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SEPARATION AND PROPERTIES OF MOLECULAR FORMS OF α-GALACTOSIDASE AND α-*N*-ACETYLGALACTOSAMINIDASE FROM BLOOD LYMPHOCYTES AND LYMPHOID CELL LINES TRANSFORMED BY EPSTEIN-BARR VIRUS

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Summary

(1) 4-Methylumbelliferyl-α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) deficiency is demonstrated in lymphoid cell lines established by Epstein-Barr virus transformation of B-lymphocytes from a Fabry patient, as in blood whole leukocytes and lymphocytes. The residual activity was heat-stable. 4-Nitrophenyl-α-*N*-acetylgalactosaminidase (2-acetamido-2-deoxy-α-D-galactoside acetamidodeoxygalactohydrolase, EC 3.2.1.49) was present at normal level in leukocytes, lymphocytes or lymphoid cells from normal and Fabry patients and was heat-stable in all cases.

(2) In normal blood lymphocytes and lymphoid cell lines, 4-methylumbelliferyl-α-galactosidase was separated by electrofocusing into three molecular forms: form I (p*I* 5.0), form II (p*I* 4.5) and form III (p*I* 4.3). Form IV, a major form in whole leukocytes (Salvayre, R., Maret, A., Nègre, A. and Douste-Blazy, L. (1979) Eur. J. Biochem. 100, 377—383) was lacking or only slightly detectable. A wide variability in the level of form III activity was observed in the various cell lines. 4-Nitrophenyl-α-*N*-acetylgalactosaminidase electrofocusing profiles presented one major peak (p*I* 4.5) and one minor peak (p*I* 5.1).

(3) Blood lymphocytes and lymphoid cell lines from Fabry disease showed similar defects of all the forms of 4-methylumbelliferyl-α-galactosidase group A

Abbreviations: EBV, Epstein-Barr virus; MeUmb, 4-methylumbelliferone; MeUmb-α-Gal, 4-methylumbelliferyl-α-D-galactopyranoside; Nph-α-GalNac, 4-nitrophenyl-α-D-*N*-acetylgalactosaminide.

and the residual activity proceeded from the 4-methylumbelliferyl-α-galactosidase form II which is a 4-nitrophenyl-α-*N*-acetylgalactosaminidase. In the absence of animal models, those established lines seem to be an accurate cellular system for in vitro experimental studies of Fabry disease.

Introduction

4-Methylumbelliferyl-α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) displays two major molecular forms, form I (p*I* 5.0) and form II (p*I* 4.5), in almost all human tissues studied so far [1—4] but the electrofocusing profiles of the solubilized enzymes remain quite characteristic of each tissue [4]. 4-Methylumbelliferyl-α-galactosidase forms were numbered following the nomenclature of Kano and Yamakama [3]: forms I and II are identical to forms A and B of the previous standard nomenclature. We have also previously demonstrated that 4-methylumbelliferyl-α-galactosidase form IV, a new major molecular form with more acidic p*I* (p*I* 4.0), is characteristic of blood whole leukocytes [5]. Forms I and IV belong to the same isoenzymatic group A characterized by the following enzymatic and genetic properties; heat-lability, close K_m values for MeUmb-α-Gal (K_m 2.0 mM), no hydrolysis of Nph-α-GalNAc, inhibition by α-galactoside or mesoinositol and deficiency in Fabry disease (mutation of the α-galactosidase X-linked gene) [5].

α-*N*-Acetylgalactosaminidase(2-acetamido-2-deoxy-α-D-galactoside acetamidodeoxygalactohydrolase, EC 3.2.1.49) of all human tissues studied is focused in a single peak [4,5] unlike the enzyme of pig liver [6,7]. This enzyme is identical to the 4-methylumbelliferyl-α-galactosidase form II, and constitutes the isoenzymatic group B, with the following properties: heat-stability, K_m for MeUmb-α-Gal (5.5 mM) quite different from group A, hydrolysis of Nph-α-GalNAc, inhibition by α-galactoside and α-*N*-acetylgalactosaminide and normal activity in Fabry disease [5,8,9]. But the molecular forms of these enzymes have never been studied either in human blood lymphocytes or in lymphoid cell lines, although these cells have been employed in the diagnosis or in the study of several genetic diseases with lysosomal storage [10, 11].

