Gilbert’s syndrome: analytical subcellular fractionation of liver biopsy specimens. Enzyme activities, organelle pathology and evidence for subpopulations of the syndrome

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Summary

1. The hepatic organelle pathology of 12 patients with Gilbert’s syndrome was studied by analytical subcellular fractionation in combination with enzymic microanalysis of liver biopsy material.

2. All patients showed increased activities of the endoplasmic reticulum marker enzyme, neutral α-glucosidase. Seven patients showed a decrease in the modal density of the endoplasmic reticulum, from 1.20 to 1.15 g/ml. These patients also showed striking hypertrophy of the hepatocyte smooth endoplasmic reticulum on electron microscopy. The remaining five patients showed normal endoplasmic reticulum density distribution with a peak at 1.20 g/ml, and had normal appearance of the endoplasmic reticulum on electron microscopy.

3. All patients showed increased activity of three lysosomal marker enzymes: N-acetyl-β-glucosaminidase, acid phosphatase and β-glucuronidase. The distribution of these enzymes in the sucrose gradients showed less enzyme in the high-density region of the gradients, indicating a reduced equilibrium density of the lysosomes. Assays of latent and sedimentable N-acetyl-β-glucosaminidase, a measure of lysosomal integrity, were normal.

4. Marker enzyme activities and density gradient distribution of other organelles, including plasma membrane (5’-nucleotidase), mitochondria (malate dehydrogenase), biliary canaliculi (y-glutamyl transferase) and cytosol (lactate dehydrogenase) were normal. Increased catalase activities were noted.

Key words: endoplasmic reticulum, enzymes, Gilbert’s disease, hereditary hyperbilirubinaemia, liver diseases, lysosomes, subcellular fractions.

Introduction

Gilbert’s syndrome, first described by Gilbert & Lereboullet (1901), is a common familial disorder of bilirubin metabolism, characterized by an isolated unconjugated hyperbilirubinaemia in the absence of overt haemolysis (Arias, 1962). The precise underlying biochemical defect in this syndrome is currently contentious but various morphological accompaniments have been described on electron microscopy of the hepatocyte. Gross hypertrophy of the smooth endoplasmic reticulum has been noted in all patients in one small selected study (McGee, Allan, Russel & Patrick, 1975) and in a substantial subpopulation of a larger consecutive study (Dawson, Carr-Locke, Talbot & Rosenthal, 1978). Lipofuscin pigment accumulation has also been
noted as a prominent feature of many patients (Barth, Grimley, Berk, Bloomer & Howe, 1971). A variety of other inconstant abnormalities of the mitochondria, plasma membrane and microvilli have been described (Simon & Varonier, 1963; Magnenat & Paluello, 1967; Feldmann, Oudea, Domart-Oudea, Molas & Fauvert, 1968; Schaff, Lapis & Safrany, 1969; Bleuger, Krupnikova, Sondore & Semushina, 1977).

The present study applies the technique of analytical subcellular fractionation combined with enzymic microanalysis (Peters & Seymour, 1978) to liver biopsies of patients with Gilbert’s syndrome to further define the organelle pathology and quantify any abnormalities thus found.

Methods

Patients

Twelve patients with Gilbert’s syndrome were studied (age 23–55 years). These patients presented to general medical outpatient for investigation of non-specific symptoms and were found to have an isolated predominantly unconjugated serum hyperbilirubinaemia (range 22–75 µmol/l) as the only abnormal investigation. All other tests of liver function, and cholecystography where appropriate, were normal. Extensive investigations excluded all common causes of haemolysis. All patients were subsequently shown to have normal liver biopsies on light microscopy, with the exception of the presence of lipofuscin pigment in five patients. No patient gave a history of excessive alcohol intake and no drugs were being taken at the time of the biopsy.

Biochemical and morphological studies

Portions of liver tissue were obtained by percutaneous liver biopsy with a Menghini needle. A portion was taken for routine histology and a further small portion fixed in glutaraldehyde for electron microscopy. The smooth endoplasmic reticulum of the hepatocytes in each biopsy was quantified by a modification of the method of Loud (1962). A grid measuring 18 cm × 22 cm with wires separated by 1.5 cm was laid on photomicrographs of 20 000 magnification taken randomly from several regions of each biopsy. The number of crossings of the membranes of smooth endoplasmic reticulum by the wires of the grid was measured for each photomicrograph and the mean number of crossings in all the photomicrographs for each biopsy was recorded. These results were compared with results from five control biopsies.

