Plasma alpha-glucosidase increase in cystic fibrosis: only an effect of chronic tissue damage?

Aumento dell'alfa-glucosidasi nel plasma di pazienti con fibrosi cistica: è soltanto il risultato del danno parenchimale cronico?

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Introduction

Cystic Fibrosis (CF) is the most common lethal genetic disease among caucasian children and young adults. The major clinical findings include pancreatic insufficiency, chronic lung infection and hepatic cirrhosis. The activity levels of serum lysosomal hydrolases in CF patients have been previously investigated. However, the validity of the measurement of these enzymes as markers of CF is controversial. Among these enzymes, alpha-glucosidase (EC 3.2.1.20) was the most extensively investigated (see 33 for review). An increased activity of acid or neutral alpha-glucosidase has been found in sera of CF Homozygotes 2 8 23 32. Higher serum alpha-glucosidase has been observed also in CF Heterozygotes 32 but this wasn't confirmed by others 8. On the contrary, normal levels of other lysosomal hydrolases in CF patients, such as alpha-mannosidase, alpha-L-fucosidase, beta-glucuronidase, beta-N-acetylglucosaminidase, beta-N-acetylgalactosaminidase, beta-galactosidase, aryl sulphatase and acid phosphatase, have been found 4.

Different explanations have been advanced on the mechanism and the meaning of the higher serum level of alpha-glucosidase in CF. The Michaelis constant, pH optimum, thermal lability and isoelectric focusing profile of alpha-glucosidase in serum of CF patients are similar to those of the enzyme of serum of control subjects 2 8 indicating that the source of the enzyme in CF is presumably identical with that in normal subjects.

The possibility of a compensatory enzymatic induction mechanism has been advanced 8. A recent hypothesis suggests that the high serum level of alpha-glucosidase could be dependent on liver damage or another pathological process associated with CF sickness 33. The observation of higher lysozyme activity in CF Homozygotes sera 22 might indicate that the higher secretion of these enzymes is secondary to macrophage activation. In the present paper we report data on the activity and on some properties of both acid and neutral alpha-glucosidase in plasma of CF Homozygotes and Heterozygotes; on the specificity of their increase in relation to other pathological processes such as chronic bronchitis and chronic hepatic cirrhosis; on the relationship with plasma lysozyme and on the levels of alpha-glucosidase in neutrophils of CF, as a possible source of the plasma enzyme.

Patients and methods

Patients

All CF Homozygotes were diagnosed with sweat test (pilocarpine iontophoresis, triplicate test). They showed variable degrees of pulmonary and pancreatic involvement. One of them was a borderline patient (M.M., 5 years 1 month), who had several positive sweat tests but absence of chronic cough and sputum, normal chest X-ray, normal output of lipase, trypsin, chymotrypsin in duodenal juice after pancreozymin-secretin stimulation (but moderate bicarbonate reduction), normal weight and height, normal biochemical liver function (tested with serum aminotransferases, gammaglutamyl-transpeptidase,
alkaline phosphatase, prothrombin and partial thromboplastin time) (age: 5 months-25 years). CF Heterozygotes were mothers of CF patients (age: 21-46 years). Chronic hepatitis patients were adult subjects with long-standing liver pathology (hepatic cirrhosis) diagnosed with biopsy. Two of them had increased biochemical indices of hepatocellular damage and of cholestasis, one of them of cholestasis only and the others had normal biochemical indices of liver function at the moment of blood collection (age: 42-59 years). Chronic bronchitis patients were adult subjects with abundant daily purulent sputum expectoration. They had bronchiectasis clusters and chronic bacterial infection (age: 44-68 years). Control subjects were individuals without clinical signs of CF and with non known CF relatives (age: 6 months-12 years 6 months for children; 29-55 years for adults). Oral informed consent was obtained from adult subjects and from the parents of the children.

Plasma samples
Serum measurement of lysosomal enzymes may be influenced by two opposed factors. During blood clotting, there may be a rapid loss of enzymatic activity due to weak stability or there may be increased activity due to white blood cells and platelets secretion of lysosomal hydrolases\(^2\). For this reason, these enzymes have been studied in plasma.

Blood was collected in plastic tubes with sodium citrate (final concentration: 11 mmol/l). The tubes were kept in melting ice immediately after the venipuncture and then were centrifuged within 15 minutes (15 min, +4°C, 5000 xg). Then, plasma was distributed in precooled Eppendorf tubes and stored at -80°C. Freezing was performed within 40 minutes of blood collection. Plasma samples were thawed only once for enzyme assay, that was made within one week of the day of collection.

