930538 Rev. 4

Mercer

CASE REPORT

CHANGES IN BILIARY (HIGH-MOLECULAR-MASS) AND LIVER ISOFORMS OF ALKALINE PHOSPHATASE AFTER BABOON-TO-HUMAN LIVER TRANSPLANTATION

Donald Mercer (1)*, Mary Tang (1) Ignazio Marino(2), Anthony Demetris (2), John Fung (2), Thomas Starzl (2) and Vijay Warty (1,2).

Donald W. Mercer, Ph.D. Office (412) 359-3225 Fax (412) 359-3598

1. Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pa 15213
2. Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pa 15213

* Current Address: Department of Laboratory Medicine, Allegheny General Hospital
320 East North Av., Pittsburgh, Pa 15212

ABSTRACT

We report a case of hyperphosphatasemia in a 35-year old patient with hepatitis B who underwent an orthotopic xenograft liver transplant. Marked elevations in total alkaline phosphatase [ALP: orthophosphoric monoester phosphohydrolase (alkaline optimum); EC: 3.1.1.1] activity beginning five days post-transplantation (six *times human normal*) and increasing to approximately seventeen *times normal* at day 11 was observed. Elevated ALP persisted for more than 40 days then steadily increased up to seventy-five *times normal* in the last 30 days. Gel electrophoresis detected both liver (LALP) and biliary (high-molecular mass, BALP) isoforms. LALP measured with ion-exchange columns revealed an activity time course pattern similar to that of total ALP. Results for BALP also obtained with use of ion-exchange columns exhibited varying amounts of activity ranging from two to four hundred and twenty-eight *times normal.*

Key Phrases

Alkaline Phosphatase Isoforms

Biliary Alkaline Phosphatase Isoform

Liver Xeno-Transplantation

Ion-Exchange Column Chromatography

INTRODUCTION

Advances in surgical technique, organ preservation and immunosuppression have made orthotopic liver transplantation an accepted form of therapy for patients with several acute and chronic end-state diseases (1-5). Recently, surgeons at the University of Pittsburgh Medical Center performed a cross-species history-making transplantation (xenotransplant) of a baboon liver into a 35 year old man who survived 70 days after the procedure *(6)*.

In xenotransplants, as well as allotransplants, clinicians rely heavily on biochemical assays to assess the patient's status. A variety of serum markers such as hepatic enzymes (7), apolipoproteins (8), total bilirubin, delta and conjugated bilirubin (9), and ketone body ratios {acetoacetic acid (AA) to beta-hydroxybutryic acid (HBA)} (10) have been proposed to monitor liver grafts. However, no markers so far have not been sufficiently specific and sensitive to adequately monitor allograft function (11). Their value is xenotransplants has not been defined.

In this case report we describe biochemical changes seen in a patient (6) who underwent baboon-to-human liver transplantation. We also describe the use of a mini-column ion-exchange separation technique for the isolation and quantitative measurement of the biliary (high-molecular-mass, BALP) isoform and liver alkaline phosphatase (LALP) isoform..

PATIENT

A 35 year old man with B virus-associated chronic active hepatitis and human immuno-deficiency virus infection was referred to our hospital due to a deteriorating condition relating to liver failure. Hepatitis B is considered by members of the transplantation community to be relative contraindication for human liver transplantation because of the great likelihood the disease will recur in transplanted liver. Xenotransplantation was performed with hopes that the baboon liver would be resistant to hepatitis B infection (6).

Liver replacement was performed using the liver from a 15 year old male baboon (*Papio Cynocephalus*) using a "piggy-back" surgical technique (6). The immunosuppression regimen included FK 506, methyl-prednisolone, prostaglandin, as well as daily non-myelotoxic doses of cyclophosphamide. The early post-operative course was uneventful. The patient was able to eat and ambulate by post-operative day five. The baboon liver (600 grams) rapidly regenerated to the size of a human liver (1,555 grams) by day 24. Products of hepatic synthesis, including clotting factors, quickly became those of baboon origin with no obvious adverse effects. The remainder of his clinical course was complicated by short episodes of bacteremia, viremia, and candidal and cytomegalovirus esophagitis. Liver biopsies obtained on post-operative days 12, 24, 55 and 65 revealed increasing cholestasis and only minimal to mild acute cellular or humoral rejection.