The remainder of the biopsy was placed in 3 ml of ice-cold sucrose (0.25 mol/l) containing disodium EDTA (1 mmol/l, pH 7.2) and ethanol (20 mmol/l) (SVE medium). The tissue was disrupted in a Dounce homogenizer with a loose-fitting pestle, and the homogenate was centrifuged at 600 g for 10 min. The pellet was resuspended in a further 2 ml of SVE medium and centrifuged again. The postnuclear supernatants were combined, and 4 ml of this supernatant was layered on a 28 ml sucrose density gradient extending linearly with respect to volume from density 1.05 g/ml to 1.28 g/ml and resting on a cushion of 6 ml of sucrose (density 1:32 g/ml) in the Beaufay small-volume automatic zonal rotor as described previously (Peters, Muller & de Duve, 1972). Fifteen fractions were collected into tared tubes and, after re-weighing and mixing, the densities of the fractions determined indirectly by refractometry.

Enzyme activities of the fractions and homogenates were determined by microanalytical methods previously described (Peters, 1976; Seymour & Peters, 1977) and expressed as munits/mg of protein in the liver homogenates, where 1 unit is equal to 1 µmol of substrate transformed/min. Latent and sedimentable N-acetyl-β-glucosaminidase activities were determined by the method of Peters, Heath, Wansbrough-Jones & Doe (1975). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. The enzyme activities were compared with previously reported values from normal liver biopsies (Peters & Seymour, 1978). Biopsies from control subjects were studied in a similar manner and at similar times to those from the patients with Gilbert’s syndrome. These studies were approved by the local ethical committee.

Results

Biochemical studies

Table 1 shows the specific activities of marker enzymes for the major organelles in the postnuclear supernatant from liver biopsy homogenates of patients with Gilbert’s syndrome in comparison with our previously published values for normal liver biopsies (Peters & Seymour, 1978). No difference was demonstrated in the markers for plasma membrane, biliary canaliculi, mitochondria
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TABLE 1. Enzymic activities of liver biopsy homogenates in Gilbert's syndrome

Specific activity is expressed as the mean value ± se (munits/mg of protein). Control values are from Peters & Seymour (1978). N.S., Not significant.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC no.</th>
<th>Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gilbert's syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (t-test)</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral α-glucosidase</td>
<td>3.2.1.20</td>
<td>1.57 ± 0.09</td>
</tr>
<tr>
<td>Lysosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>3.2.1.30</td>
<td>2.03 ± 0.28</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>3.1.3.2</td>
<td>1.29 ± 0.81</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>3.2.1.31</td>
<td>4.92 ± 0.04</td>
</tr>
<tr>
<td>Latent N-acetyl-β-glucosaminidase (%)</td>
<td>3.2.1.30</td>
<td>67.7 ± 1.7</td>
</tr>
<tr>
<td>Sedimentable N-acetyl-β-glucosaminidase (%)</td>
<td>3.2.1.30</td>
<td>56.1 ± 2.8</td>
</tr>
<tr>
<td>Plasma membrane and biliary canaliculi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S'-Nucleotidase</td>
<td>3.1.3.5</td>
<td>13.2 ± 2.3</td>
</tr>
<tr>
<td>Leucine-2-naphthylamidase</td>
<td>3.4.11.2</td>
<td>1.27 ± 0.16</td>
</tr>
<tr>
<td>γ-Glutamyl transferase</td>
<td>2.3.2.2</td>
<td>5.08 ± 0.70</td>
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<tr>
<td>Mitochondrion</td>
<td></td>
<td></td>
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<tr>
<td>Malate dehydrogenase</td>
<td>1.1.1.37</td>
<td>3220 ± 160</td>
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<tr>
<td>Cytosol</td>
<td></td>
<td></td>
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<tr>
<td>Lactate dehydrogenase</td>
<td>1.1.1.27</td>
<td>345 ± 31</td>
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<tr>
<td>β-Glucosidase</td>
<td>3.2.1.21</td>
<td>0.831</td>
</tr>
<tr>
<td>Peroxisome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>1.11.1.6</td>
<td>228 ± 16</td>
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</table>

TABLE 2. Median densities of neutral α-glucosidase on sucrose density centrifugation of liver biopsies in Gilbert's syndrome