Long-term lymphoid lines have several advantages: continuous cultures for a long time, without or with only minor changes in their phenotypic expression [10], possibility to obtain a large amount of cellular material owing to a short doubling time and growth in suspension culture [11]. A wide variability of lysosomal acid hydrolase activities was recently reported in different lymphoid lines by Dreyfus et al. [11], who described a decrease in the whole enzyme activity for several lines. However, those studies did not discriminate between a general drop of all enzymatic forms and a specific fall of one of them.

This prompted us to compare 4-methylumbelliferyl-α-galactosidase and 4-nitrophenyl-α-*N*-acetylgalactosaminidase molecular form patterns in blood lymphocytes and in established lymphoid lines from normal subjects and patients with Fabry disease, in order to evaluate their validity as an in vitro system to study Fabry disease.

Materials and Methods

Materials. Ficoll-paque was supplied by Pharmacia (Uppsala, Sweden), Ultrodex and ampholines 3.5—10 by L.K.B. (Bromma, Sweden), MeUmb, MeUmb-α-Gal, 4-nitrophenol, Nph-α-GalNAc by Koch-Light (Colnbrook, Bucks, U.K.), Triton X-100 by Rohm and Haas (Philadelphia, PA, U.S.A.), RPMI 1640 medium and fetal calf serum by Gibco (Grand Island, NY, U.S.A.) and other reagents by Merck, Darmstadt, F.R.G.).

Isolation of lymphocytes. 300 ml freshly drawn blood (using citric acid/citrate/dextrose as anticoagulant [12]) were obtained from healthy adult volunteers or from a patient with Fabry disease as previously described [5]. The research was carried out in accordance with the principles of the World Medical Association (Helsinki 1964 and Tokyo 1975) concerning biomedical research. First, platelets were eliminated by differential centrifugation [12]: platelet-rich supernatant was obtained by blood centrifugation at 120 × *g* for 10 min and platelets were sedimented from the plasma by centrifugation at 1000 × *g* for 30 min. Then, after mixing the erythrocyte-leukocyte pellet with platelet-poor plasma and a solution of 5% glucose/0.9% NaCl (pellet-plasma-glucose/NaCl, 1 : 1 : 1, v/v), the lymphocytes were isolated on a Ficoll-paque discontinuous gradient following Boyum [13]; the lymphocyte layer was then removed, mixed with a 5% glucose/0.9% NaCl (1 : 1, v/v) solution and centrifuged at 1500 × *g* for 15 min; the pelleted lymphocytes were washed twice with the glucose/NaCl solution and the last pellet was stored at —70° C. Enzyme activities were stable for at least 3 months.

Long-term lymphoid cell lines. The lymphoid cell lines C_{49}/B_{95}, Be/B_{95} and Lag/B_{95} were established from normal cord blood lymphocytes, normal adult blood lymphocytes and adult Fabry blood lymphocytes, respectively, after incubation with the B_{95} strain of Epstein-Barr virus. Those lines containing the EBV genome only expressed Epstein-Barr nuclear antigen and did not produce late EBV antigens or viral particles. The transformed cells were cultivated in RPMI 1640 medium (containing 20% heat-inactivated fetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin), were collected in exponential growth phase, washed twice in phosphate buffered saline and stored at —70° C as frozen cell pellet until enzyme analysis.

Enzyme preparation. Cell homogenates in Triton X-100 were obtained as previously described [5], and centrifuged at 220 000 × *g* for 1 h (L 65 Beckman centrifuge, rotor 60 Ti). Enzyme solubilization was superior to 85% (compared to the whole homogenate activity 100%) for 4-methylumbelliferyl-α-galactosidase and 4-nitrophenyl-α-*N*-acetylgalactosaminidase in all cell types studied.

Preparative electrofocusing. The enzymatic molecular forms were separated from 3.0 ml of 220 000 × *g* supernatant by preparative flat-bed electrofocusing (LKB 2117 Multiphor, ampholines 3.5—10 and 4—6), following Radola [14] as previously described [5]. Each band of focused Ultrodex was eluted by 3 ml Triton X-100. Four different runs were performed with extracts from four normal subjects, three runs with C_{49}/B_{95} batches and two runs with two Be/B_{95} and two Lag/B_{95} batches.