Biochemical monitoring was remarkable only for ALP which began to rise five days post-transplant and plateaued at levels ten to twenty *times normal* from post-operative day 10 through 40. On post-operative day 40, ALP began to rise again but the patient continued to do well. On post-operative day 61, the patient underwent percutaneous cholangiogram to

investigate the rise in ALP. Shortly after the cholangiogram which was read as normal, the patient became septic. This was followed by an abrupt deterioration of liver function and increases in hepatic enzymes.

He remained in critical condition until his death on post-operative day 70 following subarachnoid hemorrhage associated with disseminated angioinvasive fungal infection. At autopsy biliary sludge, composed of precipitated bile and cellular debris, was found to occupy the entire intrahepatic biliary tree. The biliary mucosa was focally necrotic and multiple bile infarcts were present throughout the liver.The choledochojejunostomy was intact and patent. There was no histopathologic evidence of rejection at necropsy.

MATERIALS AND METHODS

Samples: Serum samples from the patient were obtained daily after surgery. All samples were stored at -70 C. Day 0 was defined as the day of surgery. A single blood sample (Red Top) was taken at the same hour of the day for 70 days. Conventional biochemical markers for liver function (AST, ALT, GGT, Bilirubin, Total ALP) were assayed using a Kodak Ektachem Analyzer 700 (Eastman Kodak Co., Rochester, NY 14654). Ketone Body AA/HBA ratios were determined with arterial samples using the Ketorex Kit (Sanwa Chemical Company) Nagoya, Japan) and a KETO-340 semi-automatic spectrophotometer (Iharadenshi Company, Kasugai, Japan).

Electrophoresis: ALP isoform electrophoresis was performed utilizing the AlkPhor poly-acrylamide stacking gel (30 g/L) and running gel (70 g/L) combination system (Quantimetrix, Hawthorne, CA 90250-3115). Isoforms were identified on the basis of their electrophoretic mobility. Samples (25 uL) were applied to the top of the polymerized stacking gel and electrophoresis was conducted for 80 minutes at 3 mA per tube with the Tris-boric acid buffer system and the use of an analytical vertical-gel apparatus (Canalco, Rockville, MD. 20852). Gels were removed from the glass tubes and stained for about 30 minutes at 37 C with the ALP substrate, 5-bromo-4-chloro-3-indoylphosphate p-toluidine salt. After completion of staining which resulted in the development of blue bands, the staining solution was drained off and the tubes were filled with 5% acetic acid to stop the reaction.

Anion-Exchange (DEAD Sepharose) Chromatographic Separation of Alkaline Phosphatase Isoforms (Liver and Biliary): ALP isoforms were isolated by a procedure recently described for separation of mucin type glycoproteins (12). We applied 25 uL of serum to the top of a 7 mm X 45 mm column (Instrumentation Industries, Pittsburgh, Pa 15102) filled to a height of 6 mm with DEAE Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ 08854), and collected the sample effluent in the first fraction (Figure 1). Subsequent fractions were collected after stepwise elution with Tris HCl (5 mmol/L) containing, successively 100 and 150 mmol of sodium chloride (pH 8.0) at 25 C. We collected one fraction of the 100 mmol/L sodium chloride buffer (2500 uL, Fraction 1) two fractions of the 150 mmol/L sodium chloride buffer (2000 uL, Fraction 2 and 4000 uL, Fraction 3) and one fraction of the 300 mmol/L sodium chloride buffer (2000 uL, Fraction 4). Excellent recoveries of total alkaline phosphatase activity from the column (Fraction 1 to 4) were obtained; analytical recoveries averaged 98% (n=16). ALP isoforms in the column eluates were identified by electrophoresis on poly-acrylamide gel (Figure 2). No carry-over of LALP into BALP (Fraction 4) or BALP into Fraction 1 (LALP) was detected following electrophoretic analysis of column fractions 1 and 4.

Measurement of Liver and Biliary ALP Isoform Activity: Liver isoform (about 95%) was eluted from the DEAE Sepharose column in Fraction 1 and biliary isoform (100%) was eluted in the high salt Fraction 4 in a manner similar to several column procedures described previously (13,14). Alkaline phosphatase activity was determined by a kinetic procedure (Beckman Instruments Inc., Carlsbad, CA 92008-4836) with p-nitrophenyl phosphate as substrate. A Cobas Fara automatic spectrophotometer (Roche Diagnostics Systems, Montclair, NJ) set at 405 mn was utilized to quantitate the concentration of isoforms in column fractions. The between-run CV's for BALP and LALP in a control sera were 10.3% (mean 98 U/L, n=10) and 5.7% (mean 597 U/L, n=10) respectively. The within-run CV's in another control sera for BALP and LALP were 4.7% (mean 338 U/L, n=12), and 5.8% (mean 4781 U/L, n=12, respectively.

