Alkaline Phosphatase Isoforms in Serum After Liver Allograft Surgery

Julie Randolph-Habecker, 1,3 John A. Lott, 1 and Raymond J. Testi 2

Orthotopic liver transplantation (OLT) is now the only available treatment for end-stage liver disease; the major postoperative complications of OLT are rejection and infection. Fractionation of alkaline phosphatase (ALP) isoforms in serum by isoelectric focusing can be used to identify patients with complications. Reference ranges for liver-function tests (LFT) and liver ALP isoforms were established for post-OLT patients with stable postoperative courses and compared with those of patients with complications. We found canalicular, hepatocyte, and high-molecular-mass ALP to be statistically higher in nearly all patients with complications as compared with patients who had a stable postoperative course; these tests may identify patients requiring a liver biopsy. When used in conjunction with LFT and other clinical findings, ALP isoforms could aid in the monitoring of complications and treatment and in the adjustment of immunosuppressive therapy in stable OLT cases.

Indexing Terms: liver transplantation/isoelectric focusing/transportation rejection/liver enzymes

There are no artificial support systems to replace liver function in patients with end-stage liver disease; the only alternative today is liver transplantation. Rejection and infections after liver transplantation are common. Biliary tract complications usually occur in the first few weeks after surgery and account for 13–30% of complications after orthotopic liver transplantation (OLT) (1). At least 70% of patients receiving OLT have one or more episodes of rejection (2-7). Infections, especially with cytomegalovirus (CMV), are other important post-OLT complications (8).

Transplant pathologies are commonly diagnosed from liver-function tests (LFT) and biopsies of the liver. Percutaneous liver biopsy is the “gold standard” for diagnosing rejection; the biopsy has a reported specificity of 69% and a sensitivity of 95% (9). The histological definition of rejection includes an inflammatory infiltration of the portal tracts, expansion of the portal tract, and injury of interlobular bile ducts (for a more detailed description of the histology of rejection, see reference 10). Liver biopsy has limitations, including sampling errors from collecting tissue from nonrepresentative areas, the inability to distinguish among transplant complications, inconsistencies among readers, the need for a skilled interpreter, and complications from the procedure itself. A blood test in lieu of a liver biopsy is highly desirable.

Standard LFT have a low specificity and sensitivity when one is diagnosing pathologies of OLT, but they are useful as screening tools. The tissue source of alkaline phosphatase (ALP; EC 3.1.3.1) in serum can be determined by separating and identifying its isoenzymes and isoforms. Isoelectric focusing (IEF) of normal human ALP in serum produces 12 or more bands. The identification, standardization, and clinical significance of these bands have been established from the examination of the band patterns of isoenzymes from normal individuals, pregnant women, and patients with various hepatic, bone, lung, renal, intestinal, and hematopoietic diseases; tissue extraction studies have also been performed (11–13). IEF has many advantages over polyacrylamide gel electrophoresis (PAGE). Although PAGE is technically simpler (14), IEF produces sharper, well-resolved bands, separates the liver from the bone fractions without extra treatments, permits the application of much larger volumes of serum, allows multiple assays in the same gel, and permits densitometric quantification (15).

Our aims were to quantify the two most anodally migrating ALP bands observed after IEF, i.e., those bands specific for biliary canaliculi (band 1), for hepatocytes (band 2), and the high-molecular-mass (HMM) ALP fraction (Fig. 1). Patients with liver disease (cholestasis or biliary obstruction (16)) may exhibit HMM ALP that appears as a nonmigrating smear over the application area in IEF (17). DeBroe et al. (18) offered biochemical, histochemical, morphological, and immunological evidence that the HMM fraction is ALP complexed with liver-cell membranes; this fraction is not found in subjects with normal liver function. An increase in bands 1 and 2 and the presence of HMM ALP may signal the onset of hepatic dysfunction owing to CMV infection, organ rejection, and biliary tract complications; a decrease could indicate the recovery from such episodes. By following the postoperative course of patients receiving liver transplants, we sought a correlation between these ALP fractions and transplant complications.

Materials and Methods

Patients and Diagnosis

We included in the study 65 patients, 33 males and 32 females, ages 14–65 years, who received liver allografts

Departments of 1 Pathology and 2 Surgery, The Ohio State University, Columbus, OH 43210. 3 Address correspondence to this author at: M-200 Starling Loving Hall, 320 West 10th Ave., Columbus OH 43210-1240. Fax 614-292-7072.

