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Impact of Antibodies That React With Liver Tissue and Donor-Specific Anti-HLA Antibodies in Pediatric Idiopathic Posttransplantation Hepatitis

Yoshihiro Hirata, MD,[1] Atsushi Yoshizawa, MD, PhD,[1] Hiroto Egawa, MD, PhD,[2] Daisuke Ueda, MD,[1] Shinya Okamoto, MD, PhD,[1] Hideaki Okajima, MD, PhD,[1,3] Kimiko Yurugi,[3] Rie Hishida,[3] Hideyo Hirai, MD, PhD,[3] Aya Miyagawa-Hayashino, MD, PhD,[4] Taira Maekawa, MD, PhD,[3] Hironori Haga, MD, PhD,[4] and Sinji Uemoto, MD, PhD[1]

Background. The cause of late graft dysfunction has not been elucidated. Although an antibody-mediated reaction is suspected as a potential mechanism, the target antigens have not been clarified. **Methods.** To clarify the etiology of idiopathic posttransplantation hepatitis (IPTH), we simultaneously examined the presence of antibodies that react with liver tissue (ARLT) by means of indirect immunofluorescence staining, as well as the presence of donor-specific anti-human leukocyte antigen antibodies (HLA-DSA). A subanalysis of the IPTH group was also performed. Within the IPTH group, the correlation between ARLT titer and clinical data were analyzed. **Results.** In the sera of patients with IPTH (30 patients), ARLT were found at a significantly higher frequency than in patients without IPTH (42 patients; $P < 0.001$). Moreover, the ARLT titer appeared to be correlated with the severity of hepatitis or hepatic injury. In contrast, the frequency of HLA-DSA was significantly lower in patients with IPTH than in patients without IPTH ($P = 0.001$). **Conclusion.** Our findings indicate that ARLT, and not HLA-DSA, profoundly influence the etiology of IPTH.

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The etiology of late graft dysfunction has been widely investigated, and various mechanisms have been proposed.[1-5] One of the potential mechanisms of chronic graft injury are humoral immune response. We previously reported that graft liver fibrosis, which is prominent in the centrilobular area and is observed in patients after liver transplantation (LTx), is caused by humoral immunity.[6]

Idiopathic posttransplantation hepatitis (IPTH) is a type of late-phase graft injury that may lead to graft dysfunction.[2] One of the pathological features of IPTH is interface hepatitis.[7] De novo interface hepatitis after LTx was first reported in 1998, and most patients showed elevation of anti-nuclear antibodies (ANA).[8] Thereafter, there were many reports of de novo interface hepatitis after LTx.[9-13] Moreover, a correlation between interface hepatitis and autoantibodies was reported.[7] However, some patients of interface hepatitis showed no elevation of autoantibodies, and the concept of IPTH was proposed to explain this discrepancy.[2] Because the pathological findings of IPTH mimic those of autoimmune hepatitis (AIH), humoral immunity has been hypothesized to be associated with IPTH etiology.[7] Herein, we encountered

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[1] Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto University Hospital, Kyoto, Japan.

[2] Institute of Gastroenterology, Tokyo Women's Medical University, Shinjukuku, Tokyo, Japan.

[3] Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, Japan.

[4] Department of Diagnostic Pathology, Kyoto University Hospital, Sakyo-ku Kyoto, Japan.

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Correspondence: Atsushi Yoshizawa, MD, PhD, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto University Hospital, 54 Kawaharacho, Shogoin, Sakyo-ku Kyoto, Japan. (ayoshi14@kuhp.kyoto-u.ac.jp).

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some patients who had refractory interface hepatitis without autoantibody elevation. We hypothesized that unidentified antibodies are profoundly correlated with interface hepatitis, and investigated these unidentified antibodies.

In this study, indirect immunofluorescence staining in rat liver tissue, which is a classical technique to detect autoantibodies, was performed to detect antibodies that react with liver tissue (ARLT) in the sera of transplanted recipients. Donor-specific antihuman leukocyte antigen antibodies (HLA-DSA) were examined simultaneously.

MATERIALS AND METHODS

This study was approved by the institutional review board of Kyoto University, and a waiver for consent was obtained for patient sera collection. All experimental protocols were approved by the Animal Research Committee of Kyoto University. All animals received humane care according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, 1985 revision). Male Wistar rats weighing approximately 200 g were obtained from Japan SLC, Inc. (Shizuoka, Japan).

Patients

Of the 851 pediatric patients (younger than 20 years) who underwent LTx in Kyoto University between June 1991 and December 2012, 48 (5.6%) patients were diagnosed with IPTH, and 30 of 48 patients were followed up in our institution from June 2011 to December 2012 and were enrolled in this study. Liver biopsies were performed during this period, and serum samples were collected at the same time from 24 of 30 patients. The other 6 patients had already undergone liver biopsy earlier (January 2010 to May 2011). For these 6 patients, the laboratory data collected at liver biopsy and serum sampling were compared. There was relatively little difference between the 2 data sets, indicating that the status of the graft liver was roughly the same and that the collected serum could be used in this examination. Sera from all 30 patients were collected and stored at −80°C until further use.

