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Analysis of Bilirubin Fraction in the Bile for Early Diagnosis of Acute Rejection in Living Related Liver Transplantation

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AKAMATSU, Y., OHKOHCHI, N., SEYA, K. and SATOMI, S. *Analysis of Bilirubin Fraction in the Bile for Early Diagnosis of Acute Rejection in Living Related Liver Transplantation.* Tohoku J. Exp. Med., 1997, **181** (1), 145-154 —— The diagnosis of acute rejection in liver transplantation usually needs hepatic biopsy, but hepatic biopsy sometimes involves severe complications. We analyzed biliary bilirubin fraction after living related liver transplantation by using high performance liquid chromatography (HPLC) and investigated availability for the early diagnosis of acute rejection retrospectively. Nine children with liver cirrhosis due to biliary atresia were inculuded in this study, who underwent living related liver transplantation at The Second Department of Surgery, Tohoku Uninversity School of Medicine. Bile was collected daily from a biliary canulae inserted into the hepatic duct of the graft under aseptic and without exposure to the light. We measured the proportion of bilirubin diglucuronide (BDG), bilirubin monoglucuronide (BMG) and unconjugated bilirubin (UCB) of bile pigments in the bile by HPLC. In three of four patients with acute rejection, BDG+BMG (=Bc) was above 85% and BDG/Bc ratio was below 0.6 at the time of hepatic biopsy. After rejection therapy, BDG/Bc ratio increased in their bile. The remaining one case with acute rejection as well as bile duct injury due to arterial thrombosis of S2, Bc was below 85%, and BDG/Bc ratio was below 0.6. In four of the other five patients who had several severe complications, i.e., arterial or portal vein thrombosis, bile stasis due to cholangitis and sepsis due to necrotizing myofascitis, Bc was below 85% and BDG/Bc ratio was below 0.6. We concluded that analysis of biliary bilirubin fraction after liver transplantation could be reliable as a noninvasive maker and valuable for the early diagnosis of acute rejection. —— acute rejection; bilirubin pigments; high performance liquid chromatography (HPLC)

Living related liver transplantation in Japan has been used to treat end-stage liver cirrhosis mainly caused by biliary atresia (Hasegawa et al. 1996). But many Kinds of complications occur after transplantation (Kalayoglu et al. 1993; Doyle

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et al. 1994), for example, primary graft nonfunction, acute rejection, infection, bile duct complications (Sanchez-Urdazpal et al. 1993), vascular stenosis (Azoulay et al. 1993) and thrombosis (Defranco et al. 1993). Among these complications, acute rejection is especially a major problem after transplantation, even though immunosuppression has progressed. The specific changes of laboratory data are not recognized in acute rejection. Therefore, the differential diagnosis of acute rejection usually needs hepatic biopsy (Williams et al. 1985; Kemnitz et al. 1989). But hepatic biopsy sometimes has severe complications.

Analysis of bile acid in bile and serum (Azer et al. 1994), interleukine in bile (Tono et al. 1992) and delta bilirubin (Wu et al. 1990) were reported as noninvasive examination in acute rejection. Bilirubin conjugated within microsome of hepatocytes acquires solubility in water and is excreted into the bile. Recently several bilirubin pigments in bile are analyzed by high performance liquid chromatography (HPLC) (Cole and Little 1982; Goresky 1990). In some hepatic disease, the changes of proportion of bile pigments were reported (Yamashita et al. 1986). Therefore we wonder whether the liver function after liver transplantation was directly indicated by analysis of bilirubin fraction of bile. In the present study, we retrospectively investigated the availability of bile pigments analysis in bile for the early diagnosis of acute rejection.

Patients and Methods

Nine children with biliary atresia, who had undergone living related liver transplantation at The Second Department of Surgery, Tohoku University School of Medicine from May 1992 to December 1995 were included in this study. There were three males and five females, raging in age from 6 months to 8 years old. Whereas all patients had performed hepatic portoenterostomy one or two times before transplantation, they were resulted in liver cirrhosis (Table 1). At the transplantation all patients were cannulated to bile duct of the graft. Immunosuppression was maintained by cyclosporin or FK5O6, and steroid, with the occasional use of azathioprine.