4 Nonstandard abbreviations: OLT, orthotopic liver transplantation; CMV, cytomegalovirus; LFT, liver-function tests; ALP, alkaline phosphatase; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; HMM, high molecular mass; OSU, Ohio State University; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, ?-glutamyltransferase; TBIL, total bilirubin; and DBIL, direct bilirubin.

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between March 1991 and September 1992 at The Ohio State University (OSU) Hospitals. We restricted the group to those receiving follow-up care at OSU. The test data cover 1-4782 days post-OLT and include the pre-OLT diagnosis, date of transplantation, age, gender, biopsy data, and diagnosis of any post-OLT complications. To maintain the consistency of the biopsy diagnoses, the same pathologist reviewed all of the OLT liver biopsies. Transplant rejection was diagnosed from histological examination of the liver, on the basis of standard criteria (10). CMV infection was diagnosed by immunohistochemistry of liver tissue (19), and cholangitis was diagnosed by the histological examination of liver (20).

Ten (15%) of the patients died, and 47 (72%) had a complicated post-OLT course. Table 1 summarizes the data according to the patient's pre-OLT diagnosis, post-OLT course, and gender.

**Laboratory Tests**

Serum specimens, left over from routine testing of in- and outpatients, were obtained from the OSU Hospital Clinical Chemistry Laboratory. LFT and IEF were performed immediately to minimize the possibility of enzyme loss. LFT on serum included ALP, aspartate aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2), γ-glutamyltransferase (GGT, EC 2.3.2.2), total bilirubin (TBIL), and direct bilirubin (DBIL); all these tests were performed on a Kodak Ektachem 700XR Analyzer (Eastman Kodak, Rochester NY).

**IEF of ALP Isoforms**

The Resolve®-ALP 90-test kit, the Resolve Omega electrophoresis unit, and a 2000-V power supply, all from Isolab (Akron, OH), were used throughout according to the manufacturer's directions with the exceptions noted below. The 1% agarose gels were 0.85 mm thick and contained 25 mL/L amphotelytes that established a pH gradient of 3–10 during electrophoresis. The application template was placed 1 cm toward the cathodal side from the center of the gel. If any HMM ALP was present in the sample, this template placement ensured that the canalicular and hepatocyte bands were not obscured by the nonmigrating HMM amudge that remained at the point of application. When HMM ALP was present, bands 6–10 were obscured. We applied 15 μL of serum to each well and allowed it to soak into the gel. The electrophoresis unit was cooled to 18°C during electrophoresis. The voltage limit was set to 1100 V, and the gel was electrophoresed at 15 W for 95 min.

The ALP isoforms were visualized by incubating the gel for four 5-min intervals with the kit substrate, α-naphthyl phosphate. After the incubation, the coupling agent, 4-aminodiphenylamine diazonium sulfate in a pH 10.5 buffer, was applied. With this treatment, a brownish-yellow insoluble azo compound forms, and the stain intensity is proportional to the ALP activity. The gels were then subjected to two 10-min washes in 250 mmol/L acetic acid and then two 10-min washes in distilled water. The gels were allowed to dry overnight at room temperature or in a gel drier at 50°C. Densitometric analysis was performed with the Pharmacia Gel-Scan® Densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ) to give quantitative values for the canalicular and hepatocyte ALP fractions and approximate values for the HMM ALP. Because the HMM smudge is superimposed over bands 6–10, only a semiquantitative estimate of this ALP is possible. We chose not to use corrected values for HMM ALP nor to treat the samples containing HMM with an agent to separate the ALP from the membrane fragments. Phospholipase C can be used to convert HMM ALP to the most acidic fractions (the canalicular band) (17); however, we chose not to do this because there is important information in a semiquantitative assessment of HMM ALP. The mere appearance of HMM ALP indicates liver pathology in

