Possible role of a defect in hepatic bilirubin glucuronidation in the initiation of cholesterol gallstones

P DUVALDESTIN, J-L MAHU, J-M METREAU, JOSETTE ARONDEL, ANNE-MARIE PREAUX, AND P BERTHELOT*

From the Unité de Recherches INSERM U-99, Hôpital Henri Mondor, Créteil, France

SUMMARY Abnormally low activity of hepatic bilirubin UDP-glucuronosyltransferase was found in 25% of 81 unselected patients with gallstones, as compared with only 3% in 35 controls. At the time of cholecystectomy, the stones were taken for analysis in 48 of 81 patients, and a bile sample was obtained in 42 of them. Among the stones, 75% were cholesterol stones, 15% pigment stones, and 10% 'intermediate' stones. Low hepatic conjugating activity was not preferentially associated with a given type of stone. No relation was found between the enzymic deficiency and the biliary cholesterol saturation index. A high proportion of biliary bilirubin monoglucuronide (over 40%) was found in four of seven patients with low transferase activity, as was earlier demonstrated in patients with overt Gilbert's syndrome. Raised biliary bilirubin monoglucuronide was also found in three patients, out of 46, who had normal transferase but raised biliary beta-glucuronidase activity. There was no evidence that deficient bilirubin conjugation could be a consequence of gallstones: the activity of another hepatic microsomal enzyme, glucose 6-phosphatase, taken as a reference, was measured in 12 patients and was always normal. Taking into account the very high frequency of a bilirubin centre in the cholesterol stones (87% of the cases in the present series), it is suggested that the increased proportion in poorly soluble biliary bilirubin monoglucuronide, which was associated with defective conjugation, could act as a trigger for gallstone initiation, regardless of the final composition of the stone.

Considerable improvements in our knowledge of gallstone formation have been obtained during the last few years, when the role of so-called supersaturated bile in the pathogenesis of cholesterol gallstones (CS)¹⁻³ was proposed. In spite of this finding, however, several points remain obscure: (1) supersaturated bile may be observed in the absence of stones;⁴ (2) analysis of biliary cholesterol saturation after chenodeoxycholic acid therapy does not differentiate between patients in whom gallstones were dissolved and those in whom no dissolution occurred.⁵ In addition, it has been observed for years that 'pure cholesterol stones are rare: crystallisation seems to come from (or point towards) a centre at which a small amount of discolouration due to pigment may often be seen'.⁶ Conversely, it is of interest that the current definition

of pigment stones (PS) does not take their absolute content of bilirubin into account.⁷ Alterations in bilirubin biotransformation could indeed contribute to stone formation and Black *et al.*⁸ have already shown that a low activity of hepatic bilirubin UDP-glucuronosyltransferase (B-GT) is a frequent finding in patients with gallstones. The ensuing increase in less soluble forms of biliary bilirubin, which occurs when the activity of B-GT is low,⁹ may play a role in the nucleation of the stones.

For all these reasons, it seemed of interest to re-examine the possible role of bilirubin metabolism and the biliary bilirubin composition in unselected patients with gallstones, using new techniques which permit the precise analysis of bilirubin.⁹

Methods

SUBJECTS

Eighty-one unselected patients with gallstones were studied. There were 20 males and 61 females; their

*Address for reprint requests: Pierre Berthelot, MD, Unité de Recherches INSERM U-99, Hôpital Henri Mondor, 94010 Créteil, France.

age varied from 20 to 80 years (median: 47.5 years). Patients with obstructed cystic or common bile ducts, acute cholecystitis, or liver disease were excluded. None had overt haemolysis. Haemoglobin and serum levels of haptoglobin and bilirubin were normal in all cases. Apparently, no patient or control took any drug known to alter the activity of their liver microsomal enzymes. Thirty-five patients without known disease of the liver or of the biliary system acted as controls; they included 29 males and six females whose ages varied from 18 to 72 years (median: 47 years). Nutritional status was comparable in all the individuals included in the present study; they were all fasted for at least eight hours before the investigation. All the patients had given their informed consent before any procedure.