In this study 5 cases had acute rejection after transplantation (Table 2). One of them, No. 8; had acute rejection after the biliary tube was removed. In one of other 4 patients, No. 3, the arterial blood flow feeding segment 2 was not detected with doppler ultrasonography after liver transplantation. We usually performed hepatic biopsy at the seventh and fourteenth day after transplantation, and when necessary. Diagnosis of acute rejection was determined by two or three pathologists. At first we treated acute rejection with pulse injection of methylprednisolone for three days. When the rejection persisted, Deoxyspergualin was administered in dosage of 3 mg/kg/day.

Two patients had thrombosis, one patient had severe cholangitis, one patient suffered necrotizing fasciitis. Three of them were dead from thrombosis or sepsis caused by necrotizing fasciitis.

TABLE 1. *Preoperative data of recipients*

Patient No.	Age	Sex	Hepatic portoenterostomy		Serum total bilirubin (mg/100 ml)
			Number	Age (days)	
1	1 y 7 m	F	2	64, 407	5.0
2	1 y 1 m	M	1	78	5.2
3	8 y	F	2	112, 162	4.3
4	5 y	F	2	76, 244	22.9
5	1 y 2 m	F	1	89	3.4
6	6 m	F	1	76	9.6
7	9 m	M	1	96	32.9
8	11 m	M	1	63	3.3
9	9 m	F	2	6, 142	18.8

Y, year; m, month.

TABLE 2. *Postoperative complications*

Patient No.	Complications
1	Thrombosis of portal vein and hepatic artery
2	Acute rejection
3	Severe cholangitis
4	Acute rejection
5	Acute rejection
6	Thrombosis of hepatic artery
7	Acute rejection, cerebral infarction
8	Acute rejection
9	Necrotizing fasciitis

Bile was collected from the tube inserted into bile duct under aseptic and without exposure to the light after the liver transplantation until the tube was removed. The bile was examined immediately or was frozen at $-20^{\circ}C$ until examined. After 100 μl of the bile was diluted with 200 μl of acetone, the dilution was centrifuged at 2,000 rpm for 5 min. The supernatant was filtered through 50 μm of pore size filter. And then filtrate was injected into the HPLC column in 50 to 100 μl volumes.

Bile pigments was analyzed by a reversed phase HPLC which was carried out using TSK-gel ODS-80TM. According to the method described by Yamashita (1987), the diluent was monitored for absorbance at 450 nm. The flow-rate was 1 ml/min. The mobile phase was acetonitrile-0.1 M sodium acetate (pH 4.0) containing 5 mM pentanesulphonic acid sodium salt. Gradient elution was done from 20 to 48% acetonitrile in 35 min, from 48 to 90% in 10 min, and after that

90% acetonitrile was maintained for another 15 min.

We measured bilirubin diglucuronide: BDG, bilirubin monoglucuronide: BMG and unconjugated bilirubin: UCB in bile. Furthermore, we calculated the amount of conjugated bilirubin Bc that equal BDG plus BMG and the ratio of BDG to the amount of conjugated bilirubin: BDG/Bc ratio.

Results

In the 4 cases with acute rejection, acute rejection was diagnosed by pathologists on the 6th or 7th day after the transplantation. The patients were treated by pulse therapy using steroid after that. At the time of hepatic biopsy, Bc was above 85% except in one case who had not detected a blood flow in A2 (87.9± 10.69. mean±S.D.), but BDG/Bc ratio was blow 0.6 (0.45±0.17). After the antirejection therapy, BDG/Bc ratio was increased (0.58±0.12) (Fig. 1).

In one case with acute rejection, total bilirubin and alkaline phosphatase were increased gradually and reached 9.7 mg/100 ml and 212 IU/liter at the time of hepatic biopsy respectively after transplantation. Pulse therapy of steroid for three days was started on 9th postoperative day. In this case, the change of Bc was above 85%. On the other hand, BDG/Bc ratio depressed below 0.6 from the 2nd day after the operation and it was 0.54 at hepatic biopsy. But BDG/Bc increased after the first pulse therapy of steroid and it rose to 0.66. In those days Bc was above 85% almost invariable. Total bilirubin in serum was approximately 10 mg/100 ml, therfore, she was subsequently treated with deoxyspergualin (Fig. 2).