The control patients were selected from among patients who underwent liver biopsies from June to December 2011. The exclusion criteria included patients whose original disease was viral hepatitis infection or an autoimmune disease. The control patients were selected to match the background of the patients, including age at LTx, follow-up period, and sex. Finally, 42 patients were selected. The control group was divided into 3 subgroups based on pathological findings (Figure 1). The details are explained in the section on pathological examination. Sera from 42 patients were collected at the same time as the liver biopsy and stored at −80°C until further use.

The characteristics of the patients are shown in Table 1. There were no significant differences in the age at LTx, follow-up period, sex, original disease, and graft type. A significantly greater proportion of patients required steroid administration in the IPTH group than in the control group ($P < 0.001$).

Immunosuppression

The immunosuppression protocol consisted of tacrolimus and low-dose steroids.[14,15] The target whole blood trough level of tacrolimus was 10 to 12 ng/mL for the first 2 weeks, approximately 10 ng/mL for the following 2 weeks, and 8 to 10 ng/mL thereafter. The trough level of tacrolimus was gradually decreased 1 year posttransplant. The tacrolimus trough level was decreased as much as possible if the transaminase and bilirubin levels were within the normal range for 2 years.

Methylprednisolone treatment was initiated at the time as graft reperfusion at a dose of 10 mg/kg, tapered from 1 mg/kg per day to 0.3 mg/kg per day during the first month, and finally withdrawn within the first 3 months.[16]

The treatment for liver injury with interface hepatitis consisted of pulsed doses of methylprednisolone, 10 mg/kg per day for 3 days, which was gradually tapered. Oral prednisolone (0.1-0.5 mg/kg per day) was continued after steroid pulse therapy. The current status of immunosuppressive drugs is described in Table 1.

Pathological Examination

For the biopsy, liver tissue was obtained percutaneously with an 18-gauge needle, and sections were stained with hematoxylin-eosin, azan, and cytokeratin 7. All liver biopsy

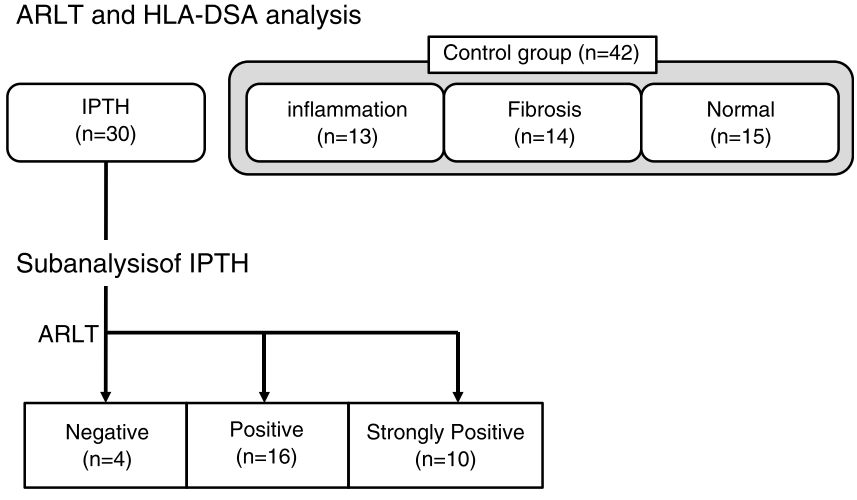

FIGURE 1. Patient classification. Thirty IPTH patients and 42 control patients with matching background, including age at LTx, follow-up period, and gender, were enrolled. The control group was classified into 3 subgroups according to the pathological findings (inflammation, fibrosis, and normal). In a subanalysis of the IPTH patients, the IPTH group was classified into 3 subgroups according to the immunofluorescence staining results (negative, positive, and strongly positive).

TABLE 1.

Patient characteristics

	IPTH (n = 30)	Control (n = 42)	*P*
Patient age at LTx	6.4 (0.2-18.7)	4.5 (0.9-19.3)	0.10
Follow-up period	11.9 (1.3-21.5)	9.7 (1.0-19.5)	0.11
Sex			
Male	8	18	0.19
Female	22	24	
Original disease			
Biliary atresia	26	33	0.37
Alagille syndrome	0	2	
Fulminant hepatitis	0	2	
Hepatoblastoma	0	3	
PFIC	1	1	
Metabolic disease	3	0	
Neonatal hepatitis	0	1	
Graft type			
Monosegment	1	0	0.50
Lateral segment	22	35	
Left lobe	6	6	
Right lobe	1	2	
Current immunosuppression			
No immunosuppression	0	3	<0.001
CNI	3	27	
CNI + MMF	1	2	
CNI + steroid	11	4	
CNI + MMF + steroid	15	6	

PFIC, progressive familial intrahepatic cholestasis; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil.

samples were assessed by the same 2 pathologists (H.H. and A.M.-H).

IPTH is defined as elevated transaminase levels in clinical laboratory data and pathologic interface hepatitis with unknown cause. Patients whose original disease was hepatitis virus infection or autoimmune disease were excluded because these diseases can recur after LTx and cause interface hepatitis. The diagnosis of IPTH does not require an elevation of autoantibodies.

IPTH relapse was defined as elevated liver function test findings and interface hepatitis (as assessed by pathological examination) in patients who had been diagnosed with IPTH previously. The possibility of hepatitis virus infection was excluded. Although the presence of autoantibodies was not included in the criteria, the autoantibodies levels were also examined.