SAMPLING PROCEDURES

In both groups a liver biopsy was obtained and divided into two pieces, one for routine light microscopic examination and one for measurement of B-GT and glucose 6-phosphatase (see below). The liver sample was stored at -20°C for a period which never exceeded two weeks; under such storage conditions, the enzyme activity was shown to remain constant for at least two months.¹⁰ A sample of tissue was obtained from a needle biopsy in 16 patients with gallstones before starting chenic acid therapy; at that time, in view of a possible toxicity of the drug, a biopsy was performed in all these cases. In 21 of the control patients, the biopsy was performed because of suspicion of liver disease, which was later ruled out because of the normal histological appearance. In 65 patients undergoing cholecystectomy and in 14 control patients operated on for peptic ulcer, a wedge liver sample was taken at time of surgery. Drugs used for premedication or anaesthesia consisted exclusively of non-inducing agents, apart from thiopental which was given shortly before the liver biopsy and would therefore not be expected to alter hepatic glucuronidation. In 50 of the patients undergoing cholecystectomy, all the stones were recovered for chemical analysis, and in 42 a sample of bile (gallbladder bile in 40 cases, common bile duct bile in the two cases of non-functioning gallbladder) was taken for bilirubin analysis and determination of the lithogenic index. It is known that there are no significant differences in bilirubin composition of gallbladder- (or duodenal-) vs. hepatic bile.¹¹ The bile sample was immediately protected from light and frozen until examined.

CHEMICAL METHODS

B-GT was assayed in digitonin-activated liver

homogenates (5% w/v) in 0.25 M sucrose containing 1 mM EDTA as previously described.^{10,12} For convenience the enzyme activity was expressed as μmol (mg) of bilirubin conjugated per hour and per gram of liver; the results were also calculated per milligram of liver protein. Abnormally low B-GT was defined as usual as activities below $1.20 \mu\text{mol}$ (0.7 mg) of bilirubin conjugated/h/g of liver.^{13,14} Glucose 6-phosphatase activity was measured in 12 homogenates (six with normal and six with low B-GT activity), according to the method described by Harper.¹⁵ This was done in order to eliminate a non-specific alteration in microsomal enzymes due either to sampling and storage conditions or to the underlying disease itself.

In the stones, cholesterol and bilirubin content was measured. The gallstones were washed with distilled water and wiped. Before analysis, their appearance was recorded by visual inspection on cross-section. The stones were then pulverised and dried to constant weight. An aliquot of the gallstone powder was extracted in isopropanol for 15 minutes. Cholesterol was measured in the isopropanol extract by the enzymic method of Roeschlau *et al.*¹⁶ For bilirubin measurement 5 mg of the pulverised sample were extracted with a chloroform-methanol (2:1, v/v) solution containing 1% of 1 N HCl.¹⁷ The portion of powdered stone which was insoluble in acid chloroform-methanol was dried, weighed, and designated the residue. One millilitre of the extract was added to 2 ml acetone-ethanol (1:1, v/v) mixture and incubated for 60 minutes at 0°C with diazotized p-iodoaniline;¹⁸ 3 ml of a solution of ascorbic acid (1% w/v in 0.1 M NaCl) were added to stop the reaction; the tubes were centrifuged and the azopigments measured in the lower phase at 530 nm. Bilirubin concentration (μg of bilirubin per mg of dry weight) was calculated from $A_{530}^{1\text{cm}} \times 25.6 \times 0.64$, where 25.6 was the extinction coefficient obtained from a calibration curve and 0.64 corresponded to the volume of the lower phase obtained at the end of the reaction. It was verified by thin layer chromatography (TLC)^{9,11} that the bilirubin in the stones was exclusively the non-conjugated pigment.