Preparation of neutrophils
8 ml of blood were poured into tubes containing sodium citrate (final concentration: 11 mmol/l). Blood was gently mixed with saline solution (NaCl 154 mmol/l) containing 6% dextran (1 part saline + 4 parts blood) and allowed to sediment. After 20-30 minutes the bulk of the red cells sedimented at the bottom of the tube and the leukocyte and platelet rich plasma was collected and diluted (1:1) with saline solution (NaCl 154 mmol/l). This leukocyte solution was carefully layered upon 20 ml of Ficoll-Paque in a 50 ml centrifuge tube. The tube was centrifuged at +20°C for 20 min (400 xg). Neutrophils and red blood cells were sedimented at the bottom. Lymphocytes, monocytes and platelets remained above the density gradient and were pipetted off with the Ficoll. The pellet was resuspended in 9 ml of ice-cold NaCl 34 mmol/l for exactly 45 seconds, after which 21 ml of NaCl 205 mmol/l were added. The tube was centrifuged at +4°C for 8 minutes (1000 xg). This step was repeated at least once again, in order to obtain a complete red cell hypothetic loss. The sedimented cells (95-98% neutrophils) were finally resuspended in 2 ml of KRP (phosphate buffer 16.6 mmol/l, pH 7.4, KCl 4.8 mmol/l, NaCl 154 mmol/l, MgCl\(_2\) 1.2 mmol/l) and kept in melting ice. In order to obtain the whole cell homogenate, the neutrophils were centrifuged at +4°C for 8 minutes (1000 xg) and the pellet was resuspended in 2 ml of 340 mol/l sucrose containing 1 mmol/l sodium bicarbonate. This suspension was sonicated in two cycles of 10 seconds (needle probe, 100 watts, Braun Labsonic sonicator) and 0.1% Triton X-100 was added last. In order to obtain granules, the pellet was homogenized and centrifuged as described\(^3\).

Alpha-glucosidase assay
Plasma aliquots (15 μl) were brought to 100 μl in 0.2 mol/l potassium acetate buffer (pH 4.50 for acid alpha-glucosidase, pH 6.00 for neutral alpha-glucosidase)\(^4\). The reactions were initiated with 50 μl of 6 mmol/l 4-methylumbelliferyl-alpha-D-glucopyranoside dissolved in the above buffer and the mixture was incubated for 60 minutes at +37°C in a shaking water bath. The reactions were blocked with 3.0 ml of 0.2 mol/l: pH 10.70 glycine-carbonate buffer. Fluorescence was read on a CGA mod. 3000/1 spectrofluorometer at excitation and emission wavelength of 365 and 448 nm respectively.

Whole cell homogenate aliquots (20 μl) were brought to 150 μl in 0.2 mol/l potassium acetate buffer and the reactions were initiated with 50 μl of 2 mmol/l substrate dissolved in the above buffer. All the determinations were performed in duplicate. In these conditions, enzyme activity was linear at least until 120 minutes and blanks were less than 5% of the samples.

One alpha-glucosidase activity unit was defined as 1 nmole of 4-methylumbelliferone (4-MU) hydrolized at 37°C for 60 minutes per ml of plasma or per mg of protein (specific activity).

Apparent Michaelis constants (Km values) were graphically determined by the Lineweaver-Burk method using 4-methylumbelliferyl-alpha-D-glucopyranoside at final concentration ranging from 0.05 to 3.0 mmol/l. Determinations were run in duplicate at pH 4.50 and pH 6.00 for 60 minutes at 37°C on 15 μl aliquots of plasma. Thermobility curves were carried out in duplicate after preincubation of undiluted plasma (300 μl) for various time intervals at 37°C and 48°C. Aliquots of plasma (15 μl) were tested for neutral alpha-glucosidase activity immediately after each inactivation time (with the methods described before).

Lysozyme assay
Lysozyme was assayed by incubating 20 μl of plasma with 980 μl of a freshly prepared suspension of Micrococcus Lysodeikticus (68 μg/ml) in 0.2 mol/l phosphate buffer, pH 6.6, containing NaCl 0.150 mol/l. The decrease in absorbance was measured at 450 nm wavelength in a Perkin Elmer mod. 576 spectrophotometer in a cuvette holder thermostated at 37°C. A calibration curve was made with standard lysozyme in every day series.

General
Protein was determined by the method of Lowry et al.\(^6\) using bovine serum albumine as standard.