Interface hepatitis was diagnosed by the presence of predominantly mononuclear portal inflammatory infiltrates associated with inflammatory spillover into the periportal zones.[7] The degree of hepatitis activity and fibrosis was evaluated by using the METAVIR scoring system.[17,18]

Inflammation was defined as inflammatory cell infiltration of the portal or centrilobular area without interface hepatitis. Inflammation included late-onset acute rejection, and late-onset acute rejection was defined as acute cellular rejection appearing later than 1 year after transplantation without interface hepatitis.[2]

Additionally, fibrosis of the portal and centrilobular area was evaluated. The METAVIR scoring system was used to evaluate portal fibrosis.[18,19] The modified staging system of nonalcoholic fatty liver disease devised by the pathology committee of the Nonalcoholic Steatohepatitis Clinical Research Network was used to evaluate centrilobular fibrosis.[7] Severe fibrosis was defined as the appearance of bridging fibrosis, portal to centrilobular or portal to portal, without inflammation.

C4d Immunostaining

C4d immunostaining was performed using a polyclonal anti-human C4d antibody (BI-RC4D; Biomedica, Vienna, Austria) diluted at 1:50. Evaluation of C4d immunostaining was performed as follows: C4d deposition in the vascular endothelium was regarded as positive staining, while C4d deposition on the elastic fibers or stroma without the vascular endothelium was regarded as nonspecific or negative staining, respectively. Portal C4d immunolabeling greater than 50% of the portal tracts was considered to indicate diffuse staining, and less than 50% represented focal staining.[6,20]

Detection of ARLT

ARLT were detected by indirect immunofluorescence, which was previously used to detect ANA.[21,22] Rat liver tissue was used instead of human liver tissue as an antigen because human liver tissue contains human IgG, and indirect immunofluorescence staining with human liver tissue would detect the IgGs in human liver tissue as well as those in the patient sera (primary antibody).

Rat liver tissue was harvested after euthanasia, frozen in liquid nitrogen, cut into 5-μm-thin sections, and mounted

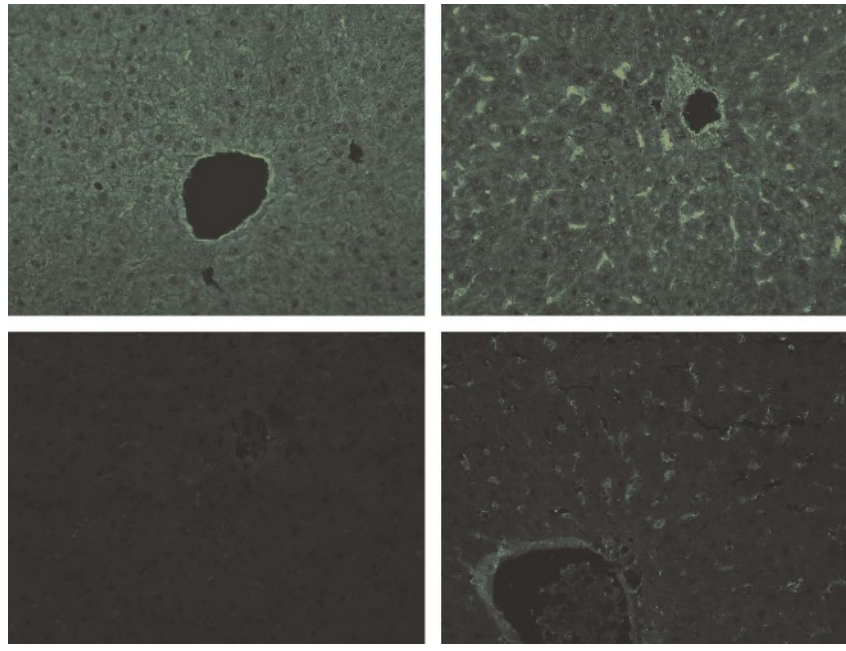

FIGURE 2. Immunofluorescence staining patterns. (1) Strongly positive, (2) positive, (3) negative, and (4) negative control (healthy volunteer). Each patient's serum served as the primary antibody, while anti-human IgG antibody conjugated to CF488A Dye served as the secondary antibody. There was no cross-reactivity of the secondary antibody to rat liver tissue. All images were obtained at 100× magnification. Staining of the hepatocyte cytoplasm was evaluated. Kupffer cells and the vascular endothelium showed higher staining intensity than hepatocytes, even with the serum of the healthy volunteer, and intense staining of Kupffer cells or the vascular endothelium was considered nonspecific.

onto glass slides. Sections were airdried and fixed in 90% ethanol and acetone, and blocked with 1% bovine serum albumin. Sections were then incubated with preserved sera from the study participants in a humidified chamber overnight at 4°C. After gentle washing, sections were incubated with anti-human IgG conjugated with CF488A dye (Biotium, Inc. Hayward, CA) for 1 hour at room temperature. Images were obtained with a Carl Zeiss AxioVision system, and all of the settings, including the magnification (100×) and exposure time (50 ms), were the same for each image.

The intensity of the stained cytoplasm within the hepatocytes was measured using ImageJ (National Institutes of Health, Bethesda, MD). The average of the intensities of 4 randomly selected areas of cytoplasm was calculated for each patient. Immunofluorescence intensity has previously been reported to show a good correlation with the antibody titer in human serum.[23] We considered the calculated intensity to reflect the ARLT titer. The cutoff value was defined as the maximum intensity based on 10 serum samples from healthy volunteers. A value over the cutoff was defined as "positive" and a value more than 2 times the cutoff value was defined as "strongly positive."