<table>
<thead>
<tr>
<th>Light density distribution</th>
<th>Normal density distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1501</td>
<td>1.1903</td>
</tr>
<tr>
<td>1.1503</td>
<td>1.1879</td>
</tr>
<tr>
<td>1.1557</td>
<td>1.1895</td>
</tr>
<tr>
<td>1.1490</td>
<td>1.1997</td>
</tr>
<tr>
<td>1.1463</td>
<td>1.1875</td>
</tr>
<tr>
<td>1.1468</td>
<td>1.1885</td>
</tr>
<tr>
<td>Mean</td>
<td>1.1493*</td>
</tr>
</tbody>
</table>

* P < 0.001 (Students t-test).

or cytosol. However, a significant increase was seen in the endoplasmic reticulum marker neutral α-glucosidase. Likewise a significant increase was seen in the three lysosomal markers, N-acetyl-β-glucosaminidase, acid phosphatase and β-glucuronidase. A small increase was seen in the peroxisome marker catalase.

Fig. 1 shows the density distribution of the endoplasmic reticulum marker α-glucosidase after sucrose-density-gradient centrifugation. Inspection of the individual data indicated that the patients quite clearly fell into two groups. In five patients the density distribution coincided with the normal controls with a mode at 1.20 g/ml. The remaining seven patients showed a different distribution with a considerably lighter peak, around 1.15 g/ml. Table 2 shows the median distribution densities of neutral α-glucosidase from the individual patients, showing the separation into two distinct populations.

Fig. 2 shows the density distribution of other organelle markers in all patients with Gilbert's syndrome, compared with normal human liver. Abnormalities were seen in the two lysosomal markers β-glucuronidase and N-acetyl-β-glucosaminidase. The β-glucuronidase showed a peak in the lighter regions of the gradient and the N-acetyl-β-glucosaminidase did not show the peak found in normal biopsies. These changes were found in all biopsies and there were no obvious subdivisions. No correlation between lysosomal enzyme activities or density distribution and presence of lipofuscin pigment was found. No significant changes in the density distribution of other organelles were seen, compared with the control data. The apparent changes in the cytosol marker lactate dehydrogenase were not significant.

Morphological studies

With the exception of the presence of lipofuscin pigment in five patients, no abnormalities were seen on light microscopy. Electron microscopy was
Fig. 1. Isopycnic centrifugation of postnuclear supernatant from liver biopsy homogenate from patients with Gilbert's syndrome (continuous line) compared with results from control subjects (dotted line). Graphs show frequency–density histograms for the endoplasmic reticulum marker enzyme neutral α-glucosidase. (a) Averaged results from seven patients with altered density distributions; (b) averaged results from patients with a normal density distribution of neutral α-glucosidase. Frequency (mean ± SD) is defined as the fraction of total recovered activity present in the individual fraction divided by the density span covered by that fraction. The activity present over the density span 1.05–1.10 represents, over an arbitrary abscissa interval, enzyme remaining in the sample layer and presumed to reflect soluble activity. Percentage recoveries (mean ± SE): (a) 107 ± 7; (b) 104 ± 7.

performed in seven patients and, with the exception of the endoplasmic reticulum, showed no abnormality of any other organelle. In four biopsies striking hypertrophy of the smooth endoplasmic reticulum was seen (group A, Fig. 3). These patients were all in the group with the lighter peak of the endoplasmic reticulum marker α-glucosidase on density gradient centrifugation. The remaining three biopsies did not differ from those of normal controls and showed the normal predominantly rough endoplasmic reticulum (group B, Fig. 3). These three patients showed normal density gradient distribution of α-glucosidase. The presence of lipofuscin in the biopsies did not correlate with changes in the smooth endoplasmic reticulum, the pigment being present in both groups.

Fig. 2. Isopycnic centrifugation of postnuclear supernatant from liver biopsy specimens from patients with Gilbert's syndrome compared with results from control subjects. Graphs show frequency–density histograms for marker enzymes. For details see the legend to Fig. 1. Percentage recoveries (mean ± SE): β-glucuronidase, 88 ± 3; lactate dehydrogenase, 92 ± 2; acid phosphatase, 82 ± 4; N-acetyl-β-glucosaminidase, 96 ± 2; catalase, 88 ± 2; malate dehydrogenase, 87 ± 2; γ-glutamyl transferase, 73 ± 5; 5′-nucleotidase, 72 ± 6.

