I Gaucher disease, three patients with type II, and two patients with type III disease were investigated. Additional information regarding these cell lines is given in Table 1. Also included in these studies were 7 fibroblast cultures designated as normals which were derived from skin biopsies from normal subjects or from patients with diagnoses unrelated to Gaucher disease.

Fibroblasts were cultured in minimal essential medium with Hanks' balanced salts containing 20% fetal calf serum and 0.01 M Hepes, pH 7.4. When the cultures were confluent, the medium was removed by washing thoroughly with 0.154 M NaCl. Cells were harvested into 10 ml of 0.154 M NaCl by scraping with a rubber policeman. They were pelleted by centrifugation and resuspended to approximately 20% (vol/vol) in phosphate-buffered saline. The suspension was sonicated at 0°C for 10 sec at 25 W with a Heat System/Ultrasonics sonicator (Plainview, NY).

Glucocerebrosidase activity was assayed with 4-methylumbelliferyl β-glucoside as a substrate (6) and protein concentration was determined by the Lowry method (7). One unit (U) of enzyme cleaves 1 μmol of substrate in 1 min at 37°C.

Estimation of Antigens by a Quantitative Radioactive "Dot" Assay. A nitrocellulose filter (BA85, 0.45-μm pores, Schleicher and Schuell) was moistened with water and placed on moistened Whatman no. 1 filter paper. Five-microliter spots of purified glucocerebrosidase diluted in 0.1% Triton X-100 containing bovine serum albumin at 3 mg/ml were made on the nitrocellulose filter to provide a standard curve. Antigen concentration was expressed in units (U), so that 1 U of antigen represents the amount of antigen in 1 U of pure enzyme: the spots represented 0–0.5 mU of enzyme activity. Fibroblast extracts were made from fibroblast sonicates to contain a final concentration of 0.1% Triton X-100 and fibroblast protein at 3 mg/ml, and 5 μl (15 μg of protein) was spotted onto the nitrocellulose filter.

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Abbreviations: CRM, cross-reacting material; U, unit.

Table 1. Origins and characteristics of Gaucher disease fibroblast lines

No.	Designation	Source	Ethnic origin	Gaucher type	Glucocerebrosidase: Enzymatic activity, mU/mg protein	Glucocerebrosidase: Antigen, mU/mg protein B8B3	Glucocerebrosidase: Antigen, mU/mg protein B6D4
1	D.W.	Patient	White/non-Jewish	I	0.8	2.0	1.8
2	W.M.	Patient	White/Jewish	I	1.3	4.4	3.7
3	S.C.	Patient	White/Jewish	I	1.4	2.7	2.7
4	GM4394	HGMCR	Black	I	0.5	2.0	1.0
5	GM1607	HGMCR	White/Jewish	I	1.6	6.5	4.6
6	GM1260	HGMCR	White	II	0.3	1.0	<0.1
7	GM877	HGMCR	White	II	0.4	1.0	0.5
8	GM2627	HGMCR	White	II (atypical)	2.1	5.6	6.9
9	GM852	HGMCR	White	III	0.5	1.4	1.1
10	GM372	HGMCR	White	III	0.5	1.0	0.5
				Normal values (mean ± SD)	8.9 ± 0.7	11.1 ± 2.2	8.4 ± 1.2

HGMCR, Human Genetic Mutant Cell Repository; U, units.

After partial air drying of the spotted nitrocellulose filters they were incubated in 2% bovine serum albumin in phosphate-buffered saline with gentle rocking for 30 min at 22°C to block unsaturated binding sites. The buffered bovine serum albumin was removed and the filter was incubated with ^{125}I-labeled monoclonal antibody against glucocerebrosidase (0.1×10^6 cpm/ml, 0.1 μg/ml) in 0.5% bovine serum albumin with 0.05% Tween-20 in phosphate-buffered saline, using 0.25 ml of antibody per cm^2 of filter. The mixture was rocked gently for 30–45 min. The filters were then washed three times for 5 min with 2 vol of 0.1% bovine serum albumin/0.05% Tween 20 in phosphate-buffered saline. After three more rinses with the same wash solution, the filters were air dried and autoradiographed on Kodak XAR x-ray film at −80°C overnight. The optical density of each spot was determined by using a laser beam scanner with an integrator (Zeineh). A standard curve was obtained and the antigen content of sample values were read from the curve.