In case with severe cholangitis, bile was not discharged from the tube, and total bilirubin increased up to 30 mg/100 ml. She received with biliary drainage and plasmapheresis. After biliary drainage Bc was below 85% and BDG/Bc was

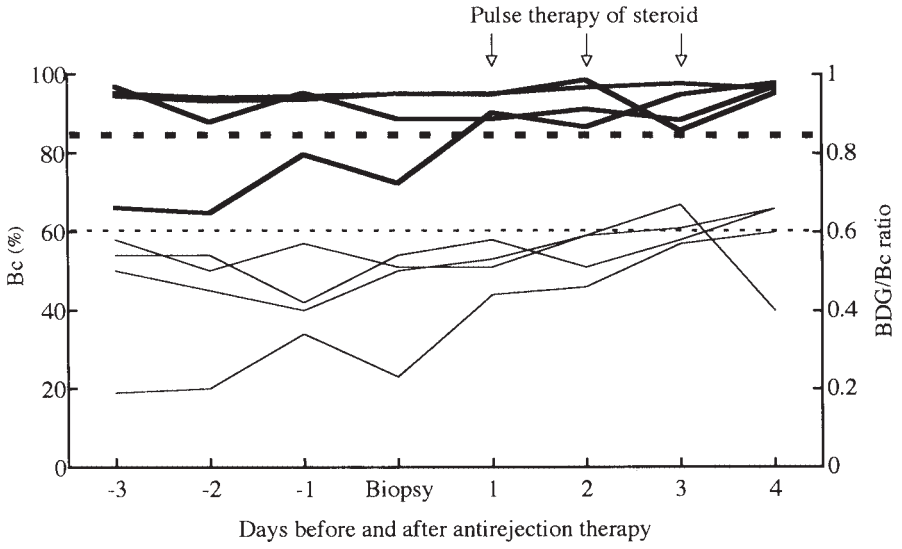

Fig. 1. Change of Bc and BDG/Bc ratio before and after antirejection therapy in acute rejecion ($n=4$)., —— Bc; —— BDG/Bc retio; ——85% on Bc; ····· 0.6 on BDG/Bc