Fig. 3. Quantification of the hepatocyte smooth endoplasmic reticulum in electron micrographs from normal controls and Gilbert's syndrome patients with (group A) and without (group B) morphological evidence of smooth endoplasmic reticulum hypertrophy.
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Clinical data

The two groups defined by density distribution of α-glucosidase did not differ in respect of age, sex, clinical presentation or mean serum bilirubin concentrations, both groups having typical Gilbert’s syndrome.

Discussion

This paper is the first systematic attempt to investigate the organelle pathology of Gilbert’s syndrome by biochemical techniques. All the patients fulfilled the accepted criteria of diagnosis of Gilbert’s syndrome (Arias, 1962), and none had evidence of overt haemolysis. The results of the fractionation experiments and analysis of enzyme activities show abnormalities of the equilibrium density distributions and total activities of markers for the endoplasmic reticulum and lysosomes. All other organelles showed normal equilibrium density distributions.

Two distinct populations of patients with Gilbert’s syndrome were seen with different density distributions of the endoplasmic reticulum marker neutral α-glucosidase. Furthermore, those patients with normal distribution of α-glucosidase had the normal, predominantly rough, endoplasmic reticulum of the hepatocyte on electron microscopy, whereas those with the light peak showed striking hypertrophy of the smooth endoplasmic reticulum. We have previously noted a decreased modal density of neutral α-glucosidase in patients with alcoholic liver damage (Seymour & Peters, 1978a), a condition associated with hypertrophy of the smooth endoplasmic reticulum. The increase in activity of N-acetyl-β-glucosaminidase, acid phosphatase and β-glucuronidase and the altered density distribution of N-acetyl-β-glucosaminidase and β-glucuronidase show that the lysosomal properties are in some way altered. Lysosomal integrity, however, as evidenced by normal latent and sedimentable N-acetyl-β-glucosaminidase activities, was unimpaired. This suggests that the abnormal distribution of lysosomal enzymes was due to lysosomes of reduced equilibrium density rather than to lysosomal disruption. Lipofuscin pigment has been reported to accumulate in excess in Gilbert’s syndrome (Barth et al., 1971). Lysosomes have been shown to fuse with precursors of lipofuscin and other residual bodies (Gordon, Miller & Bensch, 1965) and these pigment granules have been shown to be associated with lysosomal enzyme activity (Essner & Novikoff, 1960). Although we found morphological evidence of lipofuscin accumulation in less than half our patients, it is likely that the lysosomal
enzyme changes found represent early stages in the process of lipofuscin accumulation. In primary lysosomal storage diseases (van Hoof & Hers, 1968) the unaffected lysosomal enzymes, e.g. acid phosphatase, are elevated in the affected tissues. Likewise elevated enzyme activities are found in primary and secondary haemochromatosis (Seymour & Peters, 1977b) and in the Dubin–Johnson–Sprinz syndrome (Seymour, Neale & Peters, 1977), examples of secondary lysosomal storage diseases. These findings could be interpreted as frustrated attempts by the cell to remove undegradable material. A similar process may account for the lysosomal abnormalities of Gilbert's syndrome, although we can only speculate on the nature of the primary waste material.

A characteristic feature of Gilbert's syndrome is the accumulation of unconjugated bilirubin in the serum and recently it has been reported that these patients excrete a decreased proportion of bilirubin diglucuronide in the bile (Fevry et al., 1977). These changes have usually been attributed to impaired bilirubin glucuronide synthesis but, in view of the raised P-glucuronidase activity, the present study raises the possibility that enhanced hydrolysis of the conjugates may at least be partially responsible.

The current study showed no biochemical evidence of abnormalities of the plasma membrane, biliary canaliculi or mitochondria in the patients with Gilbert's syndrome. This correlates with the absence of ultrastructural abnormalities of these organelles. It also supports the findings of two previous studies (McGee et al., 1975; Dawson et al., 1978) that were unable to confirm the previous reports of abnormal plasma membrane microvilli or mitochondria (Simon & Varonier, 1963; Magnenat & Paluello, 1967; Schaff et al., 1969; Bleeger et al., 1977). This study therefore provides biochemical confirmation of previously reported abnormalities of the endoplasmic reticulum, but underlines the presence of two subpopulations. It also demonstrates altered lysosomal properties which might be associated with lipofuscin accumulation in this syndrome.

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References


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