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Correction of γ-Glutamyl Transpeptidase Deficiency in Amniotic Fluid of Some Cystic Fibrosis Fetuses by Mixing with Nondeficient Fluids

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ABSTRACT. The deficiency of γ-glutamyl transpeptidase activity, which was evident in some but not all cystic fibrosis amniotic fluids, could be corrected by mixing with either normal fluids or nondeficient cystic fibrosis fluids. Incubation of any amniotic fluid for 20 min at 62° C resulted in total loss of γ-glutamyl transpeptidase activity, but the activity could be restored by mixing with untreated nondeficient fluids. In contrast, no restoration could be obtained by mixing with untreated deficient cystic fibrosis fluids. Dialysis of amniotic fluids did not diminish their corrective capacity. Only the transpeptidation reaction was corrected and no correction was observed for the hydrolysis or autotranspeptidation of γ-glutamyl *p*-nitroanilide in the absence of the glycylglycine or methionine acceptor. Plasma specimens did not have any corrective activity, although their γ-glutamyl transpeptidase activity could be restored after heat inactivation by mixing with untreated nondeficient amniotic fluids. No correction was found for aminopeptidase or disaccharidase activities. These findings suggest that the deficient cystic fibrosis amniotic fluids probably contain normal quantities of the γ-glutamyl transpeptidase enzyme but lack a heat-labile nondialyzable activator that is necessary for its transpeptidation catalytic performance. An assay for this transpeptidase activator may provide a valuable approach to identify at least a subgroup of cystic fibrosis patients. (*Pediatr Res* 18:1340–1343, 1984)

Abbreviation

CF, cysticfibrosis

Cystic fibrosis is an autosomal recessive disorder, one of the most common genetic diseases among Caucasian populations (13). The disease involves abnormality of all exocrine glands. The major manifestations are pancreatic exocrine deficiency, chronic pulmonary disease, and abnormally high sodium and chloride concentrations in sweat. The disease is lethal and death usually occurs before or in early adulthood. Numerous biochemical abnormalities have been reported in patients with CF but the basic biochemical defect has not been as yet elucidated.

Deficiency of γ-glutamyl transpeptidase (γ-glutamyltransferase; EC 2.3.2.2), aminopeptidase M (EC 3.4.11.2), and several disaccharidase (EC 3.2.1.-) activities have been reported recently in midtrimester amniotic fluid from a number of pregnancies which had resulted in a child with CF (1, 2, 4, 16). It has been suggested that the reduced levels of these enzyme activities might be due to underdevelopment or atrophy of microvilli, to the presence of enzyme inhibitors in the amniotic fluid, or to an early intestinal obstruction. γ-Glutamyl transpeptidase catalyzes the transpeptidation and hydrolysis of glutathione (L-γ-glutamyl-L-cysteinylglycine) by transferring its γ-glutamyl moiety to amino acids, dipeptides, or water (14). Aminopeptidase M and disaccharidases are hydrolytic enzymes, catalyzing the cleavage of peptide and glycoside bonds, respectively (3, 8, 9). All these enzymes are situated on microvilli on the external surfaces of epithelial cells such as on the brush border membrane of intestinal mucosal cells where they are involved in the terminal digestion and subsequent absorption of peptides and carbohydrates.

To characterize the nature of these enzyme deficiencies in CF, we have examined the various enzymic activities in mixed CF and control amniotic fluid and plasma samples. The mixtures examined included untreated, heat-inactivated and dialyzed specimens.

MATERIALS AND METHODS

Specimens. Amniotic fluid specimens were obtained by amniocentesis between 16 and 17 wk of gestation. The cell-free supernatants were kept frozen at −20° C until used. The specimens included 43 from pregnancies at a 1:4 risk for CF in whom the outcome was known (15 CF and 28 non-CF), and 23 from pregnancies at no increased risk for CF. All specimens were collected after obtaining informed consent.

Plasma was separated from heparinized venous blood. Specimens were obtained from 5 patients with CF, 8 obligate heterozygotes, and 12 controls, and kept frozen at −20° C until used.