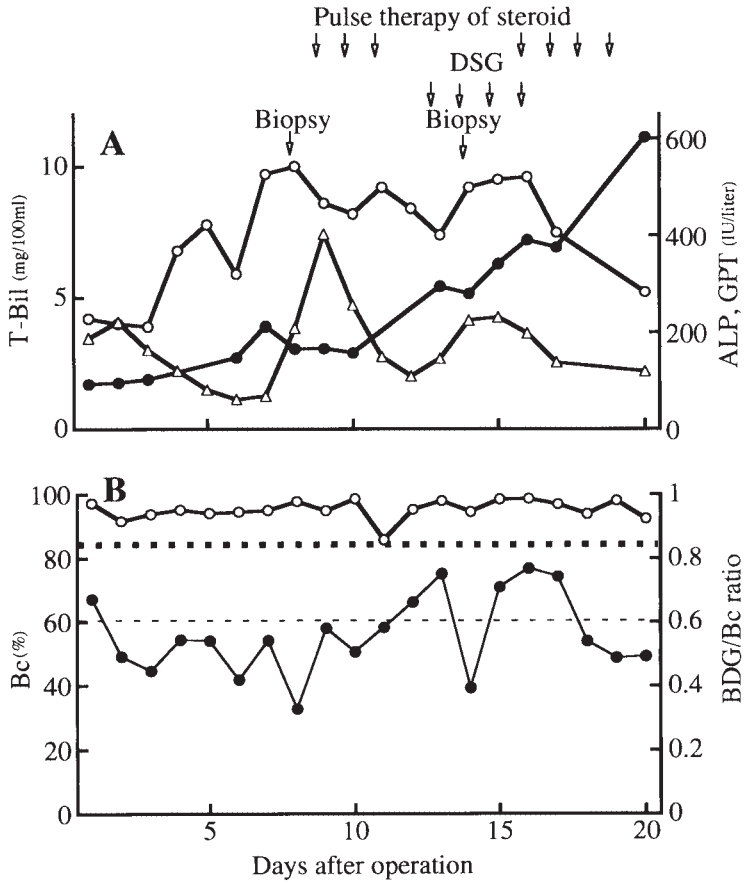

Fig. 2. Case No. 5 with acute rejection
(A) Change of laboratory data, ○—○ T-Bil; ●—● ALP; △—△ GPT,
(B) Change of Bc and BDG/Bc ratio, ○—○ Bc; ●—● BDG/Bc; ---85% on Bc;
·····0.6 on BDG/Bc
DSG, deoxyspergualin.

below 0.6, but gradually increased (Fig. 3).

One of two patients who had thrombosis, arterial thrombosis was detected and treated by thrombectomy on the third day after the transplantation. But blood flow did not improve, and she died on the 15th day after the transplantation. After thrombectomy Bc and BDG/Bc ratio had decreased remarkably (Fig. 4).

In case without hepatic compolications, Bc had changed above 85% and BDG/Bc ratio had changed above 0.6 except one time. When BDG/Bc ratio was below 0.6, central veinous catheter was removed, due to catheter sepsis (Fig. 5).

Discussion

The diagnosis of acute rejection is so difficult that the change of laboratory data in acute rejection is not specific (Azer et al. 1994) and many kind of complication with liver dysfunction can occur frequently after liver transplantation. From the begining acute rejection after transplantation was major problem. It has been the main complication especially after operative techniques advanced and postoperative managements improved (Quiroga et al. 1991). In our institu-

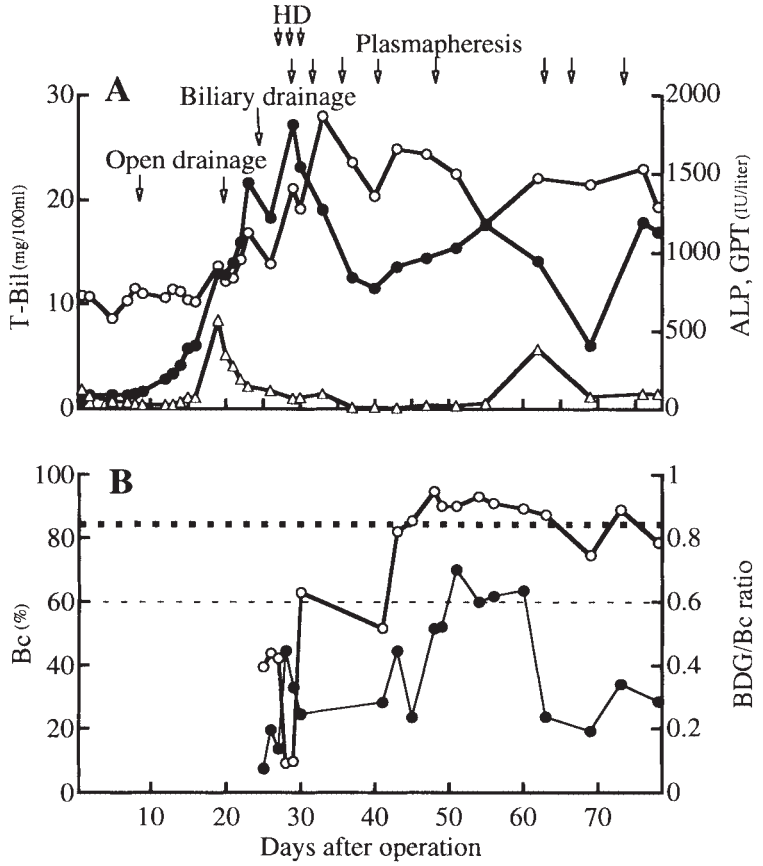

Fig. 3. Case No. 3 with severe cholangitis
(A) Change of laboratory data, ○—○ T-Bil; ●—● ALP; △—△ GPT,
(B) Change of Bc and BDG/Bc ratio, ○—○ Bc; ●—● BDG/Bc; ---85% on Bc; ·····0.6 on BDG/Bc
HD, hemodialysis.

tion fifty five percent of patients who undergone liver transplantation had acute rejection.

