Lysosomal Enzymes of Cultured White Blood Cells in Cystic Fibrosis (35595)

GUY D. GRIFFIN AND GORDON E. GIBBS

Department of Pediatrics, University of Nebraska College of Medicine, Omaha, Nebraska 68105

Cystic fibrosis is a genetic disease characterized by secretion of sweat with an abnormally high NaCl concentration, and by elaboration of an abnormally viscous mucus from various exocrine glands. The relation between the ionic and mucoid defects is unknown. Recently, much attention has been focused on certain abnormalities of metabolism which appear in cultured cells from cystic fibrosis patients. Danes and Bearn (1) reported that cultured cells from these patients showed metachromatic staining with toluidine blue. There is some evidence that these fibroblasts accumulate abnormally large amounts of acid mucopolysaccharides (2) and glycogen (3), but kinetic studies of mucopolysaccharide turnover have revealed no abnormality (4). Fibroblasts from cystic fibrosis patients also show an abnormality in collagen metabolism (5).

Cultured lymphocytes from some cystic fibrosis patients show a metachromasia with toluidine blue (6). Since white blood cell cultures can be prepared more readily than fibroblast cultures, we determined to study the activity of beta-glucuronidase (EC3.2.1.31) and other lysosomal enzymes in white blood cell cultures from cystic fibrosis. We had already found a significantly decreased activity of beta-glucuronidase in sweat gland and epidermis of these individuals (7). Other enzymes, phosphatases including ATPases, and dehydrogenases showed no abnormality in sweat gland. Beta-glucuronidase has been implicated in two physiological processes which may be of importance in cystic fibrosis, namely, mucopolysaccharide catabolism and salt transport. The enzyme has been found, along with a hexosaminidase, to degrade oligosaccharides of hyaluronic acid (8). Beta-glucuronidase activity has been shown to increase significantly in the active nasal salt gland of the salt-loaded duck (9).

We now report that beta-glucuronidase activity is significantly decreased in cystic fibrosis white blood cell cultures, at a time in culture when two other lysosomal enzymes, acid phosphatase (EC3.1.3.2) and beta-acetylglucosaminase (EC3.2.1.30) were not different from normal.

Materials and Methods. For culture of leukocytes, 10 ml of venous blood were drawn into a heparinized tube and an additional 500 units of heparin were added. The erythrocytes were allowed to sediment at 37° for 1–2 hr and the tubes were then centrifuged at 50*g* for 10 min at 5° (10, 11). The supernatant plasma and white cells were withdrawn, and an aliquot was counted in an hemocytometer. On the basis of this cell count, the plasma was diluted with medium to give a final cell concentration of 1×10^6 cells/ml. The medium used was Eagle's MEM, modified for suspension culture, and containing 20% fetal calf serum, 2% phytohemagglutinin (M form; General Biochemicals) 0.5% glutamine (200 m*M*) along with penicillin (140 units/ml) and streptomycin (50 μg/ml). After the cells were diluted to their final concentration with medium, the resulting suspension was divided into separate tubes (3-ml aliquot/tube). The tubes were placed in an upright position in a 37° incubator. After 3, 5, and 7 days the tubes were centrifuged for 5 min at 1500*g*. The medium was poured off, and the cells were washed once in phosphate buffered saline (Dulbecco's buffer), followed by recentrifugation. The wash solution was decanted, the cells were suspended in the small amount of buffer remaining in the tube, and a drop of the

resulting suspension was placed on a coverslip, air dried, and stained with toluidine blue by the procedure of Danes and Bearn (12).

For assay of beta-glucuronidase, the cells were homogenized in 0.5 ml of acetate buffer (pH 4.5, 0.2 *M*) for 2 min in an all-glass homogenizer powered by an electric motor. For assay of acid phosphatase and beta-acetylglucosaminase, the homogenizing fluid used was water. All homogenizations were carried out in an ice bath. After homogenization, the pestle was rinsed with 0.2 ml of homogenizing fluid. Beta-glucuronidase activity was assayed essentially according to the method of Talalay *et al.* (13), while acid phosphatase was determined by the method of Bessey *et al.* (14) and beta-acetylglucosaminase by the method of Borooah *et al.* (15). For beta-glucuronidase, the assay mixture contained 0.3 ml of homogenate in a total volume of 0.75 ml, which contained the following final concentrations of reagents: 2 m*M* phenolphthalein mono-beta-glucuronic acid (Sigma Chemical Co); 0.04% Triton X-100 (Rohm and Haas); and 0.16 *M* acetate buffer, pH 4.5. This total volume was subdivided into 2 aliquots which were incubated at 37° for 1.5 hr, and 2 other aliquots, which were kept in the refrigerator and used for a blank and a standard. One μg of phenolphthalein in 95% ethanol was added to the standard tube.