IgG subclasses of ARLT were examined in IPTH patients. Frozen rat liver sections were incubated with diluted serum from a patient. The secondary antibodies were anti-human IgG1, IgG2, IgG3, and IgG4 conjugated with fluorescein isothiocyanate (Binding Site, Birmingham, UK). The intensities of IgG1, IgG2, IgG3, and IgG4 staining were then compared.

Detection of HLA-DSA

The same serum samples that were used for the immunofluorescence staining analysis were also used for the HLA-DSA detection assay. The sera were analyzed for anti-HLA antibodies using LABScreen mixed beads (One Lamda, Canoga Park, CA) and a Luminex analyzer according to the manufacturer's protocol. When anti-HLA antibodies were detected, the antibody-responsive HLA was identified using a LABScreen single-antigen assay (One Lambda) to determine the donor specificity. A normalized, trimmed value for the mean fluorescence intensity (MFI) ≥ 1000 was considered positive.[20,24] Donor HLA typing of 6 patients in the IPTH group and 4 patients in the control group had not been performed before LTx, and thus, they were excluded from the HLA-DSA study.

Autoantibody Measurement

We measured ANA, liver kidney microsomal (LKM) antibodies, and antismooth muscle antibodies (ASMA) in IPTH patients. ANA and ASMA were measured using fluorescence antibody assay. The cut-off value was 1:40 for ANA and 1:20 for ASMA. LKM was measured using enzyme-linked immunosorbent assay. The cut-off value was 17, but values of 17

TABLE 2.

Result of ARLT and HLA-DSA

		IPTH (n = 30)	Control (n = 42)	
		n (%)	n (%)	*P*
ARLT	Positive	26 (86.7)	13 (30.9)	<0.001
	Negative	4 (13.3)	29 (69.1)	
HLA-DSA	Positive	3 (12.5)	19 (50.0)	0.0011
	Class I	0 (0)	3 (7.9)	
	Class II	3 (10.0)	18 (47.3)	
	DQ	2 (6.6)	14 (36.8)	
	DR	2 (6.6)	15 (39.5)	
	Negative	21 (87.5)	19 (50.0)	

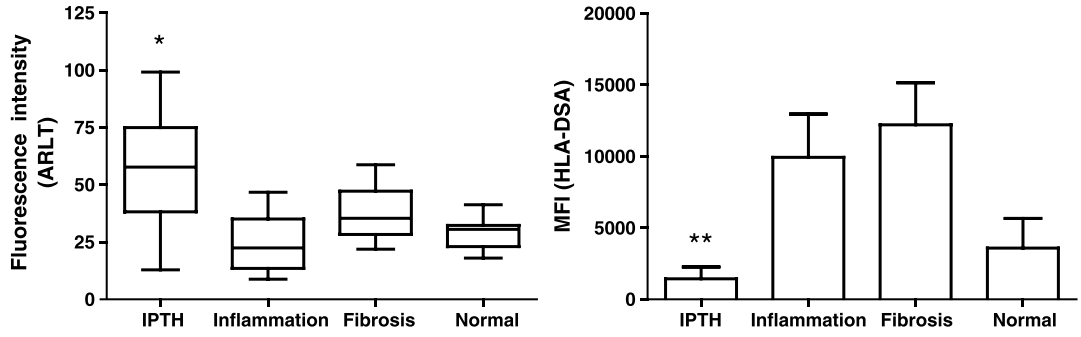

FIGURE 3. Fluorescence intensity of ARLT and HLA-DSA. (1) Fluorescence intensity of ARLT in the 4 groups classified according to the pathological findings. Patients with IPTH showed higher fluorescence intensity than other patients, and a significant difference was noted between patients with IPTH and the other groups. *$P < 0.001$. (2) MFI of HLA-DSA. The IPTH group showed a significantly lower MFI than the other groups. **$P = 0.001$.

to 51 were considered suspicious, whereas values greater than 51 were considered definitely positive.

Statistical Analysis

Clinical groups were compared using Mann-Whitney *U* test. Kruskal-Wallis test was used to compare continuous variables among multiple groups. The chi-squared test or Fisher's exact test was used to compare categorical variables. A *P* value less than 0.05 was considered to represent statistical significance. All statistical analyses were performed using JMP 11.0 (SAS Institute Inc., Cary, NC).

RESULTS

ARLT and HLA-DSA Data

Representative images of immunofluorescence staining are shown in Figure 2. First, the ARLT- or HLA-DSA–positive rate was compared between the IPTH and control groups (Table 2). The ARLT-positive rate was significantly higher in the IPTH group (86.2%) than in the control group (30.9%; $P < 0.001$), whereas the HLA-DSA–positive rate was significantly lower in the IPTH group (12.5%) than in the control group (50.0%; $P = 0.001$). The classification of HLA-DSA is shown in Table 2. Among the HLA-DSA-positive patients, HLA-DSA class II comprised 100% and 94.7% of the IPTH and control groups, respectively. In the IPTH group, DQ and DR loci were each detected in 2 patients. In the control group, the DQ locus was detected in 14 patients and the DR locus was detected in 15 patients. There were no significant differences between the presence of DR and DQ loci in both groups.