Bile samples were treated with diazotized ethyl-anthranilate followed by TLC.⁹ On such treatment, azopigments will separate mainly into unconjugated (α_0) and glucuronidated (δ) azodipyrroles. This method thus allows the respective proportions of mono- and diglucuronide to be calculated from the ratio of α_0 /total azopigment.⁹ Beta-glucuronidase activity was assayed in the bile of 44 patients with phenolphthalein glucuronide as substrate¹⁹ and incubation at pH 6.8 in order to measure mainly the bacterial enzyme. The lowest activity

that could be accurately measured was 48 nmol (15 µg) of phenolphthalein formed per h/ml.

In order to determine cholesterol saturation, the following measurements were made in aliquots of gallbladder bile: cholesterol, by the enzymic method of Roeschlau *et al.*,¹⁶ phospholipids by the method of Bartlett,²⁰ and total bile salts by an enzymic method using 3 alpha-hydroxysteroiddehydrogenase as previously described.^{21,22} The lithogenic index was calculated according to the equation proposed by Thomas and Hofmann,²³ using the maximal cholesterol solubility as proposed by Holzbach *et al.*⁴ Statistical analysis was carried out by determining the mean $\pm$ SEM for each parameter studied in each group and applying the standard Student's unpaired *t* test or the χ^2 test for two independent variables between groups.²⁴

Results

HEPATIC B-GT

No significant difference was observed when B-GT activity was assayed from a needle or from a surgical biopsy: the mean values were 2.18 ± 0.17 (SEM) µmol (1.28 $\pm$ 0.10 mg) per h/g liver in the former and 2.30 ± 0.20 (1.35 $\pm$ 0.12) in the latter. The results were therefore combined, giving for the controls a mean B-GT of 2.24 ± 0.13 µmol (1.31 $\pm$ 0.08 mg) per h/g. Among the 35 controls there was only one who had a low B-GT (0.46 µmol (0.27 mg) per h/g). This finding contrasted with the 81 patients having gallstones, 20 of whom had a low B-GT ($P < 0.01$). The activities in these 20 patients exhibited a mean of 0.71 ± 0.05 µmol (0.42 $\pm$ 0.03 mg) per h/g, the values ranging from 0.20 to 1.04 µmol (0.12 to 0.61 mg) per h/g. The presence of patients with low B-GT did not significantly influence the mean value observed in the group of patients with gallstones, which was 1.99 ± 0.12 µmol (1.17 $\pm$ 0.07 mg) per h/g.

In patients with gallstones, the B-GT deficiency occurred in 30% of the males, compared with 23% of the females; this difference, however, was not significant. Whereas in the females a similar frequency of low B-GT was noted regardless of the age, such deficiency seemed more frequent in males under 45 years of age (45%) than among those who were aged over 45 years (11%). However, this difference was not statistically significant.

STONE COMPOSITION

In 50 cases the cholesterol and bilirubin content of the gallstones was measured. Thirty-eight patients had a cholesterol content higher than 75% of the dry weight and were therefore considered as having cholesterol gallstones; they included seven males and 31 females. Macroscopically the cross-section

Table 1 *Hepatic bilirubin UDP-glucuronosyltransferase (B-GT) activity in patients having cholesterol- (CS), pigment- (PS), or intermediate stones (IS)*

Type of stone	B-GT*	Patients with low B-GT†	
		(n)	(%)
CS (n = 34)	2.12 $\pm$ 0.15 (1.24 $\pm$ 0.09)	6	16
PS (n = 7)	2.17 $\pm$ 0.46 (1.27 $\pm$ 0.27)	1	14
IS (n = 5)	2.72 $\pm$ 0.68 (1.59 $\pm$ 0.40)	0	

*B-GT is expressed as µmol (mg) of bilirubin conjugated per h/g of liver (mean $\pm$ SEM).

†Low B-GT was defined as activity below 1.20 µmol (0.70 mg) per h/g.