Enzyme assays. 4-Methylumbelliferyl-α-galactosidase and 4-nitrophenyl-α-*N*-

acetylgalactosaminidase standard assays were performed as previously described [5] and were linear with respect to time (up to 1 h), sample protein concentration (up to 0.8 mg/ml) and hydrolysed substrate (up to 250 and 200 nmol/ml, respectively). The heat-stability was determined at 50°C as previously described [5] following Kano and Yamakawa [3].

Protein determination. Protein concentration was determined by the method of Lowry et al. [15] using bovine serum albumin as standard (without removing Triton X-100), and estimated following Warburg and Christian [16] in the samples containing ampholines.

Results

Enzyme activities of leukocytes and lymphoid cells from normal subjects and patients with Fabry disease (hemizygotes)

Enzyme activities are summarized in Table I. Normal mixed leukocyte extract exhibited a slightly higher 4-methylumbelliferyl-α-galactosidase activity level than normal lymphocytes and lymphoid cell lines but deficiency of 4-methylumbelliferyl-α-galactosidase was readily demonstrated in all cell types studied from Fabry disease. The heat-stable 4-methylumbelliferyl-α-galactosidase activity was higher in blood lymphocytes and lymphoid cell lines than in whole leukocytes but always represented less than 50% of the total activity in all cell types from normal subjects. In contrast, this heat stable activity was highly predominant in all Fabry cell types. On the other hand, the activity of 4-nitrophenyl-α-*N*-acetylgalactosaminidase was similar and quite heat-stable in all cell types from normal or Fabry subjects.

Electrofocusing separation and properties of enzyme molecular forms (Figs. 1—2)

Three main molecular forms of 4-methylumbelliferyl-α-galactosidase, numbered following Kano and Yamakawa [3] were separated by electrofocusing from blood lymphocyte and from lymphoid cell line extracts of normal subjects: form I (p*I* 5.0 ± 0.2), form II (p*I* 4.5 ± 0.2) and form III (p*I* 4.3 ± 0.2) constituted the major part of the whole activity whereas form IV (p*I* 4.0 ± 0.2) was a minor form in both cell types. Forms I, III and IV were heat-labile whereas form II was heat-stable (form IV activity was too low to perform a detailed study of the heat-stability, but the peak disappeared from the heat-inactivated 4-methylumbelliferyl-α-galactosidase profiles). The substantial differences of heat stability allowed us to distinguish between form II and III in spite of a bad separation of both peaks by electrofocusing. The variability of form III activity in various normal tested subjects is to be noted.

In all cell types from normal subjects, activities of forms I and II were similar, but particularly striking differences appeared in the case of form III, whose activity in the Be/B_{95} line averaged twice that of the C_{49}/B_{95} line and 5-times that of blood lymphocytes.

In extracts of all cell types from Fabry disease, 4-methylumbelliferyl-α-galactosidase activity was mainly focused in a single broad heat-stable peak with a p*I* value slightly different from the normal form II (p*I* 4.4 ± 0.2) whereas all heat-labile peaks were hardly detectable: indeed the comparison of

TABLE I

4-METHYLUMBELLIFERYL-α-GALACTOSIDASE AND 4-NITROPHENYL-α-*N*-ACETYLGALACTOSAMINIDASE OF WHOLE LEUKOCYTES, LYMPHOCYTES AND LYMPHOID CELL LINES FROM NORMAL SUBJECTS AND FABRY PATIENTS: ENZYME ACTIVITIES BEFORE AND AFTER HEAT-TREATMENT

Enzyme activities were determined under standard conditions as described in Materials and Methods. Each determination was done in duplicate. Heat-treatment was performed for 1 h at 50°C in 30 mM phosphate buffer pH 6.5.

	4-Methylumbelliferyl-α-galactosidase		4-Nitrophenyl-α-*N*-acetylgalactosaminidase	
	before heat inactivation nmol/h per mg	after heat inactivation nmol/h per mg (%)	before heat inactivation nmol/h per mg	after heat inactivation nmol/h per mg (%)
Whole leukocytes from normal subjects (10) *	59.0 ± 17.5	14.3 ± 6.5 (25%)	44.0 ± 19.0	35.2 ± 11.5 (80%)
Whole leukocytes from Fabry patients				
-C... (4) **	5.0	4.5 (93%)	47.0	42.0 (90%)
-Ch... (3) **	12.0	8.5 (75%)	78.0	70.0 (90%)
-A... (1) **	4.2	4.0 (95%)	52.0	47.5 (92%)
Lymphocytes from normal subjects (10) *	47.5 ± 16.5	19.0 ± 7.0 (40%)	54.0 ± 16.0	49.7 ± 13.0 (92%)
Lymphocytes from Fabry patient				
-C... (2) **	12.0	11.0 (90%)	57.0	55.0 (96%)
Lymphoid cell lines from normal subjects				
-C_{49}/B_{95}	35.5	15.0 (43%)	45.0	40.0 (88%)
-Be/B_{95} (3) **	51	17.0 (38%)	55.0	51.0 (92%)
Lymphoid cell lines from Fabry patient				
-Lag/B_{95} (2) **	7.5	7.0 (94%)	42.0	40.0 (95%)