Assays. γ-Glutamyl transpeptidase activity was determined according to Naftalin *et al.* (10) in reaction mixtures containing 0.5–40 μl of amniotic fluid, plasma or mixed samples (0.5–2.5 μl amniotic fluid, 10–40 μl plasma), 5 mM L-γ-glutamyl-*p*-nitroanilide (Sigma), and 100 mM glycylglycine (Sigma) or L-methionine (Sigma) in 100 μl of 0.1 M Tris-Cl buffer, pH 9.0. Following incubation for 5–60 min at 37° C, the reaction was terminated by adding 400 μl of 1.7 N acetic acid. The liberated *p*-nitroaniline was diazotized by sequential addition of

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200 μl each of 15 mM sodium nitrite (Fisher), 90 mM ammonium sulfamate (Fisher), and 2 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride (Baker) solutions. The absorbance was measured at 540 nm with a Perkin-Elmer 35 spectrophotometer. The extinction coefficient of diazotized *p*-nitroaniline was 44,100 M^{-1} cm^{-1}.

The method of Jung and Scholz (7) for determination of aminopeptidase M activity was modified to include the diazotization steps. The reaction mixtures contained 2–40 μl of sample or mixed samples, and either 2 mM L-alanine-*p*-nitroanilide (Sigma) or 0.4 mM L-leucine-*p*-nitroanilide (Sigma) in 100 μl of 50 mM Tris-Cl buffer, pH 8.0. The reactions proceeded at 37° C for up to 2 h.

Maltase and sucrase were assayed according to Dahlquist (5) as modified for amniotic fluid by Potier *et al.* (11). Prior to the enzyme assays, 1-ml aliquots were dialyzed at 4° C for 24 h against 1 liter of deionized water or 0.15 M NaCl solution. The reaction mixtures contained 10–50 μl of sample or mixed samples and 58 mM of disaccharide (maltose or sucrose) in 100 μl of 0.1 M sodium maleate buffer, pH 6.0. Following incubation for 1–3 h, the liberated glucose was determined by the glucose oxidase-peroxidase method (12) with a kit supplied by Sigma.

Kinetic studies of γ-glutamyl transpeptidase activity were carried out in 0.1 M Tris-Cl buffer of varying pH values (7.5–9.6), at varying concentrations of donor (0.05–5.0 mM L-γ-glutamyl-*p*-nitroanilide) and acceptor (1–100 mM glycylglycine or L-methionine). The effects of untreated, heat-inactivated, and dialyzed samples on each other were examined by simple mixing at various ratios or by dialyzing one sample against another.

RESULTS

Figure 1 represents the activity levels of γ-glutamyl transpeptidase (with glycylglycine acceptor), aminopeptidase M (with L-alanine-*p*-nitroanilide), and maltase in CF and control amniotic fluids. The mean γ-glutamyl transpeptidase activity and the mean aminopeptidase M activity of CF amniotic fluid specimens differed significantly from the corresponding means of the control group, but considerable overlap was found among individual values. CF specimens with deficient levels of γ-glutamyl transpeptidase activities (<2SD below the normal mean) also demonstrated reduced aminopeptidase M, maltase, and sucrase activities. However, the disaccharidase activities were reduced to a lesser extent and the mean values of the CF group did not differ significantly from those of the controls. No significant differences were found between amniotic fluids from pregnancies at no increased risk for CF and those from pregnancies at 1:4 risk for CF, with normal outcome. Peptidase activities in plasma specimens of CF patients did not differ from those of obligate heterozygotes or controls.