RESULTS

After transplantation the concentrations of most analytes were generally within the reference range for humans (6). Special attention was given to liver function tests and other tests such as albumin, uric acid, cholesterol and total ALP whose normal ranges have been previously shown to be markedly divergent in the baboon and human (15). In general albumin averaged 25% lower than normal human, uric acid < 30 umol/L, cholesterol averaged 50% lower than normal and total ALP averaged 10 to 20 times higher than normal. At day 45 post-operative the results were as follows: Albumin, 1.9 g/L (human normal 3.5-5.0 g/L), uric acid less than 30 umol/L (human normal, 150-410 umol/L), cholesterol 1.71 mmol/L (human normal, 3.9-6.5 mmol/L) and total ALP, 2812 U/L (human normal, up to 125 U/L.

Table 1 summarizes results of conventional liver function tests (AST, ALT, GGT, Total ALP, and bilirubin) obtained on six days (day 1,5,11,20,40 and 60) during the xenograft's time course of 70 days. Abnormal and increasing total ALP was observed at day 5 (six *times normal*), day 11 (seventeen *times normal*) and day 60 (seventy-one *times normal*). However, in contrast abnormal but decreasing concentrations were demonstrated for AST, ALT and GGT during the immediate post-operative period followed by normal amounts throughout most of the post-operative course until day 40 when slightly increasing concentrations were observed. Bilirubin exhibited a similar pattern of abnormal but

decreasing concentrations except for a slight rise in concentration (up to 4.0 mg/dL) at day 25 with return to a decreasing concentration pattern from day 35 to 55. Time course plots for the entire time period (day 1 to 70) are shown in Figures 3A,3B,4A,4B and 4C.

Arterial ketone body ratios (AA/HBA) were monitored for the first 30 days and results for days 1, 5 and 11 are shown in Table 1. The ratio rose from 0.45 at day 1 (poor prognosis) to 1.5 at day 5 and values near 1.0 (good prognosis) were observed subsequently. Previously, arterial blood ketone body ratios have been shown to reflect NAD/NADH concentrations within the hepatic mitochondria and these ratios appeared to provide reliable prognostic information with regard to early functional capacity of liver allografts (10)

Gel electrophoresis of samples obtained every fourth day revealed only liver and biliary ALP isoforms with no detectable bone or intestinal isoform activity. Initially the LALP isoform band was single and sharp (up to day 5) but soon changed to multi-diffuse bands with slightly increased migration rates similar to ALP isoform bands observed in donor baboon sera. Representative sketches of gel electrophoretic patterns from a healthy individual, a donor baboon and xenograft patient at day 5 are shown in Figure 5 along with a gel photograph of the xenograft patient at day 15.

LALP quantitatively measured by anion-exchange column chromatography (Fraction 1) revealed a time course activity pattern similar to total ALP (Figure 6A). Results for BALP also obtained with the use of the same anion-exchange column (Fraction 4) demonstrated unique activity patterns (Figure 6B) with significantly greater *times normal* elevations as compared to LALP (Figure 7A and 7B).

DISCUSSION

Although orthotopic liver transplantation is now a well-established surgical procedure, complications still occur and early detection of these complications can be difficult. To assist in the detection of early complications conventional liver markers such as bilirubin, total ALP, GGT and transaminase (AST and ALT) are currently in widespread use. Unfortunately, these markers lack optimal sensitivity and specificity.

Here, we report changes in ALP isoforms following baboon to human liver transplantation. During the first 40 days, unexplainable troughs and peaks of elevated BALP isoform (Range, 2 to 90 *times normal* activity) were shown to precede the elevation of AST, ALT and GGT by 55 days and bilirubin by 16 days. Although bilirubin concentrations were abnormal during the first 20 days, they were decreasing in a manner typical of post-liver transplant patients. Thus, bilirubin concentrations during this time course were not considered alarming until the observed rise in bilirubin at day 20.

