Brief Definitive Report

ACID HYDROLASES IN LEUKOCYTES AND PLATELETS OF NORMAL SUBJECTS AND IN PATIENTS WITH GAUCHER'S AND FABRY'S DISEASE*

By E. BEUTLER, W. KUHL, F. MATSUMOTO, AND G. PANGALIS

(From the Department of Hematology, City of Hope Medical Center, Duarte, California 91010)

The glycolipid storage diseases, disorders such as Tay-Sachs disease, Gaucher's disease, generalized gangliosidosis, and Fabry's disease, are all caused by deficiencies of specific catabolic enzymes. These enzymes are lysosomal acid hydrolases; their activity can readily be demonstrated in leukocytes and in platelets. Leukocytes have therefore been used as a source of enzyme useful both in establishing the diagnosis of these diseases and in identifying carriers (1–8). In previous studies of leukocyte β-glucosidase (3, 4) we have partially separated these cells into "lymphocyte-rich," "granulocyte-rich," and platelet fractions; most studies, however, have taken no cognizance of the fact that "leukocytes" represent a heterogeneous mixture of cells which differ in morphology, lineage, and phagocytic capability. If, in reality, there were substantial individual differences in enzyme activity among these different cell types, variations in enzyme activity of the leukocyte fraction of blood might reflect the proportion of individual types of leukocytes present rather than the genotype of the individual being investigated.

Materials and Methods

Blood was obtained from six normal donors, three patients with Gaucher's disease, both parents of a patient with Gaucher's disease, and one patient with Fabry's disease. 150 ml of blood was mixed with 50 ml of 5% polyvinylpyrollidone (PVP) (PVP-40; Sigma Chemical Co., St. Louis, Mo.) and 3% sodium citrate dihydrate. The samples were placed in a 37°C water bath for 45–60 min after which the plasma layer containing the leukocytes and platelets was removed. White cells were sedimented by centrifugation at 100 *g* at 4°C for 10 min. Platelets were then sedimented by recentrifuging the supernatant plasma at 4,000 *g* for 10 min. They were washed three times with 40 ml of 0.154 M saline, and resuspended in saline. The leukocytes were resuspended in 30 ml of saline-phosphate-EDTA.[1] Equal parts of the suspension were added to two 40 ml polycarbonate tubes each containing 17 ml of Ficoll-diatrizoate mixture [12 ml of 9% Ficoll (Sigma Chemical Co.) and 5 ml of 34% meglumine diatrizoate (Renografin-60; E. R. Squibb & Sons, New York)]. These were centrifuged at 400 *g* for 25 min at 4°C. The lymphocytes and monocytes formed a layer immediately above the Ficoll-diatrizoate. The granulocytes and erythrocytes were deposited at the bottom of the tube. The lymphocyte-monocyte layer was removed with a Pasteur pipette, washed with 40 ml of 0.154 M saline, and resuspended in 3–5 ml of 0.154 M saline. The cells were counted by standard techniques, and were then added to Hanks' balanced salt solution (Flow Laboratories, Inc., Rockville, Md.) containing 20% fetal calf serum (Flow Laboratories, Inc.), 10 U/ml penicillin, and 20 μg/ml streptomycin, with the pH adjusted to 7.4 with NaOH. 20-ml por-

* This work was supported, in part, by grant no. AM 14755 from the National Institutes of Health.

[1] 0.01 M potassium phosphate-buffered 0.154 M saline with 0.27 mM EDTA, pH 7.0.