Histological examination of liver biopsy is the gold standard for diagnosis of acute rejection (Williams et al. 1985; Kemnitz et al. 1989). So majority of acute rejection were diagnosed by pathological findings and treated depend on its degree. We usually performed hepatic biopsy on seventh day after transplantation under ultrasonography and additional biopsy was curried out when rejection was suspected. We never have had critical complication after hepatic biopsy, but biliary bleeding or elevation of transaminase of the liver sometimes occurred. Actually the hepatic biopsy is a invasive examination (Bubak et al. 1991). So a reliable and non-invasive examination instead of the hepatid biopsy is hoped to be established.

Several makers in acute rejection have been reported (Avolio et al. 1992; Bao et al. 1994). But biliary pigments of bile has not been focused on diagnosis of acute rejection. Goresky et al. (1992) observed change in bilirubin pigments in bile by using HPLC after liver transplantation. They considered that the changes in bilirubin fraction of bile parallel the over all changes in liver function.

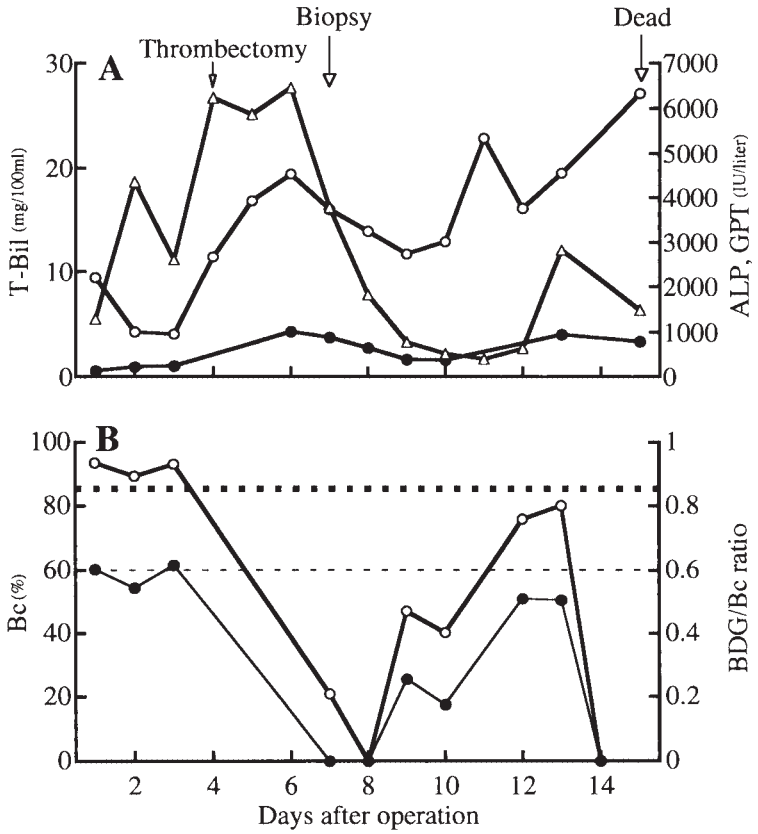

Fig. 4. Case No. 6 with arterial thrombosis
(A) Change of laboratory data, ○—○ T-Bil; ●—● ALP; △—△ GPT,
(B) Change of Bc and BDG/Bc ratio, ○—○ Bc; ●—● BDG/Bc; – – – 85% on Bc; ·····0.6 on BDG/Bc