Marked increases in total ALP and LALP isoform activities occurred shortly after BALP (2 days) with considerably less maximum *times normal* activity levels of 25 and 20 for Total ALP and LALP, respectively. Evidence that our xenograft patient was indeed experiencing a biliary disorder during this early post-operative time period was shown by biopsy at day 12 which revealed mild biliary stasis and no evidence of rejection. The several peaks and troughs of BALP activity observed here are, however not fully understood at this time.

Yeh et al (14) have recently found serum BALP to be a specific marker in a variety of liver disorders. An ion-exchange column BALP procedure similar to the one described here was utlized and results exhibited

abnormal activity in three liver disorders; primary hepatocellular carcinoma, secondary metastatic liver tumors and obstructive jaundice. BALP was absent in healthy carriers of hepatitis B virus and in patients with non-hepatobiliary diseases. Although BALP activities were detectable in some cases of liver cirrhosis and chronic hepatits, these values were generally low.

Yeh's group (14) also observed bilirubin insensitivity to biliary stasis and they speculate that only limited amounts of viable biliary treee are necessary to clear bilirubin efficiently, thus making it a poor test for early-stage biliary complications. This situation appears to have occurred in the xenograft patient i.e., unremarkable bilirubin concentration as well as hepatic enzymes AST, ALT and GGT despite biopsy-detectable biliary stasis during the early time course and widespread biliary sludge in the biliary tract as detected at autopsy (6).

Lott (16) using isoelectric focusing techniques to separate ALP isoforms, has also recently noted the clinical value of ALP isoforms as early indicators of complications in liver transplantation. Interestingly, Lott has suggested that abnormal ALP isoforms such as BALP actually precede biopsy-detectable rejection or biliary tree complications.

We suggest that BLAP release into the circulation is related to the detergent action of concentrated bile near the cell surface as previously reported in patients with cholestasis of the liver (17). A more extensive study to evaluate the clinical utility of BALP isoform as an early indicator of rejection, infection or biliary tree related complications in patients undergoing human othotopic liver transplantation is now being planned.

REFERENCES

1. Starzl TE, Iwatsuki S, Van Thiel DH, Gartner JC, Zitelli BJ, Malatack JJ, et al. Evolution of liver transplantation. Hepatology 1982; 2:614-36.
2. Sumimoto R, Kamada N, Jamieson NV, Fukude Y, Dohi K. A comparison of a solution combining histidine and lactobioate with UW solution and eurocollins for rat liver preservation. Transplantation 1991; 51:589-93.
3. Todo S, Fung JJ, Tzakis A, Demetris AJ, Jain M, Alessiani S, et al. One hundred ten consecutive primary orthotopic liver transplants under FK 506 in audlts. Transplant Proc 1991; 23:1397-1402.
4. Gordon RD, Todo S, Tzakis AG, Fung JJ, Stieber SM, Staschak S, et al. Liver transplantation under cyclosporine: A decade of experience. Transplant Proc 1991; 23:1393-96.
5. Demetris AJ, Fung JJ, Todo S, McCauley J, Jain A, Takaya S, et al. Conversion of liver allograft recipients from cyclosporine to FK 506 immunosuppressive therapy-A clinicopathologic study of 96 patients. Transplantation 1992;53:1056-62.
6. Starzl TE, Fung JJ, Tzakis A, Todo S, Demetris AJ, Marino IR, et al. Baboon-to-human liver transplantation. Lancet 1993;341:65-71.
7. Fath JJ, Ascher MD, Konstantinides BS, Bloomer J, Sharp H, Najanian JS, et al. Metabolish during hepatic transplantation: Indicator of allograft function. Surgery 1984;96:664-74.

8. Malmendier CL, Lonti JF, Mathe D, Adam R, Bismuth H. Lipid and apolipro-protein changes after orthotopic liver transplantation for end-stage liver diseases. Clin Chem Acta 1992;209:169-77.

9. Wu TW, Levy GA, Yie S, Au PD, Greig SM, Strasberg M, et al. Delta and conjugated bilirubin as complementary markers of early rejection in liver transplant recipients. Clin Chem 1990;36:9-14.

10 Taki Y, Gubernatis G, Yamaoka Y, Oellevick M, Yamamoto Y, Ringe B, et al. Significance of arterial ketone body ratio measurement in human liver transplantation. Transplantation 1990;49:535-39.