tions of the suspension each with 1.8–2.5 × 10^6 cells/ml, were poured into 8 cm Falcon plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and incubated at 37°C for 2 h under 1% CO_2 in water-saturated air. After gentle swirling to distribute the unattached cells, the medium was pipetted into 40 ml polycarbonate tubes and centrifuged at 1,200 *g* for 6 min. The lymphocytes were sedimented leaving most of the contaminating platelets in the supernate. The lymphocytes were washed three times with 40-ml portions of 0.154 M saline, and resuspended in saline for sonication.[2] The attached cells (monocytes) were washed while in the Petri dish by gently swirling a 20 ml saline solution over them three or four times. They were then detached by adding 3 ml of saline-phosphate-EDTA and gently removed with a rubber scraper ("policeman"). The monocytes were transferred to a tube, sedimented by centrifugation at 1,200 *g* for 15 min and were resuspended in saline for sonication. The granulocyte-erythrocyte pellet which had been sedimented in the Ficoll-diatrizoate centrifugation was subjected to shock lysis by mixing with 5 ml of ice-cold water for 45–60 s followed by immediate addition of 5 ml of 0.342 M sodium chloride. The granulocytes were washed three times with 40 ml of 0.154 M saline, and resuspended in 0.154 M saline for sonication. The saline suspensions (0.5–1.0 ml) of platelets, lymphocytes, monocytes, and granulocytes were enumerated by standard counting techniques, and aliquots were removed for intact-cell β-glucosidase determination. The remainder of the suspension was sonicated[3] in a 10 × 75 mm tube on ice as indicated under Results. The separation scheme is summarized in Fig. 1.

Protein determinations were done according to the method of Lowry (9). Enzyme activities were measured using 4-methylumbelliferyl (4-MU) glycosides (Pierce Chemical Co., Rockford, Ill.; or Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) using appropriate substrates at the following concentrations: 0.5 mM 4-MU-β-glucoside for β-glucosidase; 0.5 mM 4-MU-β-galactoside for β-galactosidase; 0.5 mM 4-MU-*N*-acetyl-β-D-glucosaminide for hexosaminidase; 2.5 mM 4-MU-β-glucuronide for β-glucuronidase; 4.0 mM 4-MU-α-galactoside for α-galactosidase; 0.5 mM 4-MU-α-mannoside for α-mannosidase; 2.5 mM 4-MU-α-arabinoside for α-arabinosidase; 2.5 mM 4-MU-α-glucoside for α-glucosidase; and 3.0 mM 4-MU phosphate for acid phosphatase. β-glucosidase assays were carried out in 40 mM sodium acetate buffers at both pH 4.0 and 5.0 because of the previously observed bimodality of the pH activity of curve (3, 4). All other enzymes were assayed in 0.1 M sodium citrate pH 4.4. Fluorescence was measured as previously described (4).

Results

Adequacy of Cell Separation. Preliminary studies were carried out to determine the purity of cell preparations obtained. The monocytes attached to the Petri dishes were examined after staining smears and cutout segments of the Petri dishes with Wright-Giemsa or May-Grünwald-Giemsa stains. Differential counts revealed that 90–97% of the attached cells were morphologically identifiable as monocytes. This was also confirmed by demonstrating that the great majority of cells were positive in the peroxidase and Sudan black B reactions (10). Lymphocyte and granulocyte purity were determined both on Wright-Giemsa-stained films, and by enumerating cells in a standard counting chamber. In each case, the purity of the preparations exceeded 95%.

Basophils remained in the lymphocyte-monocyte layer after Ficoll-diatrizoate centrifugation, and they adhered to the plastic Petri dishes during the 2-h period

[2] The lymphocyte preparation from splenectomized patients contained visible contamination with nucleated erythrocytes, and it was, therefore, subjected to shock lysis in the same manner as the granulocytes. To correct for any remaining erythrocyte protein contamination which was not removed by shock lysis, the hemoglobin concentration of an aliquot of the sonicate was determined by reading the optical density at 410 nm after alkalinization with a drop of NH_4OH. This correction never exceeded 10% of the leukocyte protein concentration.

[3] Sonifier, model W185, Heat Systems-Ultrasonics, Inc., Plainview, N. Y. with a special microtip, setting of 7, and a meter reading of 45–50 W.