* Number of samples from different subjects (mean ± S.D.).

** Number of samples or different culture batches from same subject.

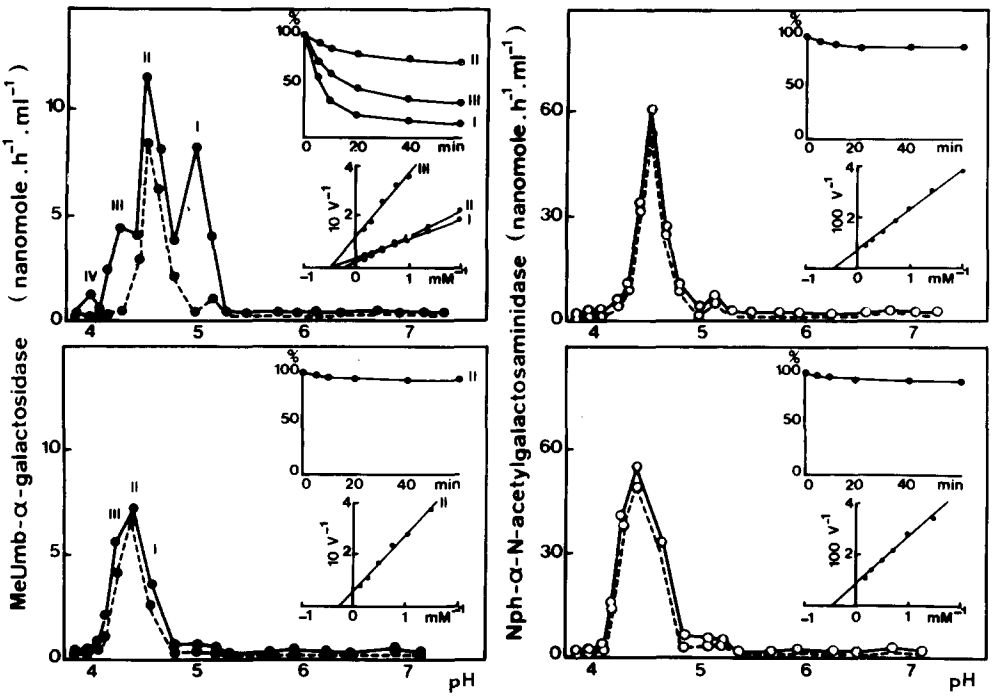

Fig. 1. Electrofocusing profiles of 4-methylumbelliferyl-α-galactosidase (●——●) and 4-nitrophenyl-α-*N*-acetylgalactosaminidase (○——○) of peripheral blood lymphocytes from normal subjects (upper panels) and from Fabry patients (lower panels) before (solid line) and after (dotted line) heat inactivation (1 h at 50°C). Flat bed electrofocusing was performed as indicated in Materials and Methods, using 3 ml of 220 000 × *g* supernatant of cell homogenized in Triton X-100, 0.25%. Total proteins of the focused supernatants were 10 mg for normal lymphocytes and 22 mg for Fabry lymphocytes. Heat inactivation curves: aliquots of electrofocusing peak fractions were incubated at 50°C in 40 mM phosphate buffer pH 6.5, sampled periodically and assayed for enzyme activities under standard reaction conditions. The reported pattern of normal lymphocytes is an average of several profiles in which was observed a variability of form III activity level. Lineweaver-Burk plots for focused forms: activities were determined under standard conditions, varying concentration of 4-methylumbelliferyl-α-galactosidase from 1.25 to 10 mM and of Nph-αGalNAc from 0.5 to 4.5 mM (4-methylumbelliferyl-α-galactosidase form II was heat-treated before use). Reciprocal plots were drawn using the method of least-squares (enzyme activities in nmol · h^{-1} · ml^{-1}).