4-methylumbelliferyl alpha-D-glucopyranoside, 4-methylumbelliferone, Triton X-100 were purchased from Sigma Chemical Co.; Ficoll-Paque from Pharmacia Fine Chemicals Co.; dextran MW 200,000 from BDH Italia.
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Tab. 1.

Plasma acid and neutral alpha-glucosidase (a-g) in CF Homozygotes and healthy controls (nmol 4-MU/60 min/ml plasma). *Alfa-glucosidasi acida e neutra (a-g) in Omozigoti CF e controlli sani (nmol 4-MU/60 min/ml plasma).*

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<tr>
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<th>Acid a-g</th>
<th>Neutral a-g</th>
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<td></td>
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<td>Mean±SD (range)</td>
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<td>Media±DS (intervallo)</td>
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<td>Controls (age: 6 months-12 years 6 months)</td>
<td>15</td>
<td>5.61±1.48 (3.62-9.38)</td>
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<tr>
<td>CF Homozygotes (age: 5 months-25 years)</td>
<td>22</td>
<td>12.67±6.36 (4.68-29.82)</td>
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Results

Acid and neutral alpha-glucosidase in plasma.—Acid and neutral alpha-glucosidase activity in plasma of CF Homozygotes are significantly higher than that of age matched healthy controls (Tab. 1).

In healthy controls, a significant correlation between age and alpha-glucosidase activity was found (acid alpha-glucosidase coefficient of correlation r = 0.62, p < 0.05; neutral alpha-glucosidase r = 0.66, p < 0.05). This concept is also shown in Fig. 1 where plasma neutral alpha-glucosidase is plotted against age. Mean values of con-
Tab. 2.
Plasma acid and neutral alpha-glucosidase (a-g) in CF Heterozygotes, hepatic cirrhosis patients, chronic bronchitis patients and healthy controls (nmol 4-MU/60 min/ml plasma).

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<th>Acid a-g</th>
<th>Neutral a-g</th>
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<tr>
<td></td>
<td>N.</td>
<td>Mean ± SD (range)</td>
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<td>Media ± DS (intervalo)</td>
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<tr>
<td>Controls (age: 29-55 years)</td>
<td>6</td>
<td>6.46 ± 0.73 (5.60-7.94)</td>
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<tr>
<td>CF Heterozygotes (age: 21-46 years)</td>
<td>6</td>
<td>6.30 ± 1.04 (4.51-7.63)</td>
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<tr>
<td>Hepatic cirrhosis (age: 42-59 years)</td>
<td>5</td>
<td>6.52 ± 2.66 (3.77-10.52)</td>
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<tr>
<td>Chronic bronchitis (age: 44-68 years)</td>
<td>5</td>
<td>4.06 ± 0.84 (2.91-4.99)</td>
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Fig. 2.
Plasma alpha-glucosidase pH 6.0. Thermal lability curves at 37°C (A) and 48°C (B).
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Tab. 3.
Acid and neutral alpha-glucosidase (a-g) in neutrophils of CF Homozygotes and healthy controls (nmol 4-MU/60 min/mg protein). *Alfa-glucosidasie acida e neutra (a-g) in neutrofili di Omozigotti CF e controlli sani (nmoli 4-MU/60 min/mg proteine).*

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<thead>
<tr>
<th></th>
<th>Acid a-g</th>
<th>Neutral a-g</th>
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<tr>
<td></td>
<td>N.</td>
<td>Mean ± SD (range)</td>
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<td>Controls</td>
<td>8</td>
<td>40.49±7.78 (33.05-53.98)</td>
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<tr>
<td>CF Homozygotes</td>
<td>10</td>
<td>43.19±7.72 (32.55-56.88)</td>
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Tab. 4.
Plasma lysozyme in CF Homozygotes, chronic bronchitis patients and healthy controls (µg/ml plasma). *Lisozima nel plasma di Omozigotti CF, pazienti affetti da bronchite cronica e controlli sani (µg lisozima standard/ml plasma).*

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<tr>
<td>Controls</td>
<td>12</td>
<td>3.22 ± 0.69</td>
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<tr>
<td>CF Homozygotes</td>
<td>12</td>
<td>3.77 ± 0.89</td>
<td>1.69 (p = n.s.)</td>
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<tr>
<td>Chronic bronchitis</td>
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<td>4.54 ± 0.86</td>
<td>2.78 ± (p &lt; 0.05)</td>
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controls aged from than 60 months correspond very strictly to the adult control values. The higher values of alpha-glucosidase in the plasma of CF Homozygotes were independent of the age of the patients. No overlap was evident between CF patients and age matched controls, except for one borderline CF subject (see Patients). No correlation was found between the plasma activity and the clinical impairment of CF Homozygotes (pulmonary infection, liver and pancreas disfunction).