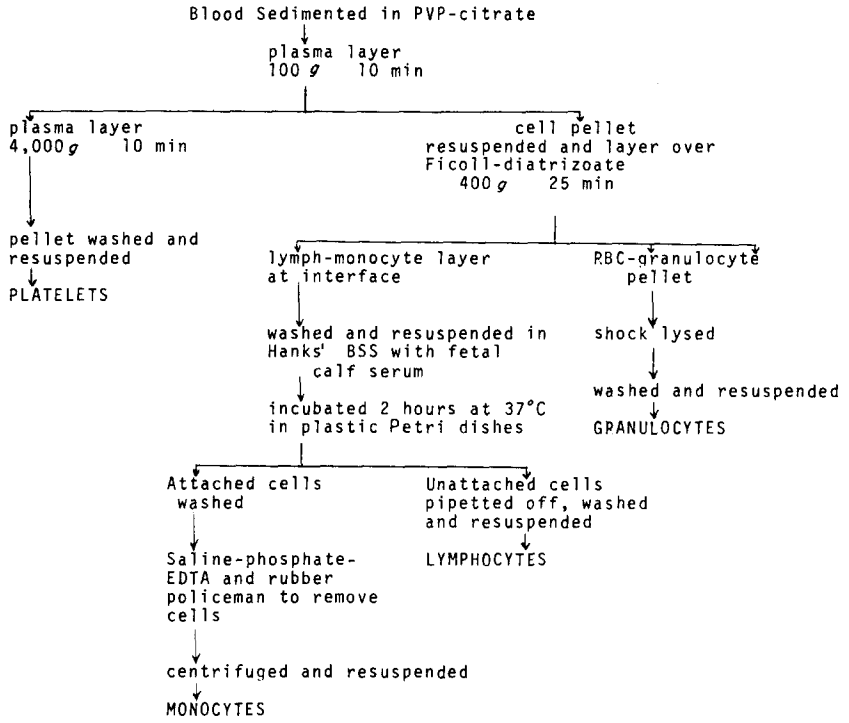

FIG. 1. Cell separation scheme for preparation of monocytes, lymphocytes, neutrophilic granulocytes, and platelets. Details described in text. RBC, erythrocytes; BSS, balanced salt solution.

of incubation. Eosinophils were found in the granulocyte layer, but their number was usually very small, indicating that many of these cells may have disintegrated in the separation procedure.

Effect of Sonication. In preliminary investigations, the enzyme activity of sonicated suspensions was compared with that of intact cell suspensions. After disruption of cells, β-glucosidase exhibited moderate to pronounced reduction in activity. All other enzymes showed slight to moderate increase in activity after sonication, with the exception of β-galactosidase. The activity of this enzyme was increased 10- to 20-fold by sonication. β-glucosidase activity was therefore studied in whole cell suspensions while the other hydrolases were measured in sonicates. The decrease of β-glucosidase activity after sonication is not surprising, in view of the suggestion that β-glucosidase activity requires three separate components, two protein and one phospholipid (11). Furthermore, dilution of fibroblast homogenate has been shown to result in marked reduction of β-glucosidase activity (12). We found no decrease in the activity of the other acid hydrolases upon dilution.

Two of the normal samples were subjected to four separate 15 s bursts of sonication separated by periods of cooling, while only a single burst was used in treating the cells of the other four normal subjects. No difference in the enzyme activity was observed with these two treatments, and the results have been pooled.

Enzyme Activity of Leukocytes and Platelets. The enzyme activities of the separated cells are summarized in Fig. 2. In most cases, the highest enzyme activities were observed in monocytes. The ratio of monocyte to lymphocyte activity in the case of different cell types differed significantly: monocytes were found to be especially rich in α-glucosidase, α-mannosidase, β-hexosaminidase, and β-glucosidase (pH 4.0) activity, when compared with lymphocytes.

Lymphocytes exhibit a higher activity level of some enzymes than do neutrophilic granulocytes, but a lower level of others. Particularly striking differences were apparent in the case of the pH 5 β-glucosidase, in which lymphocyte activity averaged seven times that of the neutrophilic granulocytes. In contrast, α-mannosidase activity was almost five times as great in neutrophilic granulocytes than in lymphocytes.

Platelets exhibited from one-half to equal amounts of activity for all the acid hydrolases when compared to lymphocytes, except for significant reductions in α-glucosidase, α-mannosidase, and β-hexosaminidase. These are incidentally the same three enzymes found to be particularly abundant in monocytes.