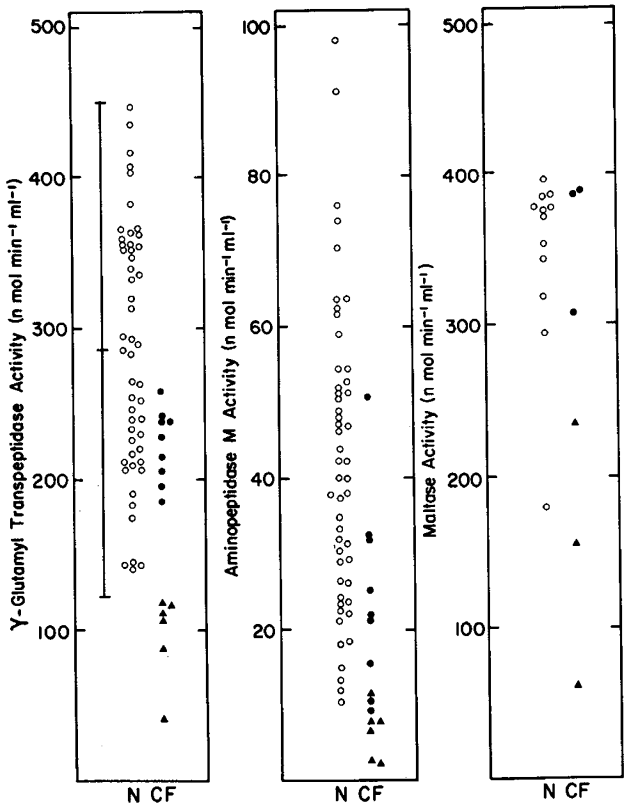

Fig. 1. γ-Glutamyl transpeptidase (glycylglycine acceptor), aminopeptidase M (L-alanine-*p*-nitroanilide), and maltase activities in normal and CF amniotic fluids. *Bar* in *left panel* represents normal mean ± 2SD. Nondeficient fluids (○, ●); deficient CF fluids, <2SD below the normal mean for γ-glutamyl transpeptidase activity (▲).

Mixing experiments were carried out in order to examine the nature of the peptidase and disaccharidase deficiencies. Table 1 presents typical results for γ-glutamyl transpeptidase activity in the various amniotic fluid mixtures. Mixing of deficient CF fluids (CF-D) with each other and any combination of nondeficient fluids (CF-N, Non-CF, and Normal) resulted in measured activities that agreed closely with the expected. In contrast, when deficient CF fluids were mixed with any of the nondeficient samples, partial or total correction of the deficiency was observed. γ-Glutamyl transpeptidase activity was proportional to the amount of amniotic fluid added, up to 2.5 μl, and linear with incubation time for at least 40 min (Fig. 2). The results were consistent and reproducible within ±15%. No correction was found for aminopeptidase and disaccharidase activities. Plasma specimens were unable to correct the γ-glutamyl transpeptidase deficiency in deficient CF fluids and additive values were obtained at various mixing ratios (1:1, 5:1, and 20:1).

Preincubation of amniotic fluid mixtures for up to 2 h at 37° C did not affect the extent of correction of γ-glutamyl transpeptidase deficiency. Similar pH profiles, with optimum activity at pH 8.5–9.0, were found for deficient CF fluids, nondeficient

Table 1. *γ-Glutamyl transpeptidase activity in mixed amniotic fluids**

Sample mixture	Activity (nmol min^{-1} ml^{-1})	
	Expected	Measured
CF-D (42) + CF-D (88)	65	61
CF-D (88) + CF-D (112)	100	95
CF-D (42) + CF-N (238)	140	204
CF-D (88) + CF-N (238)	163	211
CF-D (42) + Non-CF (252)	147	219
CF-D (88) + Non-CF (252)	170	219
CF-D (42) + Normal (283)	162	234
CF-D (88) + Normal (283)	186	223
CF-N (238) + CF-N (258)	248	238
CF-N (238) + CF-N (205)	222	209
CF-N (238) + Non-CF (252)	245	240
CF-N (238) + Non-CF (265)	252	240
CF-N (238) + Normal (283)	260	253
CF-N (238) + Normal (289)	264	255
Non-CF (252) + Non-CF (265)	258	244
Non-CF (252) + Non-CF (314)	283	268
Non-CF (252) + Normal (283)	268	257
Non-CF (252) + Normal (363)	308	301
Normal (283) + Normal (289)	286	276
Normal (283) + Normal (363)	323	314

* Samples were mixed at a 1:1 ratio. Expected activity is the mean of the individual activities given in parentheses. CF-D, deficient CF fluids (<2 SD below the normal mean); CF-N, nondeficient CF fluids; Non-CF, from pregnancies at risk with normal outcome; Normal, from pregnancies at no increased risk for CF. Underlining indicates correction.