Table 2 shows that plasma level of acid and neutral alpha-glucosidase of CF Heterozygotes, of hepatic cirrhosis and of chronic bronchitis patients is similar to that of healthy subjects.

Properties of alpha-glucosidase in plasma.—Apparent Michaelis constants (Km) of plasma and neutral alpha-glucosidase for 4-methylumbelliferyl-alpha-D-glucopyranoside were strictly similar in CF Homozygotes and healthy controls (acid alpha-glucosidase: CF Homozygotes Km 0.96 mmol/l, controls 0.91 mmol/l; neutral alpha-glucosidase: CF Homozygotes Km 1.05 mmol/l, controls 1.10 mmol/l). Thermal lability curves at 37°C and 48°C of plasma neutral alpha-glucosidase in CF Homozygotes did not differ from that of healthy controls (Fig. 2).

Alpha-glucosidase in neutrophils.—The activity of acid and neutral alpha-glucosidase measured in homogenates of blood neutrophils was similar in CF Homozygotes and age matched healthy controls (Tab. 3). Normal enzyme activities were also found in granule fractions of neutrophils (data not shown).

Lysozyme in plasma.—Lysozyme activity in plasma of CF Homozygotes was similar to that of healthy controls, while chronic bronchitis patients showed significantly higher values (Tab. 4). No significant correlation between lysozyme and alpha-glucosidase was observed in plasma of CF Homozygotes (coefficient of correlation for acid alpha-glucosidase: r = 0.07, p = n.s.; neutral alpha-glucosidase: r = 0.28, p = n.s.).

Discussion
Several investigators have reported increased activity of acid and neutral alpha-glucosidase in serum of CF patients and this work confirms these data in plasma. The present results also stress the usefulness of comparing strictly age-matched patient’s and control’s groups (Fig. 1). Conflicting data have been reported as regards alpha-glucosidase activity in serum of CF Heterozygotes. Our data, showing that the plasma level of alpha-glucosidase in CF Heterozygotes is normal, exclude the possibility of detecting CF carriers by measuring the plasma level of alpha-glucosidase. The results of the study presented in this paper deserve to be discussed in terms of the mechanism that is responsible for the increase of alpha-glucosidase in the plasma of the CF patients and that is, at present, unknown.
First of all, it is possible to rule out an apparent increase of enzymatic activity due to a higher affinity to the substrate (Km value are similar) or due to lower thermolability. Kinetic properties and isoelectric focusing properties of alpha-glucosidase in CF have been also found normal by other investigators. A possible defect in the removal of alpha-glucosidase from plasma has been claimed. Our data do not allow any conclusion on this possibility, but a first preliminary study seems to exclude it.

Secondly, the abnormal levels of alpha-glucosidase in CF could be regarded as a manifestation of chronic tissue damage. Since lung, liver and pancreas are progressively damaged in this disease, the hypothesis has been advanced that the increased alpha-glucosidase in the plasma of CF is secondary to these pathological events. The damage of parenchymal and of inflammatory cells could result in a release of lysosomal enzymes into the blood stream. To test this possibility alpha-glucosidase in plasma of non-CF subjects with severe hepatic cirrhosis and non-CF patients with longstanding chronic bronchitis was measured and no differences with healthy subjects were observed (Tab. 2). Moreover, no relationship between alpha-glucosidase levels and clinical impairment in CF patients was found. Thus, the increase of alpha-glucosidase in plasma of CF is not dependent on the process associated with cell damage. The present work doesn't consider chronic pancreatitis patients, but is unlikely that the pancreas is the source of the increased alpha-glucosidase in plasma of CF patients. In fact, even in massive pancreatic destruction such as in acute pancreatitis, alpha-glucosidase activity in serum does not change. Moreover, the level of enzymes of pancreatic origin, such as serum trypsin and serum lipase, in CF fall under control value within the age of two years (probably as a result of pancreatic fibrosis), whereas alpha-glucosidase is higher independently of age (Fig. 1).

The high level of alpha-glucosidase in CF plasma could be linked to an enhanced secretory activity of mononuclear phagocytes at the chronic inflammatory sites or could be due to a state of systemic hyperplasia of mononuclear phagocyte system caused by the chronic inflammation associated with CF sickness. The finding of an increased lysozyme activity in serum of CF patients seems to sustain the last view, as lysozyme is considered a marker of the state of activation of the macrophage phagocyte system.