As we have reported previously (3, 4), "acid" (pH 4) β-glucosidase activity was markedly decreased in leukocytes of patients with Gaucher's disease, and this decrease was most clearly evident when monocytes were examined. It is of

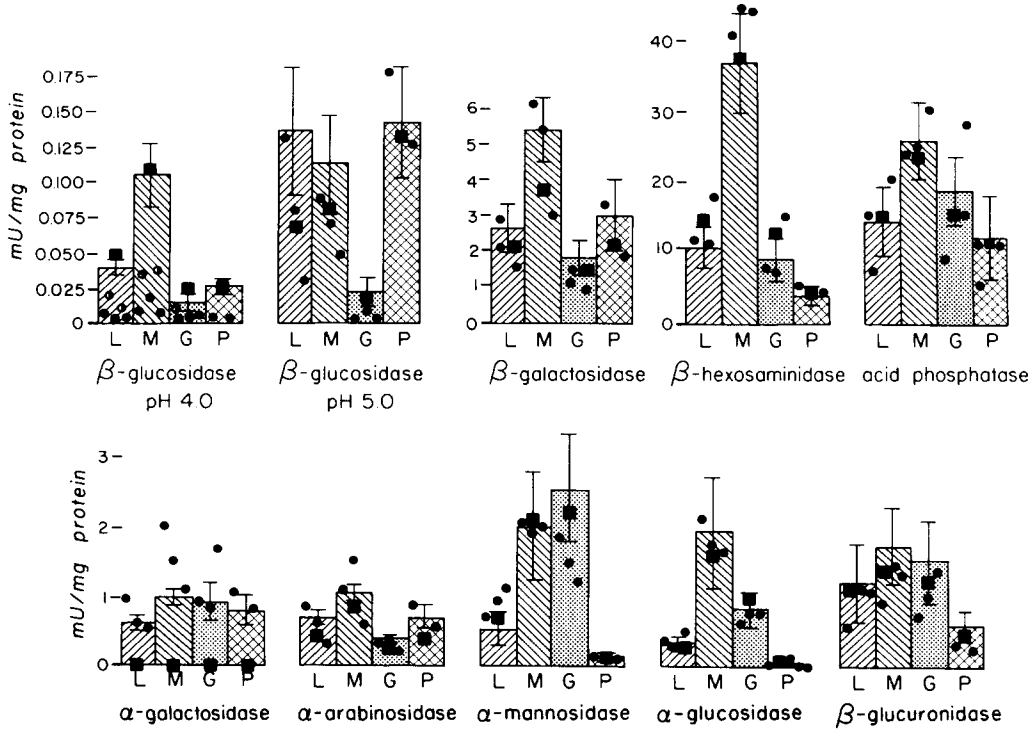

FIG. 2. Activities for the acid hydrolases in various cell types. Details of the assays are given in the text. L, lymphocytes; M, monocytes; G, neutrophilic granulocytes; and P, platelets. The bars represent the average normal activity; one standard deviation is indicated. Activities of cells from Gaucher's disease patients, (●); Gaucher's disease heterozygotes, (◑); and for the Fabry's disease patient, (■).

interest that although serum acid phosphatase activity is uniformly increased in patients with Gaucher's disease, the activity of this enzyme was normal in the various leukocyte preparations and in platelets. Only the β-glucosidase activity of the blood cells of the parents of a patient with Gaucher's disease were determined. These heterozygotes were selected for assay because the father's β-glucosidase activity had previously been found to be in the lower portion of the normal range (4). Indeed, his "lymphocyte-rich" fraction, which contained monocytes as well as lymphocytes had 57% of the activity of the average normal subject while the separated monocyte fraction had an activity of only 34% of the average normal control. It is noteworthy, therefore, that cell recovery from the lymphocyte-monocyte fraction revealed a monocyte to lymphocyte ratio which was twice that of normal. Deficiency of α-galactosidase was readily demonstrated in all cell types of the patient with Fabry's disease.

The pH Profile of β-Glucosidase. In previous investigations, we showed that the pH activity curve of leukocyte β-glucosidase was bimodal, particularly when the lymphocyte-rich fraction was examined (3, 4). Examination of the pH activity ratio of monocytes and lymphocytes from normal subjects (Fig. 2) shows that the acid (pH 4) β-glucosidase activity is equal to that of the pH 5 activity in the monocyte fraction while in contrast lymphocytes are relatively rich in the pH 5 enzymatic activity.