After incubation, 0.25 ml of glycine buffer (0.3 *M*, pH 10.4) was added to each tube, and following centrifugation, the optical density of each sample was determined at 555 mμ. The amount of phenolphthalein liberated by the enzymic reaction was calculated by comparison with the standard sample, after subtracting blank readings.

For assay of acid phosphatase and beta-acetylglucosaminase, the assay mixture contained 0.10 ml of homogenate in 0.75-ml total volume, which in the case of acid phosphatase, contained the following reagents (final conc): 8 m*M* *p*-nitrophenyl phosphate, disodium, tetrahydrate (Sigma Chemical Co.); 0.04% Triton X-100; and 0.053 *M* succinate buffer, pH 5.3. For assay of beta-acetylglucosaminase, the following reagent concentrations were used: 3.6 m*M* *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (Sigma Chemical Co.); 0.04% Triton X-100; and 0.04 *M* citrate buffer, pH 4.5.

Assay mixtures were divided in the same manner as for beta-glucuronidase, but *p*-nitrophenol (2 μg) was used as the standard. After incubation for 1 hr at 37°, 0.6 ml of 0.1 *N* NaOH was added to all acid phosphatase tubes, and 0.60 ml of 0.2 *M* borate buffer (pH 9.8) was added to beta-acetylglucosaminase tubes. After clarification by centrifugation, optical densities were determined at 430 mμ, and the amount of *p*-nitrophenol released enzymatically was calculated.

Duplicate 0.05-ml aliquots of homogenate were assayed for protein concentration using the method of Lowry *et al.* (16) with bovine serum albumin used to make a standard curve. Beta-glucuronidase activity was expressed as nanomoles of substrate hydrolyzed per 100 μg of protein, per hour of incubation at 37°. Acid phosphatase and beta-acetylglucosaminase activities were expressed using the same units.

Results. Beta-glucuronidase has been determined in 11 control patients and 12 cystic fibrosis cases. The enzyme activities on the third, fifth, and seventh days in culture are listed in Table I. Mean values for beta-glucuronidase activity in cystic fibrosis leukocytes were lower than control values at days 5 and 7 of culture. This lowering of activity was significant both on days 5 and 7 of culture. The mean age of the controls was 11 years; of the cystic fibrosis patients, 10 years. There appeared to be no significant correlation between beta-glucuronidase activity and age of the patient. The values obtained for enzyme activity on day 3 of culture are in the same range as those reported by Hirschhorn *et al.* (17) for lymphocytes grown for 72 hr in medium containing phytohemagglutinin.

Acid phosphatase and beta-acetylglucosaminase activities were determined in 11 control patients and 11 patients with cystic fibrosis. The results of these studies after 3, 5, and 7 days in culture are summarized in Table II, which also indicates

TABLE I. Beta-Glucuronidase Activity in Cultured Leukocytes.

Patient (initials)	Enzyme activity (nmoles of substrate hydrolyzed/hr/100 μg of protein) (3)[a]	(5)[a]	(7)[a]	Age (years)
		Controls[b]		
R.D.	5.64	5.92	3.55	7
D.H.	2.83	3.84	3.36	1
T.B.	3.27	5.67	5.85	9
C.C.	2.77	3.87	4.12	2
R.M.	7.86	6.98	—	13
R.S.	4.90	3.08	—	17
M.L.	3.81	4.40	4.37	9
A.F.	2.23	5.20	3.74	11
A.M.	3.08	6.00	5.29	12
D.B.	6.73	5.60	8.72	12
A.Z.	4.15	3.78	3.01	13
		Cystic fibrosis		
P.L.	6.29	3.14	2.20	4
B.V.	7.17	3.87	3.18	8
P.P.	4.78	3.55	4.40	14
P.D.	5.54	4.53	2.26	5
C.C.	4.75	3.84	2.55	9
D.S.	3.61	2.89	3.49	4
J.M.	3.27	3.08	2.76	13
M.M.	5.83	4.87	2.42	9
B.B.	2.55	3.84	5.57	17
T.C.	0.00	2.92	—	26
P.H.	4.75	3.43	1.48	7
L.G.	5.03	1.57	2.58	16

Statistical summary

Days in culture:	Mean enzyme activity ± SD 3	5	7
Controls	4.30 ± 1.80	4.94 ± 1.21	4.67 ± 1.77
Cystic fibrosis	4.46 ± 1.91	3.46 ± 0.85	2.99 ± 1.14
Standard error of the difference between the means	0.80	0.44	0.68
t	0.20	3.36	2.48
p	NS	$0.001<p<0.01$	$0.02<p<0.05$

[a] Days in culture.