To investigate the relationship between ARLT or HLA-DSA and the pathological findings, the control group was divided into 3 groups according to the pathological findings. The inflammation group comprised patients who showed inflammation with or without severe fibrosis (n = 13), the fibrosis group comprised patients who showed severe graft liver fibrosis without inflammation (n = 14), and the remaining patients who showed no typical pathological findings were included in the normal group (n = 15).

The fluorescence intensity of ARLT and HLA-DSA were compared among the 4 groups to investigate these antibodies in detail. The fluorescence intensity of ARLT of each group is shown in Figure 3, 1. The fluorescence intensity of the IPTH group (58.5 ± 23.2) was significantly higher than that of the other groups (23.7 ± 12.0, 37.4 ± 12.0, and 28.5 ± 6.4 for the inflammation, fibrosis, and normal groups, respectively; $P < 0.001$). Conversely, the MFI of HLA-DSA was significantly lower in the IPTH group (1270 ± 3,662) than in the other groups (9929 ± 10,899, 12,204 ± 11,023, and 5509 ± 8966 for the inflammation, fibrosis, and normal groups, respectively; $P < 0.001$).

The comparisons of the clinical data of the 4 pathological groups are shown in Table 3. Aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, total bilirubin, platelet, and IgG levels showed no significant differences among the 4 groups.

Location of Severe Fibrosis

Severe fibrosis of the portal or centrilobular area was compared among the groups (Table 4). Severe fibrosis of the portal area was more frequently observed in the IPTH

TABLE 3.

Clinical data of 4 pathological groups

		Control			
	IPTH	Inflammation	Fibrosis	Normal	*P*
	(n = 30)	(n = 13)	(n = 14)	(n = 15)	
AST, IU/L	55.7 ± 115.3	63.1 ± 46.8	42.6 ± 36.2	24.9 ± 9.8	0.56
ALT, IU/L	49.2 ± 84.0	66.7 ± 61.2	34.6 ± 34.1	37.9 ± 61.4	0.61
g-GTP, IU/L	118.8 ± 173.0	87.6 ± 176.2	23.6 ± 11.5	47.1 ± 103.1	0.22
T-Bil, mg/dL	0.9 ± 0.8	1.1 ± 1.3	0.9 ± 0.6	1.2 ± 1.3	0.74
Plt, $\times 10^4$/mL	17.1 ± 9.1	15.0 ± 4.9	15.3 ± 5.7	17.7 ± 7.7	0.70
IgG, mg/dL	1524 ± 864	1003 ± 387	1325 ± 413	1249 ± 293	0.16
Tacrolimus trough level, ng/mL	4.4 ± 1.8	3.5 ± 2.2	2.1 ± 1.6	3.9 ± 3.0	0.04

TABLE 4.
Location of severe fibrosis

	IPTH (n = 30)	Control			P
		Inflammation (n = 13)	Fibrosis (n = 14)	Normal (n = 15)	
	n (%)	n (%)	n (%)	n (%)	
Portal area	18 (60.0)	3 (23.1)	6 (42.8)	1 (6.6)	0.002
Centrilobular area	6 (20.0)	2 (15.3)	12 (85.7)	2 (13.3)	<0.001

group than in the other groups (P = 0.002). Conversely, severe fibrosis of the centrilobular area was more frequently observed in the fibrosis group (P < 0.001).

Subanalysis Within the IPTH Group

To investigate the effect of ARLT in IPTH, the IPTH group was divided into 3 groups according to the fluorescence intensity of ARLT (4 patients were negative, 16 were positive, and 10 were strongly positive).

The clinical data, IgG subclass of ARLT, clinical autoantibodies, and C4d staining of liver biopsy sample were evaluated.

ARLT and Clinical Data in IPTH Patients

We compared the AST, ALT, and g-GTP levels, the age at LTx and follow-up period, and the time of IPTH onset from LTx among the 3 groups (Table 5). Increased AST and ALT levels were observed in the strongly positive group compared with the positive or negative group; this difference was significant with respect to the ALT level (P = 0.03). There was no elevation in serum IgG titer in the positive and strongly positive groups compared with the negative group.

IgG Subclass of ARLT in IPTH Patients

We examined the levels of IgG subclasses in 26 patients with positive immunofluorescence staining (Figure 4). Twenty-four patients showed IgG1 positivity, 1 patient showed IgG3 positivity, and 1 patient showed IgG4 positivity.

Correlation Between the Fluorescence Intensity of ARLT and Liver Damage

Three patients experienced liver injury because of interface hepatitis during this study. Autoantibodies were not detected in these 3 patients when the interface hepatitis relapsed. The fluorescence intensity of ARLT, AST, ALT, and hepatitis activities were investigated. The time courses of interface hepatitis relapse in each patient are shown in Figures 5 1, 2, 3. AST and ALT levels were strongly correlated with fluorescence intensity. The Pearson correlation coefficient (R^2) between the AST level and fluorescence intensity of ARLT for patients 1, 2, and 3 were 0.74, 0.76, and 0.72, respectively. The R^2 between the ALT level and fluorescence intensity of ARLT for patients 1, 2, and 3 were 0.64, 0.99, and 0.80, respectively. The hepatitis activity score was also correlated with fluorescence intensity. Together, these results indicate that ARLT is the cause of liver injury in IPTH.

Immunofluorescence staining during IPTH progression is shown in Figure 5, 4. These images represent the time course of IPTH relapse in case 2 (Figure 5, 2), and the 4 figures in Figure 5, 4 correspond to the 4 time points in case 2.