RESULTS

Properties of the Monoclonal Antibodies. The cross-reactivity of the antibodies was investigated by treating nitrocellulose filter-bound spots of glucocerebrosidase with each of the unlabeled monoclonal antibodies and then measuring the ability of each of the labeled antibodies to bind to the spots. These investigations indicated that monoclonal antibody B8B3 was unique in that binding of this antibody was unaffected by the presence of monoclonal antibodies B6D4, 14B2, and 16B3. In contrast, the other three antibodies substantially inhibited binding of each other.

Detergents were found to have a striking effect on the binding of the monoclonal antibodies to antigen. Triton X-100 at 0.1% and phosphatidylserine at 0.2 mM each considerably increased the amount of each of the antibodies bound to antigen. Monoclonal antibody B8B3 was nearly nonreactive unless a detergent, bovine serum albumin, or both were present. Sodium deoxytaurocholate was much less effective in enhancing binding. As shown in Table 2, the binding of antibody B8B3 to antigen was considerably greater at 4°C than at 22°C or 37°C. In contrast, antibody B6D4 showed greater binding at 37°C than at 4°C. The properties of the four monoclonal antibodies are summarized in Table 2.

Reactivity of Gaucher Disease and Normal Fibroblasts with Monoclonal Antibodies. Fig. 1 illustrates a typical experiment in which nitrocellulose filters were spotted in duplicate with purified glucocerebrosidase to provide a standard curve and with normal and Gaucher disease fibroblasts. The filters were treated with radiolabeled antibodies B8B3 (filters I and II) and B6D4 (filters III and IV). To prevent nonspecific binding of immunoglobulin the filters were all treated with MOPC 21 ascites fluid at a 250-fold excess of protein over the specific radiolabeled antibody prior to the addition of the radiolabeled antibody. As a blank, a duplicate filter for each antibody was also treated with a 250-fold excess of unlabeled monoclonal anti-glucocerebrosidase antibody to block specific binding of radiolabeled antibody and allow measurement of nonspecific binding (filters II and IV).

Table 2. Properties of anti-glucocerebrosidase monoclonal antibodies

Monoclonal antibody	Cross-reactivity* B8B3	B6D4	14B2	16B3	Temp. optimum, °C	Reactivity with staphylococcal protein A	Binding efficiency: Without detergents	With detergents†	With bovine serum albumin
B8B3	++++				4	None	+	++++	+++
B6D4	None	++++			37	+	+	++++	+++
14B2	None	+++	NT		NT	++++	+	++++	+++
16B3	None	++++	NT	NT	NT	++++	+	++++	+++

*B8B3 and B6D4 antibodies were iodinated with ^{125}I and the binding of the ^{125}I-labeled antibody to antigen spotted on nitrocellulose was measured after allowing the antigen to react with unlabeled antibodies. "None" means there is complete binding of the ^{125}I as compared to controls with no competitive antibodies added and ++++ means ^{125}I binding was blocked by competing antibodies to background binding levels. NT, not tested.

†Detergents tested were 0.1% Triton X-100, 0.2 mM phosphatidylserine, and 0.5% taurodeoxycholate.