But they did not discussed in each complications. In that paper the ratio of proportions of bilirubin digucuronide/bilirubin monoglucuronide (BDG/BMG) decreased in initial days after transplantation in many cases. That results probably reflected more ischemic change and severe reperfusion injury than living related liver transplantation. We examined in proportion of BDG, and BMG, unconjugated bilirubin (UCB) and calculated BDG+BMG (Bc) and the ratio of proportion of BDG/Bc. That is reason why these bilirubins are major bile pigments and no particular findings was observed in the change of other pigments after transplantation. In our study, in cases with acute rejection, Bc was above 85% and BDG/Bc ratio was below 0.6 at the time of hepatic biopsy. The presence of the inflammatory infiltration in the portal area or interference with blood flow were likily associate with these changes of bile pigments. Adachi et al. (1991) investigated the changes of bilirubin pigments in bile with occlusion of mesenteric vein in rats. They found that BDG decreased and BMG increased after occlusion accompanied decrease of hepatic uridine diphosphate-glucuronic acid. In cases with constitutional hyperbilirubinemia diminution of BDG and increment of BMG in bile were observed with decrease of hepatic bilirubin uridine diphosphate glucuronyltransferase (BGT) in a dependent manner (Yamashita

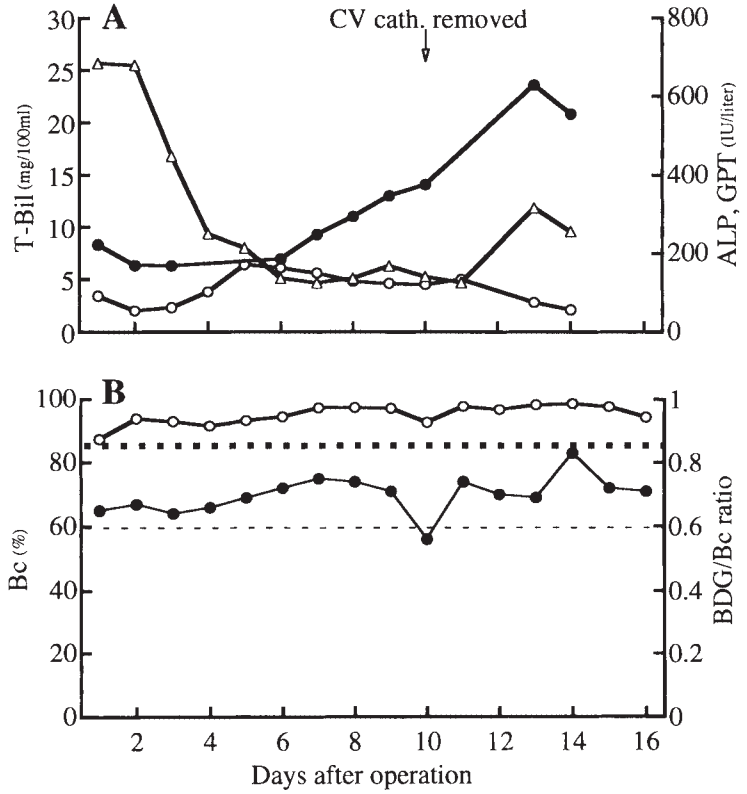

Fig. 5. Case No. 8 without hepatic complications
(A) Change of laboratory data, ○—○ T-Bil; ●—● ALP; △—△ GPT,
(B) Change of Bc and BDG/Bc ratio, ○—○ Bc; ●—● BDG/Bc; – – – 85% on Bc; ·····0.6 on BDG/Bc

1987). BGT activity may also affect decrease of BDG/Bc ratio in bile in acute rejection. It is still unclear whether bile duct injury in acute rejection effect the change of bile pigments.

β-Glucuronidase is considered to deconjugate bilirubin conjugates to unconjugated bilirubin (Saxerholt et al. 1990; Whiting et al. 1993) and this enzyme has been mainly investigated in pigment gallstone (Skar et al. 1989). In our study, in case with cholangitis Bc was below 85% and BDG/Bc ratio was below 0.6, and after cholangiodrainage, Bc and BDG/Bc ratio were increased. Decreases of Bc and BDG/Bc ratio in case with cholangitis mean increases of UCB and BMG, therefore, beta-glucuronidase might affect the changes of fraction of bile in case with cholangitis.