**Table 1. Patients' diagnoses and outcomes.**

<table>
<thead>
<tr>
<th>Pretransplant diagnosis</th>
<th>Total</th>
<th>No. of patients with complications</th>
<th>Deaths</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic cirrhosis</td>
<td>11</td>
<td>8</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
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<tr>
<td>Hepatitis C</td>
<td>9</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Non-A, non-B hepatitis</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fibrolamellar carcinoma</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>12</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Sclerosing cholangitis</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Wilson disease</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Cystic fibrosis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>α1-Antitrypsin deficiency cirrhosis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Fulminant hepatic failure</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>47</td>
<td>10</td>
<td>33</td>
<td>32</td>
</tr>
</tbody>
</table>

*Complications of transplantation include rejection, CMV infection, and biliary tract complications that occurred during the patients' post-OLT course.
OLT. Converting HMM to bands 1A and 1B (Fig. 1) and interpreting the amount of HMM ALP present from these data is problematic. In studies with Triton X-100, we observed bands more acidic than band 1 and additional fractions between bands 1 and 2 that were very different from the other ALP bands. Triton X-100 treatment also reduced the total ALP activity by ~30%; hence, we recommend against using Triton X-100.

Two pooled serum controls, one containing serum from normal females and one from normal males, were applied to each gel to estimate the between-run variability; each pool was assayed 12 times. For bands 1 and 2 from the pooled female serum we found means (SD) of 11.4 (3.5) U/L and 18.3 (4.5) U/L, respectively; for bands 1 and 2 for the pooled male serum, the means were 6.5 (2.2) U/L and 14.7 (4.3) U/L, respectively.

Results and Discussion

Post-OLT Analyte Ranges for Stable Patients

Ranges were determined for ALP bands 1 and 2 and HMM ALP as well as total ALP, GGT, AST, TBil, and DBil in stable post-OLT patients defined as those having no complication, i.e., free of biopsy-diagnosed rejection, CMV infection, and biliary tract complications. The test values from these complication-free periods were organized according to three post-OLT time intervals: 60–180 days, 181–365 days, and >365 days. The analyte ranges of the stable patients along with reference values from controls are shown in Table 2.

Routine Laboratory Data in Stable Post-OLT Patients

The majority (67% at 60–180 days, 67% at 181–365 days, and 74% at >365 days) of the post-OLT total ALP values were within the normal range of 36–126 U/L. Also, for these OLT cases, the mean ALP decreased during the 365-day period. The mean GGT values from the three post-OLT time groups were always higher than the normal range. There was a consistent increase of the mean AST beginning during the 60–180-day period through >365 days. Of the TBil values, 67–94% were within the normal range of 3.0–22 μmol/L. The mean post-OLT TBIL values decreased from 60–180 days to >365 days. Of the DBIL values, 50–88% were within the normal range of <5.0 μmol/L.

ALP Isoforms in Stable Post-OLT Patients

The activities of ALP bands 1 and 2 and HMM ALP at 60–180 days and 181–365 days post-OLT are shown in Fig. 2. For all the patients, 83% of the values for the canicular ALP isoform (band 1) were greater than the normal range of <4.5 U/L, but the mean decreased with time after OLT. Of the values for the hepatocyte ALP isoform (band 2), 44%–100% were within the normal range of <30 U/L. The mean values fluctuated but generally decreased slightly with time after OLT.

The majority of the HMM values for the stable post-OLT patients were either undetectable or present in trace amounts. None of the patients from the 181–365-day group showed any HMM ALP; 61% of the values from the 60–180-day group and 32% of the >365-day group were greater than normal for the HMM ALP.

Data from Patients with Complications of OLT

There were 27 episodes of post-OLT complications available for analysis, and 21 of the 27 complications occurred within 60–180 days post-OLT. There were no instances of complications after 365 days; these typically occurred within the first 4 months after OLT (21–24).

Statistical differences for the various tests between the patients with a stable outcome and those with complications are shown in Table 3. In 35% of the patients, the increases in the ALP fractions occurred as early as 3–10 days before the diagnosis of a post-OLT complication. During other episodes, the increases were present at or after the diagnosis of complications. Several patients exhibited both increased LFT and ALP isoforms, but there was no coincident biopsy or associated diagnosis; thus, post-OLT complications may have been missed.

Rejection group. All patients in this group had rejection episodes at 60–180 and 181–365 days post-OLT (Fig. 2 and Table 3). The proportions of patients having abnormal routine LFT and ALP isoforms are shown in Table 2. Reference ranges (and means) from serum of controls and stable post-OLT patients.