4-methylumbelliferyl-α-galactosidase profiles, before and after heat-inactivation, showed the complete heat-stability of the form II whereas minor shoulders disappeared upon heat-treatment and could thus represent residual (less than 30%) forms I and III.

4-Nitrophenyl-α-*N*-acetylgalactosaminidase was focused in a single main peak coinciding with 4-methylumbelliferyl-α-galactosidase form II and displayed the same heat-stability. A minor peak (p*I* 5.1) represented less than 10% of the whole 4-nitrophenyl-α-*N*-acetylgalactosaminidase activity. In Fabry blood lymphocytes and in Lag/B_{95} line the broad peak was also quite similar to the heat-stable 4-methylumbelliferyl-α-galactosidase residual peak.

Optimum pH (in 0.1 M acetate buffer) was 4.5 ± 0.1 for all forms of 4-methylumbelliferyl-α-galactosidase and for 4-nitrophenyl-α-*N*-acetylgalactosaminidase.

According to the apparent K_m for 4-methylumbelliferyl-α-Gal (Table II), the

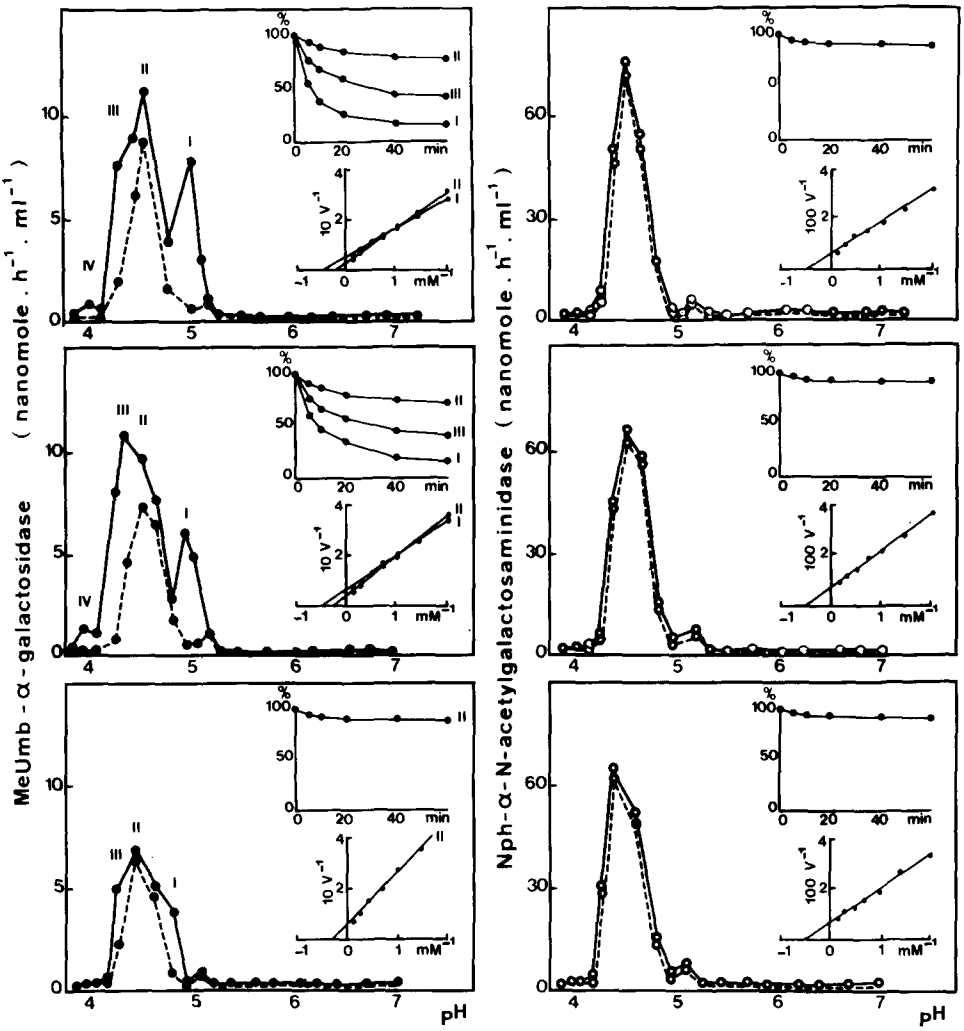

Fig. 2. Electrofocusing profiles of 4-methylumbelliferyl-α-galactosidase (●——●) and 4-nitrophenyl-α-*N*-acetylgalactosaminidase (○——○) of lymphoid cell lines from normal subjects (C_{49}/B_{95} (upper panels) and Be/B_{95} (middle panel) and from Fabry patients (Lag/B_{95} (lower panel)). Total proteins of the focused supernatants were 11 mg, 9 mg and 24 mg respectively. See legend to Fig. 1 for other experimental conditions.