Table 2 *Stone composition in 50 cases of cholesterol- (CS), pigment- (PS), or intermediate stones (IS)*

	Cholesterol*	Bilirubin*	Residue*
CS (n = 38)	98.08 $\pm$ 2.61	1.39 $\pm$ 0.20	0.17 $\pm$ 0.12
PS (n = 7)	6.47 $\pm$ 2.46	19.56 $\pm$ 8.10	32.24 $\pm$ 6.02†
IS (n = 5)	53.52 $\pm$ 5.21	9.57 $\pm$ 8.58	nil†

*Expressed as percentage of the dry weight (mean $\pm$ SEM).

†Addition of the three parameters measured did not reach 100%; these therefore do not reflect all possible constituents of these types of stones, as previously observed by Nakayama.¹⁷ The high residue also reflects the presence of unidentified, amorphous, material in the PS.

showed a pigment centre in 33 of the 38 cholesterol gallstones (87%). Seven patients had pigment stones, defined on the basis of a cholesterol content lower than 25%;⁷ they were three males and four females. Five patients, all females, had intermediate stones (IS). B-GT was measured in 46 of 50 patients. There was no significant difference in B-GT distribution according to the type of the gallstone (Table 1). Low B-GT was observed in six patients with cholesterol gallstones, in one with pigment stones, and in none of the five with intermediate stones. Bilirubin accounted for 19.6, 1.3, and 9.6% of the dry weight of PS, CS, and IS, respectively (Table 2). In addition, in six unselected CS having a dark centre, bilirubin content was measured separately in the centre and at the periphery. In each case, bilirubin content was much higher in the centre, with a mean concentration ratio of 7.5 (values ranging from 2.2 to 19.5).

BILIARY BILIRUBIN

The composition of the bile was assessed in 42 patients, 30 with CS, seven with PS, and five with IS. A bilirubin monoglucuronide/total conjugates ratio equal to, or higher than 40% was found in

nine patients (Table 3). In seven of them, such a high proportion of bilirubin monoglucuronide appeared to be a consequence of either a low B-GT (four cases),⁹ or a beta-glucuronidase activity exceeding 318 nmol (100 µg) per h/ml (three cases), compared with 48 ± 4.2 (15 ± 1.3) in the remaining patients. In the two patients whose percentages of bilirubin monoglucuronide/total conjugates were slightly raised (43.6 and 41.4) in spite of a normal B-GT (2.60 and 4.19 µmol (1.52 and 2.45 mg) of bilirubin conjugated per h/g, respectively, and almost undetectable beta-glucuronidase activity 48 and 64 nmol (15 and 20 µg) per h/ml respectively), no explanation was found. When the seven patients with either a low B-GT or a biliary beta-glucuronidase activity greater than 318 nmol (100 µg) per h/ml were excluded, then the mean percentage of bilirubin monoglucuronide was 31.6% in the group of CS, 27.4% in that of PS, and 29.0% in that of IS. These three values are all of the same order of magnitude as the mean value ($27.2 \pm 7.8\%$) that we found previously in healthy individuals.⁹

Among the five patients having 40% or more bilirubin monoglucuronide, there were two CS, two PS, and one IS; there was no obvious relation between the bilirubin monoglucuronide content and the chemical type of the stones.

LITHOGENIC INDEX

The mean lithogenic index was determined in the bile of 40 patients. It was of course higher in the 29 patients with CS than in the seven having PS (Table 4). Among the patients with CS, the lithogenic index of the six with low B-GT activity did not differ from that observed in the 25 individuals with normal enzymic activity. Nor did the lithogenic index of the single PS patient having low B-GT activity differ from that of the other six individuals.