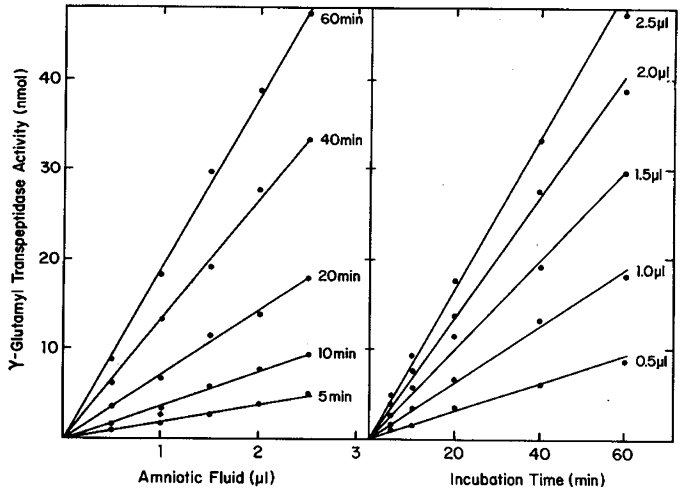

Fig. 2. γ-Glutamyl transpeptidase activity (glycylglycine acceptor) as function of the amount of amniotic fluid (*left panel*) and incubation time (*right panel*).

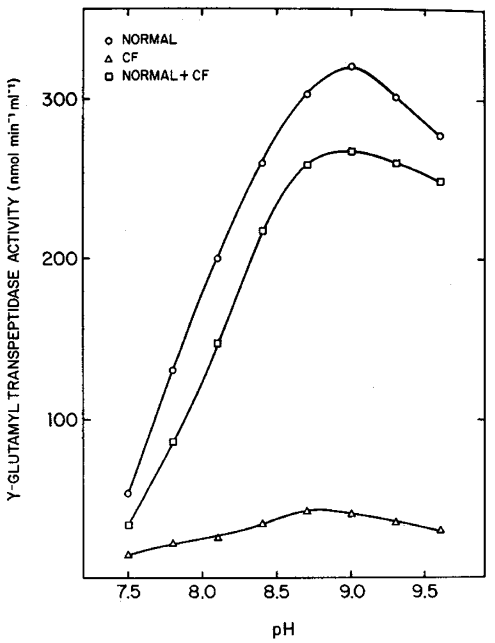

Fig. 3. pH dependence of γ-glutamyl transpeptidase activity in normal (○) and CF (△) amniotic fluids, and in their mixture at a 1:1 ratio (□).

fluids, and the corrected mixtures (Fig. 3). Amniotic fluid γ-glutamyl transpeptidase activity was affected by the ratio of donor to acceptor substrate and by their final concentrations in the reaction mixture. Varying the concentration of the donor substrate in the presence of optimal concentration of the acceptor substrate had essentially no effect on the correction, whereas a decrease in the concentration of the acceptor substrate in the presence of optimal donor concentration, or proportional decrease of both, markedly reduced the extent of correction (Table 2). Correction was also found at pH 9.0 when L-methionine served as the acceptor substrate but no correction was observed for the hydrolysis of L-γ-glutamyl-*p*-nitroanilide in the absence of glycylglycine or L-methionine.

The thermostability of γ-glutamyl transpeptidase activity in deficient CF fluids was identical to that with nondeficient fluids, the essentially total loss of activity following incubation for 20 min at 62° C (Fig. 4). The activity could be restored in any of the heat-inactivated amniotic fluid samples by mixing with either untreated normal fluids or untreated nondeficient CF fluids. In contrast, no restoration could be obtained by mixing with untreated deficient CF fluids (Table 3). γ-Glutamyl transpeptidase activity could also be restored in heat-inactivated plasma specimens by mixing with untreated nondeficient amniotic fluids but could not be restored by mixing with untreated plasma specimens.