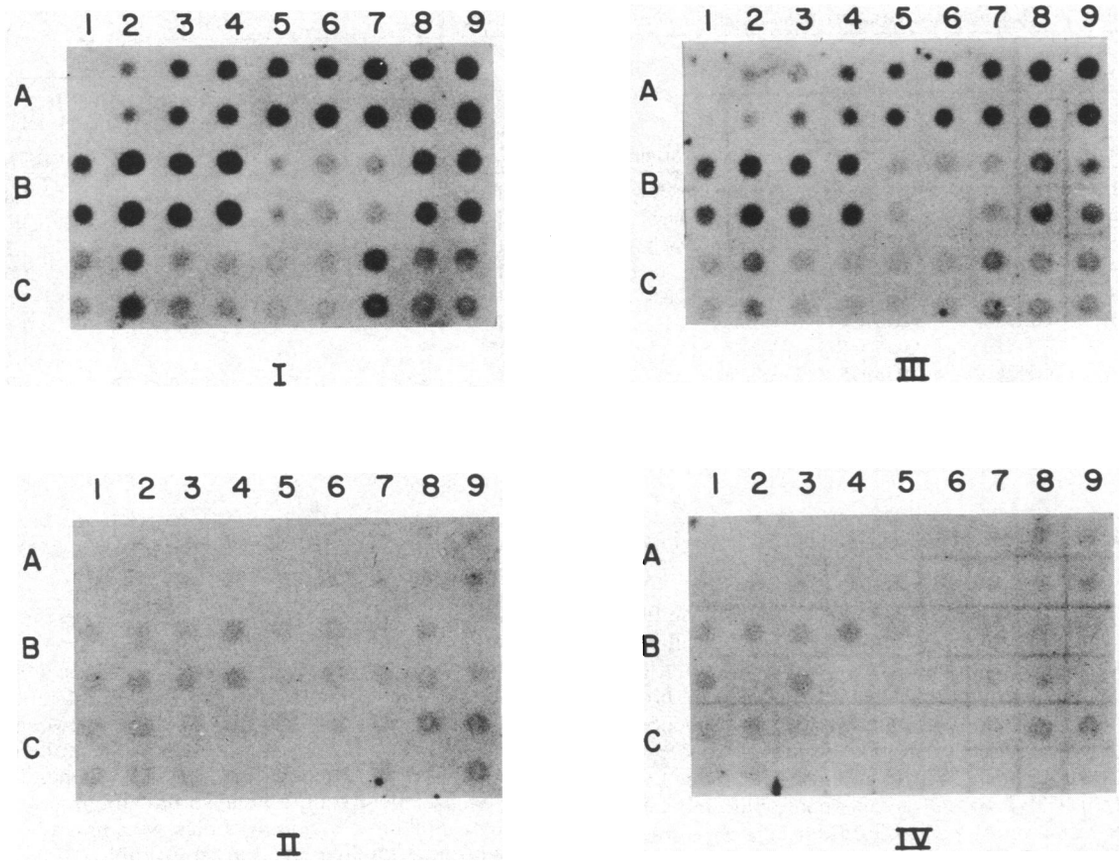

FIG. 1. Autoradiographs of a typical experiment quantitating glucocerebrosidase antigen in fibroblasts from normal subjects and patients with Gaucher disease. Duplicate rows are designated with letters; columns are designated with numerals. A-1 through A-9 represent purified glucocerebrosidase spotted as a standard curve of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, and 0.50 mU, respectively. Normals: B-2, B-3, B-4, B-8, and B-9. Gaucher disease type I: B-5, B-7, C-3, C-4, C-7, C-8, and C-9. Gaucher type II: B-1, C-2, and C-6. Gaucher type III: B-6, C-1, and C-5. Filters I and II were treated with radiolabeled B8B3 monoclonal antibody; filter I was pretreated with MOPC 21 and filter II was pretreated with MOPC 21 and unlabeled B8B3 for measurement of nonspecific binding as described in text. Filters III and IV were treated with radiolabeled B6D4 monoclonal antibody; filter III was pretreated with MOPC 21 and filter IV was pretreated with MOPC 21 and unlabeled B6D4 for measurement of nonspecific binding as described in text.

A standard curve obtained after laser scanning of the spotted standards is illustrated in Fig. 2. As shown in Fig. 3 and in Table 1, the amount of antigen in Gaucher disease fibroblasts was decreased in all cases. Excellent correlation was present between measurements made with antibodies B8B3 and B6D4, which do not cross-react and are therefore measuring separate epitopes. The quantity of antigen detected in type I Gaucher disease was greater than that found in type II and type III disease, except for one case of the type II disease that gave unusually high antigen levels when compared to all other Gaucher disease fibroblasts. This case (GM1607) had been classified as type II, atypical.