In case with arterial and venous thrombosis Bc was below 85% and BDG/Bc ratio was below 0.6. Conjugation of bilirubin may be disturbed with ischemic injury of the graft.

In case without liver complication Bc was above 85% and BDG/Bc was above 0.6 except one time when he had catheter sepsis. In sepsis some kinds of inteleukin from Kupffer and leukocyte activated by bacteria or endotoxin injury hepatocyte (Funatsu et al. 1994). Hyperbilirubinemia is observed in sepsis. But from this study, conjugation of bilirubin was not disturbed remarkably as

reported (Roelofsen et al. 1994).

Although the number of cases is limited in this study, in each complication the bilirubin fraction demonstrated different patterns after liver transplantation. We concluded that analysis of the biliary bilirubin fraction after liver transplantation could be reliable as a noninvasive index and helpful for the early diagnosis of acute rejection.

References

1) Adachi, Y., Kamisako, T. & Yamamoto, T. (1991) The effects of temporary occlusion of the superior mesenteric vein or splenic vein on biliary bilirubin and bile acid excretion in rats. *J. Lab. Clin. Med.*, **118**, 261-268.
2) Avolio, A.W., Gozzo, M.L., Forni, L., Agnes, S., Colacicco, L., Barbaresi, G., Magalini, S.C. & Castagneto, M. (1992) Mitochondrial/cytoplasmic enzyme ratio for the diagnosis of acute rejection after liver transplantation: Sensitivity and specificity. *Transplant. Proc.*, **24**, 2572-2573.
3) Azer, S.A., McCaughan, G.W. & Stacey, N.H. (1994) Daily determination of individual serum bile acids allows early detection of hepatic allogfaft dysfunction. *Hepatology*, **20**, 1458-1464.
4) Azoulay, D., Castaing, D., Ahchong, K., Adam, R. & Bismuth, H. (1993) A minimally invasive approach to the treatment of stenosis of the portal vein after hepatic transplantation. *Surg. Gynecol. Obstet.*, **176**, 599-601.
5) Bao, Y.M., Adam, R., Samuel, D., Castaing, D. & Bismuth, H. (1994) Influence of high aminotransferase levels on rejection in liver transplantation. *Transplant. Proc.*, **26**, 259-260.
6) Bubak, M.E., Porayko, M.K., Krom, R.A.F. & Wiesner, R.H. (1991) Complications of liver biopsy in liver transplant patients: Increased sepsis associated with choledochojejunostomy. *Hepatology*, **14**, 1063-1065.
7) Cole, K.D. & Little, G.H. (1982) Isocratic high-performance liquid chromatography of bile pigments. *J. Chroniatogr.*, **227**, 503-509.
8) Defranco, J., Trotteur, G. & Dondelinger, R.F. (1993) Duplex ultrasonographic evaluation of liver transplants. *Acta Radiol.*, **34**, 478-481.
9) Doyle, H.R., Marino, I.R., Jabbour, N., Zetti, G., McMichael, J. Mitchell, S., Fung, J. & Starzl, T.E. (1994) Early death or retransplantation in adults after orthotopic liver transplantation. *Transplantation*, **57**, 1028-1036.
10) Funatsu, K., Nishida, J., Itsuji, S., Ueno, M., Arakawa, S., Takagi, S., Ebihara, Y., Mizuno, Y., Orita, M. & Isii, H. (1994) Role of heptic mactrophages and endotoxin in liver injury — Involvement of active oxygen radicals produced by hepatic macrophages in liver injury —. *J. Abdominal Emergency Med.*, **14**, 1007-1016.
11) Goresky, C.A. (1990) High-performance liquid chromatographic separation of bilirubin conjugates: The effects of change in molarity and pH. *J. Chromatogr.*, **528**, 123-141.
12) Goresky, C.A., Gordon, E.R., Sanabria, J.-R., Strasberg, S.M. & Wayne Flye, M. (1992) Changes in bilirubin pigments secreted in bile after liver transplantation. *Hepatology*, **15**, 849-857.
13) Hasegawa, S., Tanaka, K., Egawa, H., Inomata, Y., Murakawa, M.,Terada,Y., Matsui, T., Arai, T., Yamaoka, Y. & Mori, K. (1996) Perioperative respiratory management with fiberoptic bronchoscopy in pediatric living-related liver transplantation. *Surgery*, **119**, 198-201.
14) Kalayoglu, M., D'Alessandro, A.M., Knechtle, S.J., Eckhoff, D. E., Pirsch, J.D., Judd, R., Sollinger, H.W., Hoffmann, R.M. & Belzer, F.O. (1993) Long-term results of liver