various focused forms could be divided into two groups: forms I and III with low K_m values and form II with high K_m values (K_m of form II was quite similar before and after heat treatment). In all the cell types studied, the apparent K_m (for Nph-α-GalNAc) of the focused 4-nitrophenyl-α-*N*-acetylgalactosaminidase had almost the same value (Table II). Moreover, a reciprocal inhibition of the hydrolysis rate of each substrate was observed for form II, in assays containing both substrates, MeUmb-α-Gal and Nph-α-GalNAc (Table III), and therefore demonstrated that they were probably hydrolysed in a single enzymatic site.

TABLE II

MICHAELIS CONSTANTS OF FOCUSED MOLECULAR FORMS OF 4-METHYLUMBELLIFERYL-α-GALACTOSIDASE AND 4-NITROPHENYL-α-*N*-ACETYLGALACTOSAMINIDASE IN LYMPHOCYTES AND IN LYMPHOID CELL LINES FROM NORMAL SUBJECTS AND FABRY PATIENTS

K_m values were calculated from the Lineweaver-Burk double-reciprocal plots (Figs. 1 and 2) using the least-squares method. Results are expressed in mM.

Sources	K_m for MeUmb-α-Gal 4-methylumbelliferyl-α-galactosidase			K_m for Nph-α-GalNAc 4-nitrophenyl-α-*N*-acetyl galactosaminidase
	I	II	III	
Peripheral blood lymphocytes				
normal subjects	2.4	5.1	—	2.5
Fabry patients	—	5.0	—	2.2
Lymphoid cell lines				
C_{49}/B_{95} (normal subject)	2.6	4.9	—	2.4
Be/B_{95} (normal subject)	2.7	5.0	3.1	2.2
Lag/B_{95} (Fabry patient)	—	5.1	—	2.4

TABLE III

RECIPROCAL INHIBITION OF 4-METHYLUMBELLIFERYL-α-GALACTOSIDASE FORM II-4-NITROPHENYL-α-*N*-ACETYL GALACTOSAMINIDASE BY BOTH SUBSTRATES

Enzyme assays and enzyme activities ($V_{det.} = V$ determined) (1): Single substrate assays were performed under standard conditions using MeUmb-α-Gal or Nph-α-GalNAc at indicated concentrations. Both substrates assays contain MeUmb-α-Gal and Nph-α-GalNac together and were done under standard conditions. Lag/B_{95} lymphoid cell line supernatant was used as enzyme source after heat inactivation (1 h at 50°C in 30 mM phosphate buffer, pH 6.5). For each assay, one incubated blank without enzyme was substracted. Liberated MeUmb was fluorimetrically measured at λ excitation 375 nm/λ emission 460 nm (λ was chosen to minimize interferences). 4-Nitrophenol liberated was spectrophotometrically measured at 410 nm. V calculated (2): V_c = in the hypothesis of competitive inhibition

$$V_c = \frac{V\,(S)}{(S) + K_m(1 + \frac{(I)}{K_i})}$$

V_{nc} = in the hypothesis of non-competitive inhibition

$$V_{nc} = \frac{V\,(S)}{(K_m + (S))(1 + \frac{(I)}{K_i})}$$

V and K_m were determined for enzyme preparation. V for 4-methylumbelliferyl-α-galactosidase = 24 nmol · h^{-1} · ml^{-1}. K_m for MeUmb-α-Gal = 5.0 mM. V 4-nitrophenyl-α-*N*-acetylgalactosaminidase = 100 nmol · h^{-1} · ml^{-1}. K_m for Nph-α-GalNac = 2.4 mM. Calculations were done with the hypothesis in which substrate fixation sites were the same in single substrate and both substrates assays and thus, K_m values for MeUmb-α-Gal = K_i values for MeUmb-α-Gal and K_m values for Nph-α-GalNAc = K_i values for Nph-α-GalNac.