Table 4 Mean lithogenic index in the bile of patients having cholesterol- (CS) or pigment stones (PS) according to hepatic bilirubin UDP-glucuronosyltransferase (B-GT) activity

Type of stone	Mean lithogenic index* (n of patients)		
	All	With normal B-GT†	With low B-GT‡
CS	1.36 ± 0.08 (29)	1.38 ± 0.09 (23)	1.31 ± 0.08 (6)
PS	0.76 ± 0.03 (7)	0.76 ± 0.04 (6)	0.75 (1)

*Lithogenic index $\pm$ SEM was calculated from the equation given by Thomas and Hofmann.²³

†B-GT is expressed as µmol (mg) of bilirubin conjugated per h/g of liver $\pm$ SEM.

‡Low B-GT was defined as activity below 1.20 µmol (0.70 mg) per h/g.

GLUCOSE 6-PHOSPHATASE

There were no significant differences between the values of glucose 6-phosphatase activity in six patients with and six without low B-GT: 74.1 ± 3.1 nmol per min/mg protein and 72.6 ± 1.7 , respectively.

Discussion

In the present series, 20 out of the 81 patients with gallstones had a low B-GT activity. A similar finding has already been made by Black *et al.*⁸ who found five cases with enzymic deficiency among 34 patients undergoing cholecystectomy for cholelithiasis. In contrast, such a defect existed in only one of our 35 controls, and in one of 26 of the patients of Black *et al.*⁸ who were operated on for causes other than biliary lithiasis. The lowest limit of 'normal' B-GT activity is difficult to assess, and it has been arbitrarily fixed at 1.20 µmol

Table 3 Chemical type of stones, hepatic bilirubin UDP-glucuronosyltransferase (B-GT) activity, and biliary bacterial beta-glucuronidase activity in nine patients with high bilirubin monoglucuronide content in bile

Patients	Type of stone*	Percentage of bilirubin monoglucuronide†	Hepatic B-GT‡	Biliary β-glucuronidase§
1	CS	41.2	0.58 (0.34)	48 (15)
2	CS	40.0	0.53 (0.31)	60 (19)
3	CS	53.0	0.97 (0.57)	48 (15)
4	CS	46.4	0.75 (0.44)	48 (15)
5	CS	41.4	4.19 (2.45)	73 (23)
6	CS	61.6	1.28 (0.75)	429 (135)
7	PS	43.6	2.60 (1.52)	48 (15)
8	PS	63.2	1.73 (1.01)	1768 (556)
9	IS	89.2	5.08 (2.97)	843 (265)
Normal value (mean $\pm$ SEM)		27.2 ± 7.8	2.19 ± 0.17 (1.28 ± 0.10)	47.7 ± 4.1 (15.0 ± 1.3)

*Cholesterol- (CS), pigment- (PS), or intermediate stones (IS) as defined from their cholesterol content.⁷

†Calculated from the α_z-azopigment—that is, the unconjugated azodipyrrrole, formed from coupling with diazotized ethylanthranilate.⁹

‡B-GT is expressed as µmol (mg) of bilirubin conjugated per h/g of liver.

§β-glucuronidase activity is expressed as nmol (µg) of phenolphthalein formed per h/ml bile.

(0.7 mg) per h/g liver. It is of interest that the mean B-GT activity in the 20 patients with gallstones who had low levels of transferase was significantly higher than the activity we observed in a group of 37 adults patients with overt Gilbert's syndrome:¹⁴ the means were 0.72 ± 0.05 μmol (0.42 ± 0.03 mg) per h/g and 0.43 ± 0.05 (0.25 ± 0.03), respectively ($P < 0.001$). This strongly suggests that, although B-GT was reduced in both groups, they both represent different degrees of a common biochemical defect. In accordance with such a hypothesis, it should be noticed that among our subjects with B-GT deficiency (20 with cholelithiasis and one control), none had an unconjugated hyperbilirubinaemia. Similarly, the B-GT deficiency found recently by Felsher and Carpio²⁵ in chronic persistent hepatitis was comparable with that of our 20 patients in those who had normal serum bilirubin, whereas the enzymic activity was as low as in Gilbert's syndrome when unconjugated hyperbilirubinaemia was present.