Evaluation of the Presence of Autoantibodies

Next, clinical autoantibodies, which included ANA, LKM, and ASMA, were investigated. The relationship between ARLT and the clinical autoantibodies is shown in Table 6. Seven patients showed ANA positivity, 6 patients had a titer of 1:40, and 1 patient had a titer of 1:80. Three patients showed LKM positivity; all were classified as suspicious, not definite. None of the patients was ASMA positive. ARLT presence did not result in a significant difference in clinical autoantibody levels between the positive and negative groups. This indicates that the presence of ARLT is independent of the presence of clinical autoantibodies.

C4d Immunostaining

None of the patients in the IPTH group showed diffuse endothelial C4d staining. The ratio of focal endothelial C4d-positive staining was 50.0% (2/4) in the negative ARLT group. 12.5% (2/16) in the positive ARLT group, and 30.0% (3/10) in the strongly positive ARLT group. There was no significant difference in C4d positivity among the 3 groups (P = 0.28).

DISCUSSION

The complications caused by immunological responses after LTx differ between the early and late phases.[2,25] In the early phase, the primary complication is acute cellular rejection caused by HLA mismatch, and thus, inactivation of T lymphocytes by immunosuppressants is crucial to control acute cellular rejection.[26-28] The development of immunosuppressive drugs has enabled us to overcome this complication.[29] The most recent 5-year survival rate of pediatric LTx recipients is greater than 90%.[30]

TABLE 5.
Clinical data of IPTH patients classified with ARLT

	ARLT			
	Negative (n = 4)	Positive (n = 16)	Strongly positive (n = 10)	P
Age at LTx, y	9.1 ± 5.9	9.9 ± 8.7	9.6 ± 8.9	0.98
Follow-up period, y	12.1 ± 8.1	11.8 ± 6.3	12.8 ± 1.7	0.91
IPTH onset from LTx, y	6.5 ± 9.4	6.7 ± 7.8	7.8 ± 5.7	0.87
AST, IU/L	24.5 ± 5.6	27.8 ± 13.3	56.7 ± 61.6	0.12
ALT, IU/L	22.3 ± 15.8	23.7 ± 14.4	56.3 ± 51.3	0.035
g-GTP, IU/L	97.3 ± 94.9	79.7 ± 112.3	163.8 ± 234.8	0.41
T-Bil, mg/dL	0.8 ± 0.2	0.8 ± 0.3	0.9 ± 0.5	0.57
IgG, mg/dL	941 ± 230	1640 ± 1049	1508 ± 464	0.43

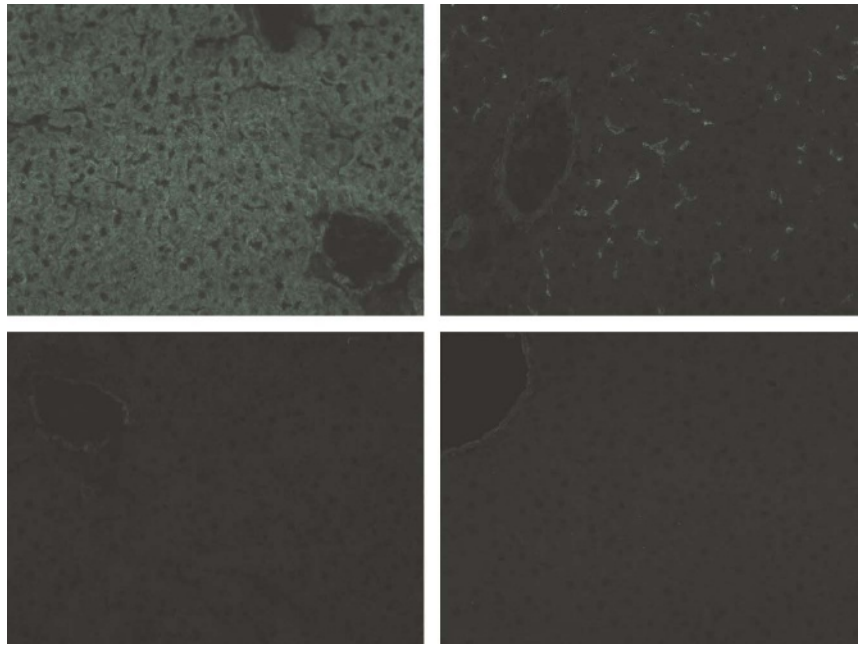

FIGURE 4. IgG subclass immunofluorescence staining of IPTH patients.(1) IgG1, (2) IgG2, (3) IgG3, (4) IgG4. Twenty-four of 26 ARLT-positive patients were IgG1-positive.

The etiology of late phase complications has not yet been determined; however, recent studies have suggested that antibody-mediated reaction is one of the causes of late phase complications.[6,31-33] We hypothesized that an antibody-mediated response against liver tissue causes late graft dysfunction, and therefore, we conducted an immunofluorescence analysis with rat liver tissue in the present study. As for the detection of ARLT, it was suspected that the fluorescence intensity was affected by serum IgG level. However, the IPTH group showed higher fluorescence intensity than the control group, without a significant elevation of serum IgG level. Among the patients with IPTH, both the positive and strongly positive groups showed no significant elevation of serum IgG titer, indicating that this immunofluorescence staining was not affected by serum IgG titer and detects only ARLT.