[b] Diagnoses of the controls were: R.D. and D.H., allergy; C.C., diarrhea; R.M., congenital heart defect; R.S., injury to nose; M.L., traumatic paraplegia; A.F., hemiplegia; A.M., euthyroid, postthyroidectomy; A.Z., epilepsy; others were normal.

the mean ages of each of the groups. The diagnosis of the normal patients is also noted in Table II. No significant difference in acid phosphatase activity was seen between the normal and cystic fibrosis leukocyte cultures. In the case of beta-acetylglucosaminase activity, a significant decrease in cystic fibrosis was observed only after 7 days of culture.

At days 5 and 7, the leukocytes appeared microscopically to be mostly mononuclear, presumably lymphocytes. Other studies have shown rapid disappearance of the granulocytes in such cultures. Rabinowitz (18) found that cultures of purified neutrophils to

TABLE II. Acid Phosphatase and Beta-Acetylglucosaminase Activities in Cultured Leukocytes.

Days in culture:	Mean enzyme activity ± SD (nmoles of substrate split/hr/100 μg of protein) 3	5	7	No. of individuals
	Acid phosphatase			
Controls[a]	114 ± 43	102 ± 86	109 ± 53	11
Cystic fibrosis	146 ± 77	112 ± 44	78 ± 45	11
t	1.18	0.35	1.48	
p	NS[b]	NS	NS	
	Beta-acetylglucosaminase			
Controls[a]	104 ± 59	64 ± 25	77 ± 23	11
Cystic fibrosis	68 ± 21	66 ± 41	53 ± 16	11
t	1.90	0.14	2.86	
p	NS	NS	<0.01	
	Mean ages (years)			
Controls	12			
Cystic fibrosis	12			

[a] Controls had the following diagnosis: epilepsy, hernia, deviated nasal septum, tonsillitis, acanthocytosis, diabetes (2), chronic hepatitis, and abscess of right axilla; all the rest are normal.

[b] NS = not significant at the level of $p < 0.05$.

which phytohemagglutinin was added did not develop blast-like cells, while in purified lymphocyte cultures, under the same conditions, 90–100% of the lymphocytes transformed into blasts by day 3 in culture. Bloom *et al.* (19) observed that after 48 hr in culture with phytohemagglutinin, almost all the cells in leukocyte cultures were lymphocytes or transformed lymphocytes, and these retained their viability over 7 days in culture.

Discussion. While the activity of beta-glucuronidase in control leukocytes in culture increased from days 3 to 5, and then decreased again by day 7, beta-glucuronidase activity in leukocytes from cystic fibrosis patients decreased at each succeeding time it was measured. In preliminary experiments, we found that beta-glucuronidase activity in freshly isolated human leukocytes was not significantly different in cystic fibrosis or control leukocytes, but that the activity in these fresh preparations was about three times that at 3 days in culture. Apparently, the decreased activity of beta-glucuronidase in cystic fibrosis cultured leukocytes, in relation to control patients, is a phenomenon which appears only as the culture is extended beyond 3 days.

It might be argued that the decreased activity of beta-glucuronidase found in cystic fibrosis leukocytes is simply a consequence of decreased growth and viability of the cystic fibrosis cultures. Protein content of the leukocyte cultures, however, indicated similar amounts of protein in both control and cystic fibrosis cultures on days 3 and 5, and only on day 7 did the cultures of cystic fibrosis cells show a very apparent decrease in protein content. Mean cellular protein content of control cultures on days 3, 5, and 7 was 672, 574, and 588 μg, while cellular protein content in cystic fibrosis cultures for the same intervals was 630, 560 and 490 μg.

A further indication that the decrease in beta-glucuronidase observed is a rather specific phenomenon is the fact that acid phosphatase activity in cystic fibrosis cultures was not significantly altered from control values. The activity of beta-acetylglucosaminase, however, was significantly decreased in cystic fibrosis cultures after 7 days of culture. Since this enzyme, along with beta-glucuronidase, is known to function in the

breakdown of acid mucopolysaccharides, this may indicate that a block in mucopolysaccharide catabolism develops in cystic fibrosis cultured cells after a certain time in culture. Whether the decreased activities of these enzymes are a primary cause or secondary result of this block remains to be seen. No explanation of the lowered beta-glucuronidase activity is apparent. Possible causes could be: accumulation of an inhibitor; altered enzyme structure; impaired synthesis of the enzyme; accelerated destruction of the enzyme.

Summary. White blood cells, mainly lymphocytes, in culture from patients with cystic fibrosis, showed a diminution of the activity of beta-glucuronidase. The mean activity of this enzyme in cystic fibrosis was 30% lower than in the controls at 5 days of culture and 35% lower than the controls at 7 days. Acid phosphatase activity in cystic fibrosis cultures was not significantly different from control cultures, while beta-acetylglucosaminase activity was significantly decreased in cystic fibrosis cultures of 7 days compared to control cultures.

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