Dialysis of nondeficient amniotic fluids against distilled water, 0.15 M NaCl, or 0.1 M Tris-Cl buffers neither diminished γ-glutamyl transpeptidase activity nor their corrective capacity. Correction of deficient CF fluids and restoration of the activity in heat-inactivated preparations could not be obtained by dialysis of these samples against nondeficient amniotic fluid samples.

Table 2. *Effect of donor and acceptor concentrations on γ-glutamyl transpeptidase activity in mixed amniotic fluids**

Concentration (mM)			Activity (nmol min^{-1} ml^{-1})			
Donor	Acceptor				Normal + CF-D	
(*p*NA-γ-Glu)	(Gly-Gly)	(Met)	Normal	CF-D	Expected	Measured
0.50	100		68	12	40	58
1.00	100		112	19	66	94
2.00	100		170	30	100	148
5.00	100		246	42	144	217
5.00	40		185	36	110	139
5.00	20		124	23	74	81
5.00	10		84	16	50	52
1.00	20		234	38	136	198
0.20	4		70	10	40	54
0.05	1		21	3	12	13
5.00		100	75	11	43	60
1.00		20	41	7	24	29
0.20		4	13	3	8	10
0.05		1	4	1	2	2

* Samples were mixed at a 1:1 ratio. Expected activity is the mean of the individual activities. CF-D, deficient CF fluid (<2SD below the normal mean); *pNA-γ-Glu*, *L-γ-glutamyl-p*-nitroanilide; Gly-Gly, glycylglycine; Met, L-methionine.

DISCUSSION

The reduced levels of peptidase activities and the lower, although less distinctive, levels of disaccharidase activities determined retrospectively in this study, in midtrimester amniotic fluids from pregnancies that have led to the birth of children with CF, confirm the recent reports by Carbarns *et al.* (4), Baker and Dann (1), van Diggelen *et al.* (16), and Brock *et al.* (2). However, considerable overlap exists between the values of the affected and the normal groups, and thus, determination of the enzyme activities *per se* cannot be used for accurate prediction of the outcome in pregnancies at risk for CF.

The results of the mixing experiments in respect to γ-glutamyl transpeptidase activity, singled out several CF amniotic fluids that were neither capable of correcting the deficiency of each other, nor were they capable of restoring the activity in heat-inactivated samples. The restoration of the activity in any heat-inactivated sample (amniotic fluid or plasma) by mixing with an untreated nondeficient amniotic fluid (normal or CF) indicated that a heat-labile factor is probably responsible for the correction of γ-glutamyl transpeptidase activity. The corrective factor was found to be nondialyzable as indicated on one hand by retaining essentially full corrective capacity in nondeficient amniotic fluids following dialysis against distilled water, and on the other hand by failure to achieve correction when samples were dialyzed against one another. The corrective factor was present in amniotic fluids with nondeficient levels of γ-glutamyl transpeptidase activity but not in plasma samples. All plasma samples examined (normal, CF, and obligate heterozygotes) were devoid of any corrective activity. Only γ-glutamyl transpeptidase activity could be affected by mixing of samples and no correction was observed

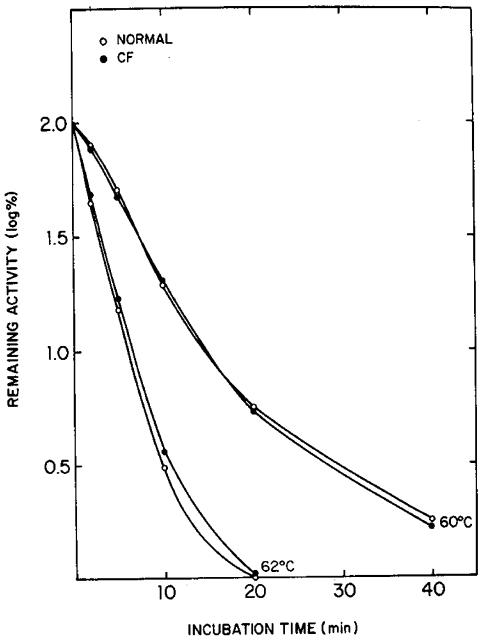

Fig. 4. Thermostability of γ-glutamyl transpeptidase activity at 60 and 62° C in normal amniotic fluid (○; initial activity, 314 nmol min^{-1} ml^{-1}) and in deficient CF amniotic fluid (●; initial activity, 42 nmol min^{-1} ml^{-1}).