In the subanalysis of the IPTH patients, the ALT level was significantly elevated in the strongly positive group compared with the negative group. During the period when 3 patients experienced a relapse of IPTH, the AST and ALT levels, and the intensity of immunofluorescence staining, demonstrated a strong correlation in each patient. These results imply that the degree of liver injury is proportional to the ARLT titer.

Much attention has been paid to HLA-DSA as a cause of antibody-mediated rejection and late graft dysfunction.[20,24,34-36] Posttransplantation HLA-DSA has been observed in >40% of all pediatric patients who received LTx, and has a deep correlation with graft liver progressive fibrosis.[20] Weaning of calcineurin inhibitors is a risk factor of HLA-DSA.[37-39] In the present study, the IPTH group showed higher tacrolimus trough level and lower HLA-DSA presence than the control group, consistent with the findings of previous reports. The lower incidence of HLA-DSA in the IPTH group indicated that HLA-DSA has negligible impact on the etiology of IPTH.

The primary immunological difference between HLA-DSA and ARLT is the antigen site and manner of activation. The targets of HLA-DSA are class II HLA, which are expressed in the central vein endothelium and not in the portal tract or hepatocytes.[40] Furthermore, the central vein endothelia function as antigen-presenting cells.[41] We hypothesize that central vein endothelia present the donor HLA as alloantigens. T cells then recognize the antigen and activate B cells, which produces HLA-DSA. HLA-DSA presence is treated by increasing the calcineurin inhibitor dosage. In contrast, the antigens of ARLT exist in the cytoplasm of hepatocytes, according to our study. Antigen-presenting cells must raise an immunological reaction against the ARLT antigen, which may partially explain why the treatment for IPTH requires steroid or metabolic antagonist administration.

Progressive centrilobular-fibrosis has been observed in HLA-DSA–positive patients, whereas fibrosis around the portal tract has been observed in IPTH patients.[11,20] In this study, the control group showed higher HLA-DSA positivity, and the fibrosis score of the centrilobular area was significantly higher than that of the portal area. Conversely, the IPTH group showed a higher rate of ARLT, and the fibrosis score of the portal area was significantly higher than that of the centrilobular area.

HLA-DSA against HLA class II are present in greater than 90% of HLA-DSA-positive patients. In the liver tissue, HLA class II antigens are expressed in the central vein endothelium[40] and HLA-DSA react mainly with the central vein endothelium, ultimately leading to centrilobular-fibrosis. In IPTH, the limiting plate, which is the location of hepatocytes at the junction of the portal tract and hepatic parenchyma, is the initial target of ARLT, because ARLT react with hepatocytes. Consequently, IPTH patients show interface hepatitis and fibrosis in the portal area. HLA-DSA are not correlated with IPTH because class II antigens are not expressed in hepatocytes.

C4d is a complement 4 split product that has been used as a marker of antibody-mediated rejection in liver allografts.[42,43] In the C4d staining of liver tissue, staining of the vascular