<table>
<thead>
<tr>
<th>Time post-OLT</th>
<th>ALP, U/L</th>
<th>GGT, U/L</th>
<th>AST, U/L</th>
<th>TBil, μmol/L</th>
<th>DBil, μmol/L</th>
<th>Band 1, U/L</th>
<th>Band 2, U/L</th>
<th>HMM, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>60–180 days</td>
<td>36–126°</td>
<td>70–265 (135.3); 8/18°</td>
<td>23–951 (197); 9/17</td>
<td>14–81 (34.9); 5/18</td>
<td>6.8–32.5 (13.6); 2/7</td>
<td>0–12.0 (4.9); 7/17</td>
<td>4–38 (13.0); 15/18</td>
<td>0–79 (18.5); 11/18</td>
</tr>
<tr>
<td>181–365 days</td>
<td>75–198 (114.3); 2/6</td>
<td>25–176 (101.3); 4/6</td>
<td>25–84 (48.5); 3/6</td>
<td>5.1–30.8 (14.8); 2/6</td>
<td>3.4–20.5 (8.8); 3/6</td>
<td>0–8.6 (3.1); 2/17</td>
<td>3–15 (9.0); 5/6</td>
<td>0 (0); 0/6</td>
</tr>
<tr>
<td>&gt;1 year</td>
<td>50–203 (105.5); 5/19</td>
<td>20–460 (125.2); 8/17</td>
<td>13–292 (56.4); 4/19</td>
<td>5.1–23.9 (11.6); 1/17</td>
<td>0–8.6 (3.1); 2/17</td>
<td>2–27 (9.1); 16/19</td>
<td>6–80 (27.4); 7/19</td>
<td>0–50 (11.3); 6/19</td>
</tr>
</tbody>
</table>

* Persons with no transplants or pathologies.
° Reference range (mean); and no. of patients with values greater than the reference range for normals all patients in group.
* Values obtained from Eastman Kodak, Rochester, NY.
* ALP isoforms.
* Values calculated from reference ranges established by Griffiths and Black (11).
Table 3. ALP and GGT were increased in nearly all patients (P <0.01 and P <0.05, respectively, vs stable patients), and band 1 was increased in all (P <0.01 vs stable patients). These data suggest that rejection involves the biliary canaliculi, a fairly consistent finding in biopsies. The magnitudes of the test results for rejec-

**Fig. 2.** Post-OLT distribution of canalicular ALP (panels A and D), hepatocyte ALP (panels B and E), and HMM ALP (panels C and F) fractions in serum determined by IEF in stable patients and those with complications at 60–180 days (panels A–C) and 185–365 days (panels D–F) post-OLT. Values on the x-axis were chosen to expand the results in the lower ranges. Groups A and B, stable patients; groups C and D, patients with rejection; group E, patients with rejection and CMV infection; group F, patients with cholangitis; and group G, patients with other biliary complications.
tion episodes occurring at 60–180 days and 181–365 days post-OLT are not the same (Table 3). The mean values for the later time are all higher, suggesting that late rejection is a more severe process.

Rejection and CMV. Compared with the stable group, these patients had the highest mean values and statistically significant differences (Table 3) for all tests, suggesting that they had the most serious biochemical derangements, a finding consistent with the clinical picture. These individuals tended to be much sicker. The high activity and concentrations of HMM ALP in these cases suggest a more severe cholestatic process than in the other groups.

Cholangitis and biliary complications. On the basis of the data in Table 3, these patients are indistinguishable from those with a diagnosis of rejection. Bands 1 and 2 show good sensitivity (P < 0.001 vs the stable group), suggesting both canalicul and hepatocyte involvement in the disease process.

When the data from all the patients are considered, the ALP isoforms and HMM ALP can indicate a post-OLT complication, but there does not appear to be a “typical” ALP isoform profile for a given type of post-OLT complication. The ALP fractions increased before the LFT in a number of cases, which could lead to an earlier liver biopsy, diagnosis, and treatment. These fractions also decreased after successful treatment and remained high in cases with unsuccessful treatment. When used in conjunction with LFT and other clinical symptoms, ALP isoforms could aid the practitioner in monitoring complications and treatment. This technique may also find a use in the adjustment of immunosuppressive therapy in stable post-OLT patients.

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