Table 3. *γ-Glutamyl transpeptidase activity in mixtures of untreated and heat-inactivated amniotic fluids**

		Activity (nmol min^{-1} ml^{-1})	
Untreated	Inactivated	Expected	Measured
Normal (333)	Normal (1.8)	167	303
Normal (265)	Normal (1.8)	133	204
Normal (333)	CF-N (1.1)	167	278
Normal (265)	CF-N (1.1)	133	190
Normal (333)	CF-D (0.3)	167	262
Normal (265)	CF-D (0.3)	133	186
CF-N (258)	Normal (1.8)	130	265
CF-N (185)	Normal (1.8)	93	142
CF-N (258)	CF-N (1.1)	130	224
CF-N (185)	CF-N (1.1)	93	131
CF-N (258)	CF-D (0.3)	129	230
CF-N (185)	CF-D (0.3)	93	133
CF-D (88)	Normal (1.8)	45	42
CF-D (42)	Normal (1.8)	22	20
CF-D (88)	CF-N (1.1)	45	39
CF-D (42)	CF-N (1.1)	22	19
CF-D (88)	CF-D (0.3)	44	40
CF-D (42)	CF-D (0.3)	21	19

* Samples were mixed at a 1:1 ratio. Expected activity is the mean of the individual activities given in parentheses. CF-D, deficient CF fluid (<2SD below the normal mean); CF-N, nondeficient CF fluid. Underlining indicates correction.

for aminopeptidase M or disaccharidase activities. Furthermore, the correction appeared to be specific for the transpeptidation reaction of γ-glutamyl transpeptidase as indicated by determination of additive values when mixed samples were incubated with L-γ-glutamyl-*p*-nitroanilide in the absence of the glycylglycine of L-methionine acceptor substrate. This point requires further investigation since the presently employed assay for γ-glutamyl transpeptidase only measures free *p*-nitroaniline which is the common product of the hydrolysis, transpeptidation, and autotranspeptidation reactions. Discrimination between these reactions can be achieved by determination of specific products such as L-γ-glutamylglycylglycine, L-γ-glutamyl-L-methionine (transpeptidation), and L-γ-glutamyl-L-γ-glutamyl-*p*-nitroanilide (autotranspeptidation), and by using D-γ-glutamyl-*p*-nitroanilide (rather than the L isomer) which is a suitable substrate for the hydrolysis reaction but is inactive as an acceptor for autotranspeptidation (15).

The corrective factor implicated by the results of the present study may be an activator for γ-glutamyl transpeptidase which is necessary for the catalytic performance of the transpeptidation reaction. CF amniotic fluids with deficient levels of γ-glutamyl transpeptidase activity probably contain normal quantities of the enzyme but insufficient quantities of the activator. In contrast, nondeficient amniotic fluids probably contain an excess of activator and, thus, are capable of providing the missing activator to deficient CF fluids as well as to heat-inactivated samples. The amounts of this activator in plasma samples seem to be sufficient but not excessive. The deficiency of γ-glutamyl transpeptidase activator in some CF amniotic fluids does not account for the reduced levels of aminopeptidase M and disaccharidase activities and may be another expression of the microvillar abnormality hypothesized by Carbarns *et al.* (4). This abnormality which probably appears at a very early stage in at least some CF fetuses, may affect directly the levels of some microvillar enzymes (aminopeptidase M and disaccharidases) and indirectly the levels of others (reduced γ-glutamyl transpeptidase activity due to deficient levels of its activator). Isolation and characterization of the γ-glutamyl transpeptidase activator may provide a valuable approach to identify at least a subgroup of cystic fibrosis patients. Other abnormalities with depressed amniotic fluid γ-glutamyl transpeptidase activity such as trisomy-18 (6) should also be examined.

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