Fig. 4 presents the glucocerebroside specific activities (i.e., catalytic activity/antigen) in cells of all patients in which sufficient antigen were detected to allow quantitation. The specific activity was decreased in all cases.

DISCUSSION

There are few data available regarding the presence of CRM in tissues of patients with Gaucher disease. Using parallel purification of normal and Gaucher spleen, Pentchev *et al.* (8) reported that the decrease in specific activity of the enzyme isolated from the Gaucher spleen was almost exactly the same as the decrease in enzymatic activity in the whole spleen. This implies that a normal amount of Gaucher antigen was present in the spleen. Subsequently, using a competition assay in which sedimentation of a glucocerebrosidase standard served as an indicator of the amount of antibody bound, Pentchev *et al.* (9) concluded that the amount of

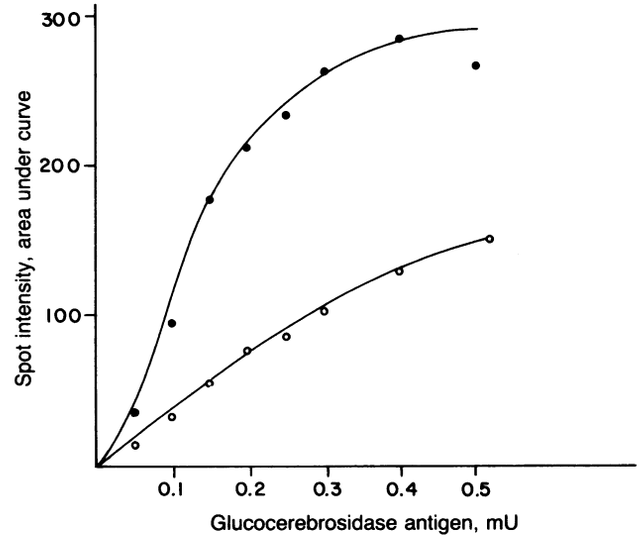

FIG. 2. Standard curves obtained by laser beam scanning of the dots on the autoradiographs. The area under the curve was plotted against the quantity of purified glucocerebrosidase in each dot. ●, B8B3; ○, B6D4.

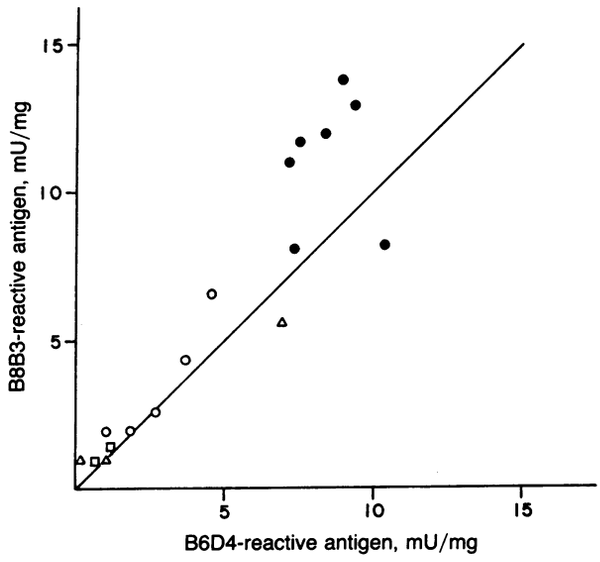

FIG. 3. Quantity of glucocerebrosidase antigen in fibroblasts as measured by two different monoclonal antibodies that react with different epitopes. Solid circles (●) represent normals. Open symbols represent Gaucher disease: type I (○), type II (△), and type III (□).

CRM in Gaucher disease spleen was about one-half of normal for all three types.

We have produced four monoclonal antibodies against glucocerebrosidase. On the basis of competition studies, it appears that one of the antibodies reacts with an epitope that is quite separate from the epitope that reacts with the other antibodies. All antibodies react with glucocerebrosidase with much greater affinity in the presence of detergent or bovine serum albumin. This finding is of interest, since detergents strongly stimulate the activity of glucocerebrosidase (10, 11). It seems possible that the antibodies react with epitopes that are revealed or created only when the enzyme is activated by detergents.