Enzyme activities (1)	Substrate concentration (mM)		Enzyme activities (nmol · h^{-1} · ml^{-1})			
	MeUmb-α-Gal	Nph-α-GalNac	single substrate assay $V_{det.}$ (1)	both substrates assay		
				$V_{det.}$ (1)	V calculated (2)	
					V_c	V_{nc}
4-Methylumbelliferyl-α-galactosidase	2.5	2.2	9	4.7	5.1	4.2
	5.0	2.2	12	7.7	8.3	6.3
4-Nitrophenyl-α-*N*-acetylgalactosaminidase	5.0	1.5	39	22.5	22	19
	5.0	2.5	51	29.5	31.5	24

Discussion

4-Methylumbelliferyl-α-galactosidase activities that we observed in leukocytes and lymphocytes from normal blood were similar to those reported by Desnick et al. [17], Beutler et al. [18] and by Svennerholm et al. [19]. In contrast, the values reported by Dreyfus et al. [11] were slightly lower because the substrate concentration used by these authors was weaker, but surprisingly the values found by Tanaka [20] were very low. No data were available in the literature for 4-nitrophenyl-α-*N*-acetylgalactosaminidase in leukocytes or lymphoid cells.

Comparison between whole activity of 4-methylumbelliferyl-α-galactosidase in blood lymphocytes and in lymphoid cell lines from normal subjects demonstrated similar activity level, as reported for this enzyme in other lymphoid cell lines by Dreyfus et al. [11]. Moreover, the amount of heat-stable activity was similar in both cell types and was stable in the various batches of lymphoid cells collected 2 or 3 days after medium change, during the exponential growth phase (at that time, viable cell counts were good and lysosomal enzyme activities were at the maximum [21]). Finally lymphoid cell lines from Fabry disease patients showed a deficiency of 4-methylumbelliferyl-α-galactosidase similar to that of Fabry blood lymphocytes and it seems useful to study Fabry disease, as previously, for other genetic metabolic diseases [10,22—24]. However, in these previous studies, the possibility of heterogeneity with respect to enzyme molecular forms was not investigated, although many lysosomal storage diseases are known to result from the absence of a specific isozymic form of these enzymes. It is noteworthy that 4-nitrophenyl-α-*N*-acetylgalactosaminidase activity level was quite identical in all cell types from normal and Fabry disease cell lines, as described for whole leukocytes [5].

4-Methylumbelliferyl-α-galactosidase molecular forms I and II of normal blood lymphocytes were quite similar to those of other tissues [1—4]. One could also see the presence of a minor form III badly separated from form II and the very little amount or lack of form IV, that we previously demonstrated to be characteristic of whole leukocytes [5] and more precisely of polymorphonuclears [25]. Leukocytic electrofocusing profile, reported by Christomanou et al. [26], showed a single peak (p*I* 4.5) with a shoulder (p*I* 5.0) and is comparable to our lymphocyte profile, for which a better separation due to the flat-bed electrofocusing technique was attained. Moreover, differences in leukocyte extraction procedure might have influenced their results.

In lymphoid lines C_{49}/B_{95} and Be/B_{95} established from normal subjects, 4-methylumbelliferyl-α-galactosidase profiles presented also forms I to III, but the level of form III activity was different. Identification of form III, badly separated from form II, was accurately settled by heat-inactivation (form II heat-stable vs. form III heat-labile), by substrate specificity (form II only hydrolysed Nph-α-GalNAc) and by genetics (form II only remained in Fabry cells). Therefore, form III belongs to the isoenzymatic group A, previously defined, like forms I and IV [5]. Relationships between the various forms of α-galactosidase group A are not known today, but the difference of p*I* could be explained by a microheterogeneity of the carbohydrate moiety; as described for other hydrolases, microheterogeneity may result from a different amount

of sialylation [27—30] or from phosphomannose residues [31—34]. The first hypothesis is in agreement with the presence of sialic acid residues in α-galactosidase A as reported by Kusiak et al. [35] and Bishop and Sweeley [36]. However, other structural hypotheses might be proposed: either polymeric complexes [37—38] or precursors of higher molecular weight [39]. Moreover, form III variability does not seem to be explained by the EBV transformation since the same B_{95} EBV strain was used in all experiments and since each EBV transformed cell line contains a similar amount of EBV genomes or Epstein-Barr nuclear antigen (unpublished data). But form III variability might be due to differences in lymphoid cell population or donors; first, EBV transformed cells proceed from B-lymphocytes which represent a minor section in peripheral blood lymphocyte population; on the other hand, differences in C_{49}/B_{95} and Be/B_{95} lines might be age-dependent (C_{49} line proceeds from the cord blood and Be/B_{95} from the peripheral blood of an adult woman about 40-years-old), by analogy with the increase of acidic forms in α-fucosidase electrofocusing pattern during fetal development, as reported by Alhadeff et al. [40]. However, the hypothesis of a clonal selection can also not be rejected.