However, our results (Tab. 4) showed that plasma lysozyme was increased in chronic bronchitis patients but not in CF patients. A possible explanation of the discrepancy between our results and those of Hughes et al. could be the different clinical impairment of the patients of the two series. Furthermore, the level of other lysosomal enzymes (alpha-L-fucosidase, alpha-mannosidase, beta-N-acetyl-glucosaminidase, beta-glucuronidase, beta-galactosidase, arylsulphatase, acid phosphatase) is not increased in CF serum, as expected in the case of hypersecretory activity of inflammatory cells at the sites of inflammation.

So, at the moment, it is unlikely that the increase of alpha-glucosidase in plasma is the result of a state of systemic activation of the macrophage system and of the secretory activity of mononuclear phagocytes at the inflammatory sites.

A third possibility to be considered is a primary cellular alteration, either in the synthesis, assembly or secretion of the enzyme. Higher plasma activity could reflect higher intracellular activity, as a result of compensatory enzymatic induction. Assuming that CF basic defect consists in an impairment of glycoprotein catabolism, the block of the catabolic pathway could result in a compensatory oversynthesis of some glycosidic hydrolase such as alpha-glucosidase in some cells. In fact, an increased alpha-glucosidase activity in lysosomes of cultured lymphoid cells from CF patients was reported. Since neutrophils have the greater amount of alpha-glucosidase among the blood cells, they could be one of the cell type where the process of induction takes place. Our data, showing no differences between the intracellular activity of granulocytes of CFs and of controls (Tab. 3), indicate that in these cells the compensatory induction of alpha-glucosidase does not occur. At first sight these data seem also to indicate that the granulocytes are not the sources of the increased alpha-glucosidase in plasma of CF because in this case the intracellular content of the enzyme should be decreased. However, the calculation of the enzyme content of blood granulocytes and that of the plasma shows that an increase of the enzyme of plasma similar to that found in CF and due to a release from granulocytes would be accompanied by a decrease of the enzyme in granulocytes so small that is would be difficult to demonstrate.

It has been suggested, on the basis of an increased leakage of lysosomal hydrolases from fibroblasts stimulated by Tamm-Horsfall protein and of studies on membrane receptors and Ca++ content and influx in fibroblasts and neutrophils, that the cells of CF behave as hypersecretory cells. Thus, in spite of the content of alpha-glucosidase of neutrophils and considering that these cells represent a polydispersed secretory organ, it is likely that they are the source of the enzyme in plasma of CF. Studies are in progress in our laboratory on the spontaneous and stimulated secretory activity of CF granulocytes.

In conclusion, the reasons for the increase of alpha-glucosidase in the plasma of CF Homozygotes remain obscure. In our opinion the exclusion of some possible trivial explanations such as the association with secondary pathological events is relevant because it stresses the fact that this phenomenon is specific and proper to the CF Homozygotes state. It is likely that studies at molecular level on the mechanism that controls the stimulus-secretion coupling allow us to understand the reasons for the high level of alpha-glucosidase in the plasma.

Summary

Increased activity levels of acid and neutral alpha-glucosidase (EC 3.2.1.20) in plasma of Cystic Fibrosis (CF) patients have been confirmed. CF Heterozygotes didn't show abnormal levels. Apparent Michaelis constants and lability curves at 37°C and 48°C showed similar values in CF Homozygotes versus controls. In order to understand the mechanism responsible for the increase of alpha-glucosidase the following analyses have been carried out: a) the activity of alpha-glucosidase in plasma of non-CF patients with chronic inflammation; b) the plasma level of lysozyme: a marker of activation of macrophage system; c) the alpha-glucosidase activity in neutrophils, as a possible source of plasma alpha-glucosidase. Chronic bronchitis and hepatic cirrhosis patients had alpha-glucosidase activity within the normal range. Plasma
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lysozyme was found increased in chronic bronchitis patients but not in CF homozygotes. Specific activity of acid and neutral alpha-glucosidase in neutrophils from healthy subjects and from CF homozygotes were similar. The results show that the increased alpha-glucosidase of plasma of CF patients: a) does not derive from the damaged parenchymal cells of lung and liver; b) is not related to an increased secretory activity of the inflammatory cells at the inflammatory sites; c) is not due to a systemic hyperplasia of macrophage system. These results, which exclude that the increase of alpha-glucosidase is due to secondary pathological processes, are discussed in term of a phenomenon specific to alpha-glucosidase and proper to the CF homozygote state, whose mechanism at cellular and molecular level remains to be clarified.

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