11. Sankary HN, Williams JW, Foster PF. Can serum liver function tests differentiate rejection from other causes of liver dysfunction after hepatic transplantation. Transplantation Proced 1988;20:669-70.

12. Mercer DW, Jacobs SA, Herberman RB. Detection of tumor associated mucin CA M26 isoform in sera of patients with breast cancer. J of Tumor Oncology 1993;8:43-54.

13. Mercer DW, Talamo TS. Multiple markers of malignancy in sera of patients with colorectal carcinoma: Preliminary clinical studies. Clin Chem 1985;31:1824-28.

14. Yeh C-T, Wei JS, Liaw Y-F. Biliary alkaline phosphatase measured by mini-column chromatography on DEAE-cellulose: Application to detection of hepatobiliary disease. Clin Chem 1989;35:1684-87.

15. Phillips CF, Nicolalde AA, Wolfson SK. Biochemical values in the normal and hypothermic baboon *Papio cynocephalus/anubis.* J. Med. Primatol. 1975; 4:308-319.

16. Randolph JA, Lott JA, Tesi RJ, Thomas TM. Serum alkaline phosphatase (ALP) isoforms as an early detection of complications in liver transplantation (TX). Clin Chem 1992; 33:978 (Abstract).

17. DeBroe ME, Roels F, Nouwen EJ, Claeys L, et al. Liver plasma membrane: The source of high molecular weight alkaline phosphatse in human serum. Hepatology 1985; 5:118-128.

TABLE 1 SERUM LIVER FUNCTION TESTS

Sample	Day	Total ALP U/L	Total Bilirubin Mg/dL	AST U/L	ALT U/L	GGT U/L	AA/HBA
Donor Baboon	-	387	<0.1	54	23	36	*ND
Patient (Pre-Op)	15	158	3.7	102	47	56	*ND
Patient (Post-Op)	1	155	12.7	205	139	49	0.45
	5	784	9.5	180	72	43	1.52
	11	2035	4.7	28	36	55	0.87
	20	2693	1.3	22	24	64	*ND
	40	3512	2.4	31	58	90	*ND
	60	7246	7.4	113	136	225	*ND
Normal Human Range	Up to	125	1.0	40	40	65	0.70

*ND: Not Done

FIGURE LEGENDS

Figure 1 Ion-Exchange Column Chromatographic Separation of LALP and BALP Isoforms.

Figure 2 Gel Electrophoresis of Column Isolated ALP Isoforms

Left side (Fraction 1, LALP); Right side (Fraction 4, BALP)

Figure 3 Time Course for Total Bilirubin (3A) and Total Alkaline Phosphatase (3B) After Baboon Liver Transplant

Figure 4 Time Course for AST (4A), ALT (4B), and GGT (4C) After Baboon Liver Transplant

Figure 5 Typical Gel ALP Electrophoretic Isoform Patterns for Human, Baboon, and Xenograft Patient

Left Side (Schematic Diagram); Right Side (Gel Photograph)

Figure 6 Time Course Results for LALP and BALP (Activity) After Baboon Liver Transplant

Figure 7 Time Course Results for LALP and BALP (*Times Normal*) After Baboon Liver Transplant

Times normal calculations are based on pre-transplant (Day 0) activity measurements; LALP (146 U/L), BALP (4.9 U/L).

FOOTNOTES

Nonstandard abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALP, alanine aminotransferase; GGT, gamma glutamyltransferase; AA, acetoacetic acid; HBA, beta-hydroxybutryic acid; BALP, biliary isoform of alkaline phosphatase; LALP, liver isoform of alkaline phosphatase.

FIG 1

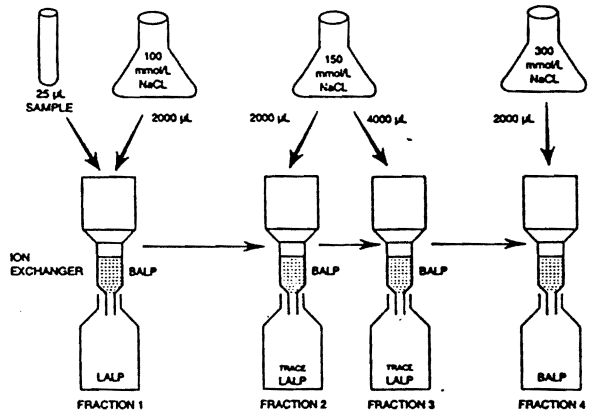

25 µL
SAMPLE
100
mmol/L
NaCL
2000 µL
150
mmol/L
NaCL
2000 µL
4000 µL
300
mmol/L
NaCL
2000 µL
ION
EXCHANGER
BALP
BALP
BALP
LALP
TRACE
LALP
TRACE
LALP
BALP
FRACTION 1
FRACTION 2
FRACTION 3
FRACTION 4