In all electrofocusing profiles, the main peak of 4-nitrophenyl-α-*N*-acetylgalactosaminidase always corresponds to 4-methylumbelliferyl-α-galactosidase form II and both activities had the same properties and seemed to be supported by the same enzyme. These results are in good agreement with those previously reported in placenta by Kusiak et al. [35], in liver by Sweeley's group [7,9] and Schram et al. [41] and in leukocytes by Salvayre et al. [5]. A minor form of 4-nitrophenyl-α-*N*-acetylgalactosaminidase (less than 10% of the whole activity) with a less acidic p*I* value (p*I* 5.1), exists in blood lymphocytes and lymphoid cells. This form does not seem to be due to autolysis or storage as described by Schram et al. [42] for a minor form from liver, since it exists in freshly prepared cells; likewise the purification procedure was intentionally reduced to avoid modifications or loss of native forms. However, as in other human tissues [4,8,9], 4-nitrophenyl-α-*N*-acetylgalactosaminidase did not show the heterogeneity of the pig liver enzyme reported by Weissman et al [16] and by Sung et al. [7].

In blood lymphocytes and lymphoid lines from Fabry patients, the isozymic patterns were quite similar: heat-labile forms were very low or absent and the heat-stable 4-methylumbelliferyl-α-galactosidase peak corresponded to that of 4-nitrophenyl-α-N-acetylgalactosaminidase by the electrofocusing profile and by the enzymatic properties. So the main residual 4-methylumbelliferyl-α-galactosidase peak seems identical to the normal form II as reported in other Fabry tissues [5,41,43] and also identical to the 4-nitrophenyl-α-*N*-acetylgalactosaminidase major peak as previously demonstrated [5,7,9,35]. However, in Fabry lymphoid cells, the residual form II was slightly broader and more acidic than in normal cells: this shift of p*I* may not be explained by an anomaly of the ampholine gradient (because pH gradient was regular and hexosaminidase peaks had a normal p*I*). Similar data were reported by Mueller and Rosenberg [44] for residual β-glucosidase of Gaucher fibroblasts. This minor modification of p*I* could disturb the uptake-release mechanism of lysosomal hydrolases [29] and perhaps explain some secondary deficiencies in lysosomal storage diseases. The molecular basis of the p*I* minor shift is not known: normal

α-galactosidase form II contains no sialic acid residue, neither in human placenta nor in pig liver (Sung quoted by Bishop et al. [36]). So, a modification of sialylation seems unlikely, but a modification in the content of phosphomannose residues, recognition markers essential for uptake [31—33], might not be excluded.

In conclusion, in lymphoid cell lines and blood lymphocytes from normal subjects, 4-methylumbelliferyl-α-galactosidase molecular forms may be separated into two groups defined by enzymatic and genetic properties:

-group A is constituted by forms I and III (as for form IV of polymorphonuclears), heat-labile, hydrolysing MeUmb-α-Gal but not Nph-α-GalNAc with low K_m for MeUmb-α-Gal, lacking in Fabry disease (α-galactosidase X-linked gene);

-group B is constituted by 4-methylumbelliferyl-α-galactosidase form II-4-nitrophenyl-α-*N*-acetylgalactosaminidase hydrolysing both substrates with high K_m for MeUmb-α-Gal, heat-stable, remaining in Fabry disease.

Lymphoid cell lines presented a variability of the microheterogeneity of 4-methylumbelliferyl-α-galactosidase forms (variability of the level of form III). However, lymphoid cell lines established from a Fabry patient presented the same deficiency of 4-methylumbelliferyl-α-galactosidase group A and the same conservation of the group B as blood lymphocytes and therefore may constitute a useful model of Fabry disease for in vitro experimental studies.

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