The B-GT deficiency was a slightly more frequent finding in males aged under 45 years, whereas cholelithiasis affects predominantly women who are over 45 years; however, probably in view of the relatively small number of males who were investigated, this was not significant. In contrast with the patients with gallstones, our control group predominantly consisted of males. None of the previously published series of B-GT measurements in man have ever mentioned any sex differences. Our own results, although dealing with only six female controls, also failed to suggest any significant sex-linked difference in the normal hepatic B-GT activity: 2.14 ± 0.21 μmol (1.25 ± 0.13 mg) per h/g in the females and 2.26 ± 0.15 (1.32 ± 0.09) in the males.

Three main explanations for the association between a low B-GT activity and the presence of gallstones can be offered. Firstly, fortuitous coincidence; this however appears to be unlikely because of the statistical significance which existed for the low B-GT activity in our series compared with normal subjects as well as the frequency of the enzymic defect observed in the patients of Black *et al.*⁸ Secondly, there is no evidence to support the hypothesis that cholelithiasis caused the low B-GT activity. If this were the case, one would expect that the enzymic deficiency would be found more frequently and be associated with other microsomal enzymic defects. This latter possibility was ruled out because the glucose 6-phosphatase activity was similar in patients with or without B-GT deficiency. For these reasons, it is more tempting to speculate that B-GT deficiency played a role in the formation of gallstones. In this regard, it is of interest that

no clear relation could be found between B-GT deficiency and the composition of the stones. Such an enzymic deficiency was encountered as frequently in cholesterol gallstones as in pigment stones, which suggests that the B-GT deficiency favoured the formation of both types of stones. This obviously was not the result of the influence of B-GT on biliary cholesterol saturation. In fact, 16% of our patients with CS had a lithogenic index of 1.00 or less, thus indicating that cholesterol supersaturation is not always the cause of CS formation.

Abnormal bilirubin metabolism could be responsible for the initiation of CS, especially bearing in mind the fact that 87% of the CS of the present series had a bilirubin centre. Biliary unconjugated bilirubin should, however, not be implicated for the following reasons: (1) only very small amounts of this pigment exist in bile (approximately 1% total), which cannot be simply related to the biliary deconjugating activity;²⁶ (2) when we analysed the bile of numerous adults with low hepatic B-GT activity, no increase in biliary unconjugated bilirubin was found.⁹

An increase in the proportion of biliary monoglucuronide could offer a reasonable explanation for gallstone formation, as it is less water-soluble than the diglucuronide which normally predominates in human bile.¹¹ At present, two main causes are known to produce an increased proportion of bilirubin monoglucuronide in bile. The first is a raised biliary bacterial beta-glucuronidase activity; this was observed in three instances in this work, although it must be remembered that deconjugation of phenolphthalein does not necessarily reflect that of bilirubin. The second, as we have shown in previous work, is B-GT deficiency, which is very frequently associated with a high proportion of biliary bilirubin monoglucuronide.⁹

It should, however, be noted that the frequency of a bilirubin centre in our cholesterol stones seems to far exceed that of patients having conjugation deficiency. Moreover, in Gilbert's syndrome, although an increased incidence of gallstones has been suggested,²⁷ convincing evidence needs to be obtained from a prospective, controlled study. Nevertheless, it remains tempting to speculate that decreased B-GT activity may contribute to gallstone formation, whatever their type, *via* an increase in biliary bilirubin monoglucuronide. Such a hypothesis is not invalidated by the limited number of cases in which we found raised biliary monoglucuronide values. The measured B-GT and biliary composition may well be only a poor reflection of more important alterations which existed at the time of stone nucleation. Even though of brief

duration, an increased proportion of biliary mono-glucuronide could act as a trigger for stone initiation in a given saturation biliary cholesterol, but different bile nucleation times.²⁸

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