Fig 2

COLUMN FRACTIONS

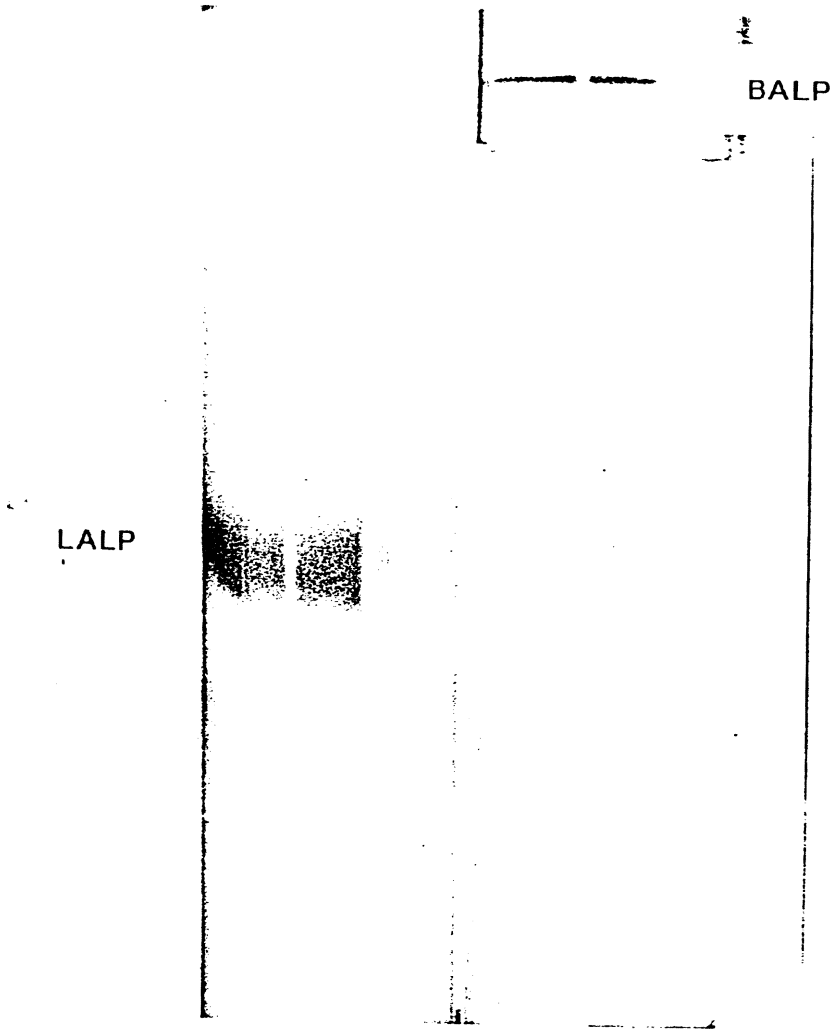


FIGURE 3 MERCER

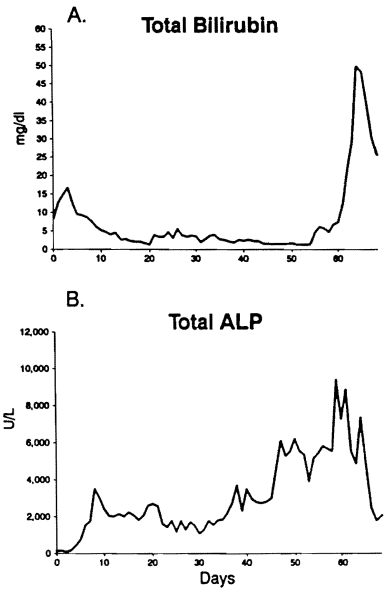


Fig 4

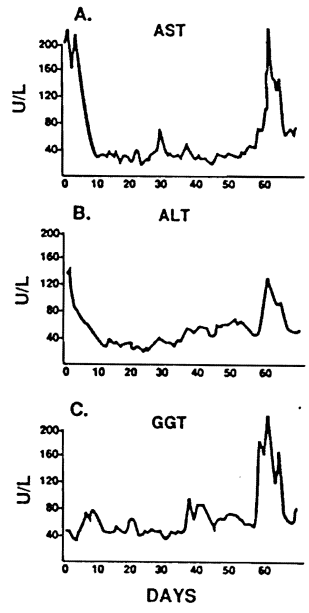
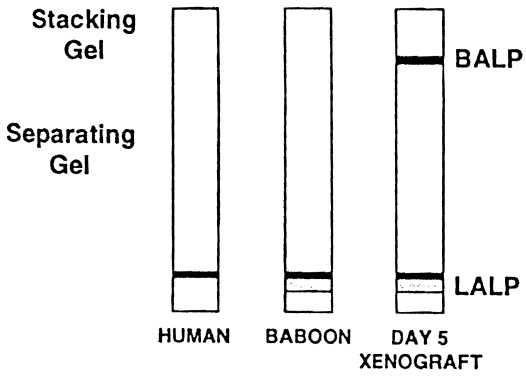
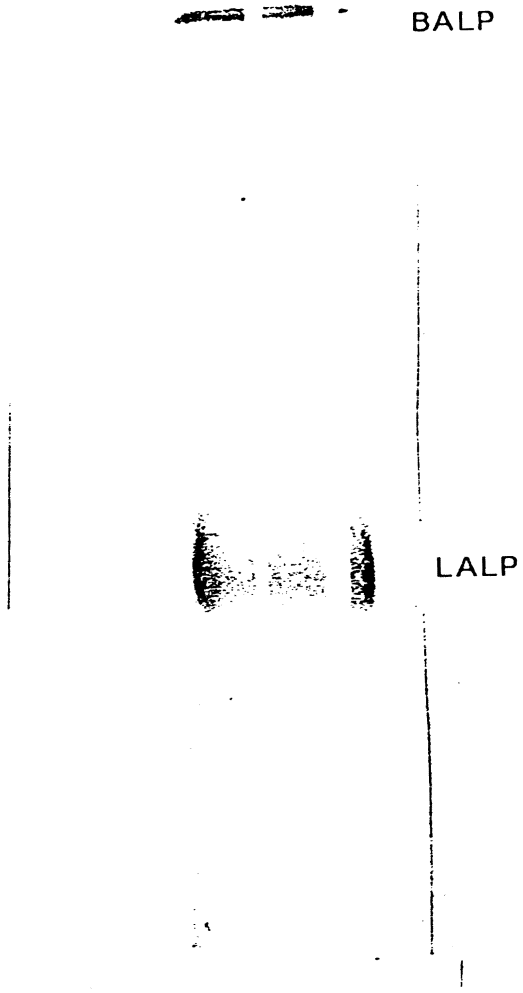

A.
AST
U/L
200
160
120
80
40
0 10 20 30 40 50 60
B.
ALT
U/L
200
160
120