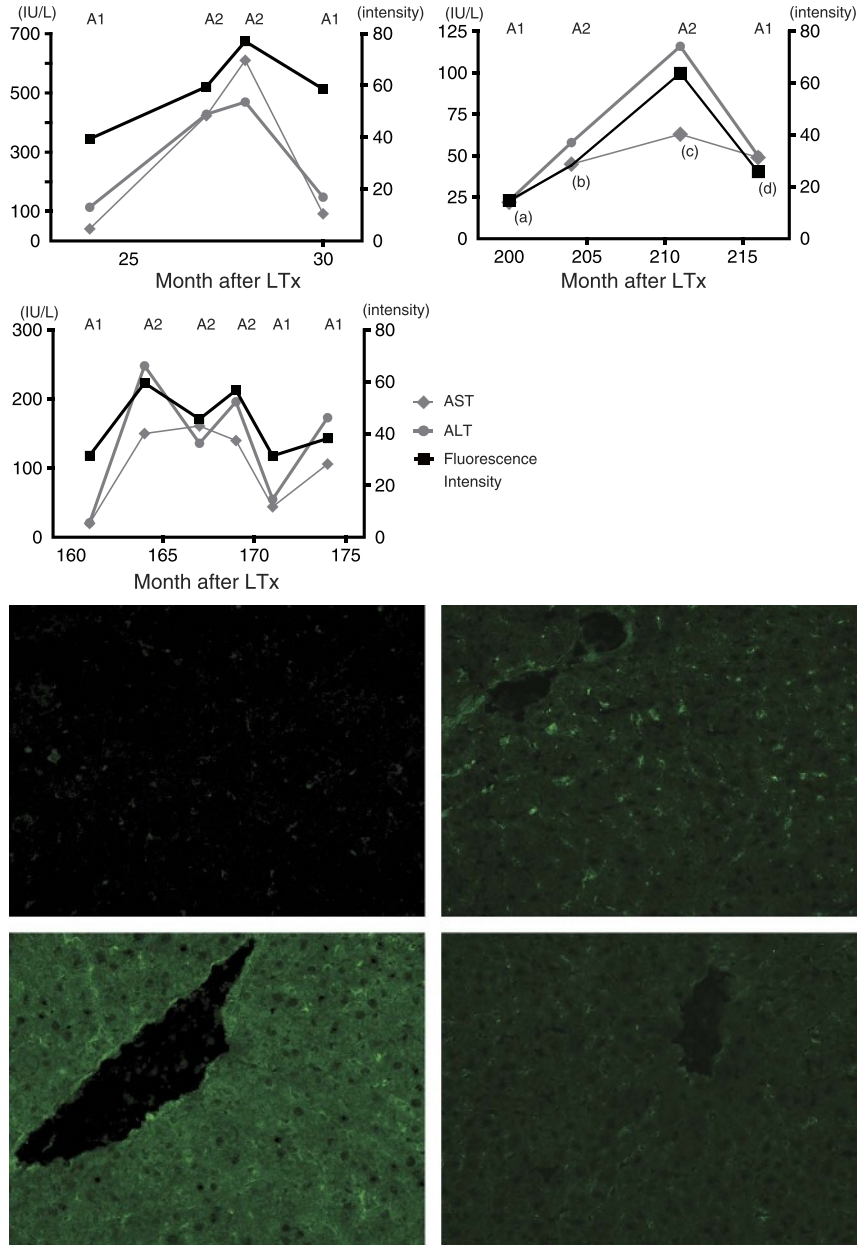

FIGURE 5. Time course of the IPTH relapse patients. Panels 1, 2, and 3 show the time course of the pathological findings, AST level, ALT level, and the fluorescence intensity of ARLT. Three patients experienced relapse and remission of IPTH. The fluorescence intensity of ARLT was correlated with the AST level, ALT level, and pathological findings. The R^2 between AST and ARLT and between ALT and ARLT for patients 1, 2, and 3 were 0.74, 0.76, and 0.72 and 0.64, 0.99, and 0.80 respectively. The X-axis represents months after LTx. The left Y-axis shows the levels of AST and ALT, and the right Y-axis shows the intensity of immunofluorescence staining. A1 and A2 illustrate the hepatitis activity score according to the METAVIR scoring system. Panel 4 shows the immunofluorescence staining during IPTH progression in a patient. The 4 figures (A), (B), (C), and (D) correspond to the time points (A), (B), (C), and (D), respectively, in panel 2.

endothelium is evaluated, while staining of hepatocytes is considered nonspecific. As for HLA-DSA, C4d staining is correlated with HLA-DSA presence.[20] However, to our knowledge, C4d staining in IPTH patients has not been reported previously. In our study, C4d staining had no correlation with IPTH or ARLT. HLA-DSA recognize the antigens expressed on the vascular endothelium, and C4d staining is positive when the HLA-DSA and antigen reaction occurs on the vascular endothelium. Our immunofluorescence staining findings suggest that the antigens of ARLT existed in the cytoplasm of hepatocytes. The antigen-antibody reactions would thus occur in the hepatocytes, not in the endothelium. This may

TABLE 6.

Relation of clinical autoantibodies and ARLT

		Clinical autoantibodies		
		Negative (n = 20)	Positive (n = 10)	
		n (%)	n (%)	*P*
ARLT	Negative	3 (15.0)	1 (10.0)	0.72
	Positive	10 (50.0)	6 (60.0)	
	Strongly positive	7 (35.0)	3 (30.0)	

explain why C4d and ARLT were not correlated in this histopathological study.

With regard to the etiology of IPTH, it remains unclear whether an autoimmune disorder or rejection against alloantigens is involved. The presence of autoantibodies, which would suggest an autoimmune disorder, was previously detected in patients with IPTH.[1,8,44-46] Additionally, the presence of anti-cytokeratin 8/18 antibody was previously detected in AIH patients.[47] Conversely, it has also been reported that the cause of IPTH is a humoral rejection against an alloantigen.[10,48,49]

The mechanism of de novo ARLT production remains unknown. In a study of AIH, presumed environmental agents were found to include viruses, drugs, or immunization agents.[50,51] Recipients with episodes of acute cellular rejection have been reported to be at a greater risk of IPTH.[11] We hypothesize that acute cellular rejection leads to the disruption of hepatocytes; intracellular antigens present in hepatocytes that are normally absent in the peripheral blood are then drained into the bloodstream, where they are exposed to the immune system, resulting in the production of ARLT.

A long interval after LTx is reported to be a risk factor for autoantibody production.[44] In our study, although the period after LTx in the control group patients matched that in the IPTH patients, ARLT was detected at a significantly higher rate in the IPTH group. Within the IPTH group, there were no significant differences in the interval between LTx and the onset of IPTH, or in the follow-up period after LTx. These findings indicate that ARLT is only marginally correlated with the period after LTx.

There are some limitations associated with the present study. Firstly, the specific ARLT were not identified. Secondly, the immunofluorescence staining analysis showed similar hepatocyte staining patterns; however, it is possible that different antibodies show the same staining pattern, and all the patients who showed positive or strongly positive staining may not have the same ARLT. Further studies using SDS-PAGE are currently underway to clarify the precise role of the antibodies in IPTH.

In conclusion, the etiology of IPTH is antibody-mediated, and ARLT, not HLA-DSA, are correlated with IPTH and reflect the activity of IPTH. The pathogenic immunogenicity in patients with IPTH does not decline because the allo-antigens present in the graft liver never disappear. This may explain why therapy for patients with IPTH consists of steroid and/or antimetabolites (eg, mycophenolate mofetil and mizoribine) and why a dose reduction of these immunosuppressants is difficult in patients with IPTH.

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