Using either of the noncompetitive antibodies, we found that the amount of antigen in Gaucher disease fibroblasts was

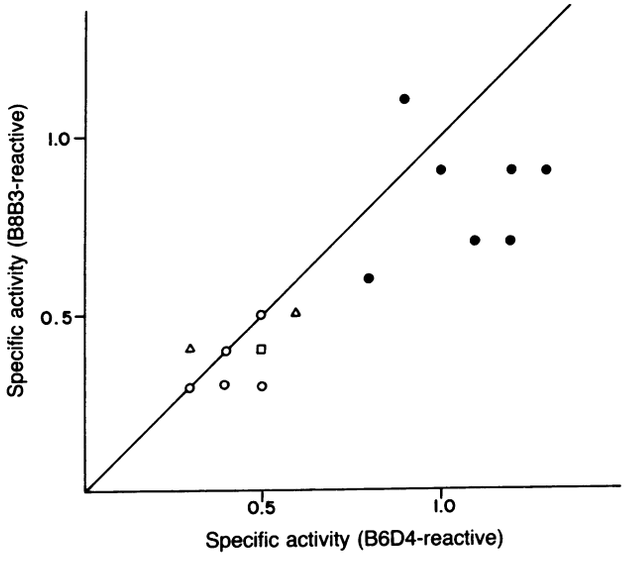

FIG. 4. Correlation of the specific activity (enzymatic activity of glucocerebrosidase per antigen) as quantitated with two different noncompetitive monoclonal antibodies (B8B3 and B6D4). Symbols are as in Fig. 3.

decreased in all cases studied. Although this finding does not agree with the preliminary data of Pentchev *et al.* (8) on a single spleen, it is in general agreement with their subsequent studies. However, using the more direct approach outlined here, we find a much more profound decrease in the amount of CRM in the majority of Gaucher fibroblasts and evidence of heterogeneity in this disease state. Moreover, the degree of antigen loss seems to be much greater in type II and type III disease than in type I. The amount of residual antigen was less than 13% of the mean normal in all but one of such patients. The single exception, with approximately 50% and 82% residual CRM (B8B3- and B6D4-reactive antigen, respectively) had been designated as "atypical."

When a normal amount of CRM is formed in a genetically determined disorder, the measurement of enzyme specific activity (catalytic activity/antigen) potentially provides a sensitive means of heterozygote detection. Heterozygote detection in Gaucher disease is not totally reliable, and a powerful means for detecting heterozygotes would be of considerable value. However, our finding that the amount of CRM is variably reduced in all forms of Gaucher disease (from <5% to 82% of normal) precludes the estimation of enzyme specific activity as a means to more accurate diagnosis.

The heterogeneity in the amount of cross-reacting material and specific activity of the residual enzyme in patients with Gaucher disease is also of special interest. The marked variability in the amount of residual antigen within the common type I disease supports the concept of genetic heterogeneity of this disorder, even within the Ashkenazi Jewish population. The fact that such heterogeneity exists has already been deduced from the marked variability of expression that is observed in different patients and the apparent concordance of disease expression among sibs (12). Of interest, also, is that, although the amount of antigen present in types II and III disease is, in general, less than that observed in type I disease, there is some overlap between the two groups. One cell line, designated as atypical type II Gaucher disease by the Genetic Mutant Cell Repository, was found to have half to three-fourths of the normal amount of CRM. While it has been suggested (13, 14) that the different types of Gaucher disease may be discriminated from one another by immunoblotting, we have detected so-called "processed" bands of glucocerebrosidase only irregularly with our monoclonal antibodies, with no relationship to disease type. It would clearly be highly desirable to have a reproducible means of differentiating neuronopathic Gaucher disease from the more common type I variety. It is hoped that the use of monoclonal antibodies as described in this paper and the development of additional such reagents such as those recently described by Barneveld *et al.* (15) may prove helpful in gaining improved understanding of the pathophysiology of Gaucher disease.

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