80
40
0 10 20 30 40 50 60
C.
GGT
U/L
200
160
120
80
40
0 10 20 30 40 50 60
DAYS

FIG 5


Stacking Gel
Separating Gel
BALP
LALP
HUMAN
BABOON
DAY 5 XENOGRAFT


BALP
LALP
XENOGRAFT PATIENT
DAY 15

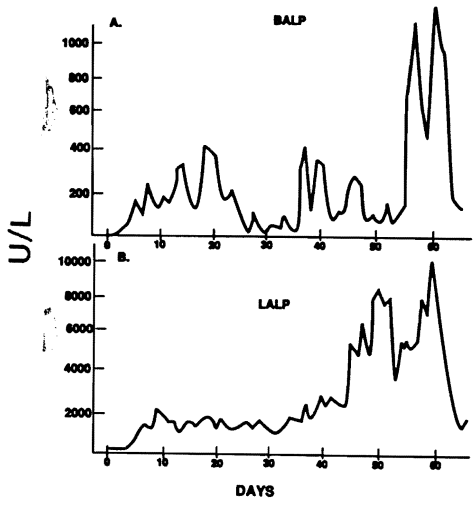

A.
BALP
1000
800
600
400
200
U/L
B.
LALP
10000
8000
6000
4000
2000
0
10
20
30
40
50
60
DAYS

Fig 7