transplantation for biliary atresi. *Surgery*, **114**, 711–718.
15) Kemnitz, J., Gubernatis, G., Bunzendahl, H., Ringe, B., Pichlmayr, R. & Georgii, A. (1989) Criteria for the histophathological classification of liver allograft rejection and their clinical relevance. *Transplant. Proc.*, **21**, 2208–2210.
16) Quiroga, J., Colina, I., Demetris, A.J., Starzl, T.E. & Vand Thiel, D.H. (1991) Cause and timing of first allograft failure in orthotopic liver transplantation; A study of 177 consecutive patients. *Hepatology*, **14**, 1054–1062.
17) Roelofsen, H., Van Der Veere, C.N., Ottenhoff, R., Schoemaker, B., Jansen, P.L.M. & Oude Elferink, R.P.J. (1994) Decreased bilirubin transport in the perfused liver of endotoxemic rats. *Gastroenterology*, **107**, 1075–1084.
18) Sanchez-Urdazpal, L., Batts, K.P., Gores G.J., Breanndan Moore, S., Sterioff, S., Wiersner, R.H., & Krom, R.A.F. (1993) Increased bile duct complications in liver transplantation across the ABO barrier. *Ann. Surg.*, **218**, 152–158.
19) Saxerholt, H., Skar, V. & Midtvedt, T. (1990) HPLC sepatation and quantification of bilirubin and its glucuronide conjugates in faeces and intestinal contents of germ-free rats. *Scand. J. Clin. Lab. Invest.*, **50**, 487–496.
20) Skar, V., Skar, A.G., Bratlie, J. & Osnes, M. (1989) Beta-glucuronidase actibity in the bile of gallstone patients both with and without duodenal diverticula. *Scand. J. Gastroenterol.*, **24**, 205–212.
21) Tono, T., Monden, M., Yoshizaki, K., Valdivia, L.A., Nakano, Y., Gotoh, M., Ohzato, H., Doki, Y., Ogata, A., Kishimoto, T. & Mori, T. (1992) Biliary interleukin 6 levels as indicators of hepatic allograft rejection in rats. *Transplantation*, **53**, 1195–1201.
22) Whiting, J.F., Narciso, J.P., Chapman, V., Ransil, B.J., Swank, R.T. & Gollan J.L. (1993) Deconjugation of bilirubin-IXα glucuronides: A physiologic role of hepatic microsomal β-glucuronidase. *J. Biol. Chem.*, **268**, 23197–23201.
23) Williams, J.W., Peters, T.G., Vera, S.R., Britt, L.G., Van Voorst, S.J. & Haggitt, R.C. (1985) Biopsy-directed immunosuppression following hepatic transplantation in man. *Transplantation*, **39**, 589–596.
24) Wu, T.-W., Levy, G.A., Yiu, S., Au, J.-X., Greig, P.D., Strsberg, S.M., Ettles, M., Abecassis, M., Superina, R.A., Langer, B., Blendis, L.M., Phillips, M.J. & Taylor, B.R. (1990) Delta and conjugated bilirubin as complementary markers of early rejection in liver-transplant recipients. *Clin. Chem.*, **36**, 9–14.
25) Yamashita, M. (1987) Biliary bilirubin fractionation in hepato-biliary diseases. *Med. J. Kinki Univ.*, **12**, 141–154.
26) Yamashita, M., Adachi, Y. & Yamamoto, T. (1986) Analysis of bilirubin conjugates in human bile by column liquid chromatofraphy. *J. Chromatogr.*, **375**, 386–391.