Discussion

The finding of substantial differences in acid hydrolase activities in different blood cell types is of substantial importance in the diagnosis of hereditary deficiencies of these acid hydrolases, and particularly in the diagnosis of the carrier states. In the study of Gaucher's disease, for example, the degree of monocyte contamination of the lymphocyte-rich fraction obtained with Ficoll-diatrizoate centrifugation may play a major role in obscuring the diagnosis of the heterozygous state. Our findings also shed further light on the bimodal pH optimum curves previously observed in peripheral blood leukocytes. Dissection of the lymphocyte-rich layer into monocytes and lymphocytes indicates that the enzyme with the lower pH optimum is primarily present in monocytes, while the pH 5 enzyme is predominantly the lymphocytic enzyme.

Summary

Lymphocytes, monocytes, neutrophilic granulocytes and platelets were each separated to greater than 95% purity from six normal subjects, three patients with Gaucher's disease, two heterozygotes for Gaucher's disease, and one patient with Fabry's disease. Activities of the following acid hydrolases were determined: "acid" (pH 4.0) β-glucosidase, pH 5.0 β-glucosidase, α-galactosidase, α-arabinosidase, α-mannosidase, α-glucosidase, β-glucuronidase, β-galactosidase, β-hexosaminidase, and acid phosphatase. Enzymatic activity varied greatly with cell type and the enzyme being measured; the importance of assaying pure preparations especially for heterozygote detection is emphasized. Gaucher's disease patients' cells were found to be deficient in the pH 4.0 acid β-glucosidase, variable in the pH 5.0 β-glucosidase, and normal in all other acid

hydrolases tested, including acid phosphatase, the activity of which is known to be elevated in plasma. Blood cells of a patient with Fabry's disease were deficient in α-galactosidase and normal in all other acid hydrolases tested.

The authors express their gratitude to Doctors Martin Cline and Robert Lehrer, U. C. L. A. School of Medicine, Los Angeles, Calif., for their very helpful suggestions.

Received for publication 12 January 1976.

References

1. Snyder, R. A., and R. O. Brady. 1969. The use of white cells as a source of diagnostic material for lipid storage diseases. *Clin. Chim. Acta* **25**:331.
2. Kampine, J. P., R. O. Brady, and J. N. Kanfer. 1967. Diagnosis of Gaucher's disease and Niemann-Pick disease with small samples of venous blood. *Science (Wash. D.C.)*. **155**:86.
3. Beutler, E., and W. Kuhl. 1970. Detection of the defect of Gaucher's disease and its carrier state in peripheral-blood leucocytes. *Lancet.* **1**:612.
4. Beutler, E., and W. Kuhl. 1970. The diagnosis of the adult type of Gaucher's disease and its carrier state by demonstration of deficiency of β-glucosidase activity in peripheral blood leukocytes. *J. Lab. Clin. Med.* **76**:747.
5. Padeh, B., and R. Navon. 1971. Diagnosis of Tay-Sachs disease by hexosaminidase activity in leukocytes and amniotic fluid cells. *Isr. J. Med. Sci.* **7**:259.
6. Losman, M. J. 1974. β-glucosidase activity as a diagnostic index of Gaucher's disease. *S. Afr. Med. J.* **48**:1150.
7. Klibansky, C. H., J. Hoffman, J. Pinkhas, D. Algom, M. Dintzman, M. Ben-Bassat, and A. De Vries. 1974. Leukocyte glucocerebrosidase deficiency diagnostic in adult Gaucher's disease with negative bone marrow biopsy. Some properties of the enzyme in leukocytes and spleen. *Eur. J. Clin. Invest.* **4**:101.
8. Kint, J. A. 1970. Fabry's disease: alpha-galactosidase deficiency. *Science (Wash. D. C.)*. **167**:1268.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
10. Lillie, R. D. 1965. Histopathologic Technic and Practical Histochemistry, McGraw-Hill Publishing Co. Ltd., London, England. 3rd edition.
11. Ho, M. W., and M. Rigby. 1975. Glucocerebrosidase: stoichiometry of association between effector and catalytic proteins. *Biochim. Biophys. Acta.* **397**:267.
12. Ho, M. W., J. Seck, D. Schmidt, M. L. Veath, W. Johnson, R. O. Brady, and J. S. O'Brien. 1972. Adult Gaucher's disease: kindred studies and demonstration of a deficiency of acid β-glucosidase in cultured fibroblasts. *Am. J. Hum. Genet.* **24**:37.