## Clinical Transplantation

# Characterization of CD4, CD8, CD56 positive lymphocytes and C4d deposits to distinguish acute cellular rejection from recurrent hepatitis C in post-liver transplant biopsies

Abstract: Introduction: Hepatitis C viral (HCV) infection is the most common cause for liver transplantation (LTx) in USA. Hepatitis C viral recurrence in liver allograft is almost universal, which is often difficult to distinguish from acute cellular rejection (ACR).
Aim: Aim of the present study is to examine the differences between distribution of CD4, CD8, CD56 positive lymphocytes, and C4d deposits in patients with ACR and recurrent HCV.
Patients and methods: As a pilot project, a group of five post-LTx HCV RNA negative patients, strongly suspicious for ACR based on clinical findings and history of medication non-compliance and another group of five post-LTx HCV positive, medication compliant patients with abnormal liver function were retrospectively selected. Liver biopsies of these patients were stained with monoclonal CD4, CD8, CD56, and polyclonal C4d antibodies and compared.
Results: Mean CD4, CD8, and CD56 counts in ACR group were $156.7 \pm 17.6$, $35.4 \pm 8.8$, and $1.0 \pm 1.8$/HPF, respectively and were $89.7 \pm 41.3$, $20.3 \pm 23.2$, and $0.6 \pm 0.9$/HPF, respectively in HCV recurrence group. Biopsies of four of five patients with ACR demonstrated moderate to strong C4d staining, whereas all patients with recurrent HCV had none to mild C4d staining.
Conclusion: Mean CD4, CD8, and CD56 were similar for acute rejection and recurrent HCV infection. However, 80% of patients with ACR showed moderate to strong staining for C4d and all recurrent HCV patients showed none to mild C4d staining.

**Ashok Jain[a], Charlotte Ryan[b], Ravi Mohanka[a], Mark Orloff[a], Peter Abt[a], Joan Romano[a], Leah Bryan[a], Pam Batzold[a], Parvez Mantry[c] and Adel Bozorgzadeh[a]**

[a]Department of Surgery, Division of Transplantation, [b]Department of Pathology and [c]Department of Gastroenterology and Hepatology, University of Rochester Medical Centre, Rochester, NY, USA

Corresponding author: Ashokkumar Jain MD, University of Rochester Medical Centre, Department of Surgery, Division of Transplantation, Box SURG, 601 Elmwood Avenue, Rochester, NY 14642, USA.
Tel.: +1 (585) 275-2924; fax: +1 (585) 506-0054; e-mail: ashok_jain@urmc.rochester.edu

Hepatitis C viral (HCV) infection in the most common cause of end stage liver disease leading to liver transplantation (LTx) in USA. However, recurrent HCV infection of the allograft is uniformly observed in all cases after successful LTx (1–3).

When HCV patients develop hepatic dysfunction after LTx, a liver biopsy with Hematoxylin and Eosin (H&E) stain is usually performed to determine the cause of hepatic dysfunction. It is often difficult to distinguish between recurrent HCV and acute cellular rejection (ACR) as the cause of hepatic dysfunction on H&E stain on liver biopsy. Both these conditions show lymphocytic cellular infiltrate, in porta hepatis (predominantly ACR) and hepatic lobules (predominantly hepatitis). Mostly, overlapping features are observed, making the differentiation difficult (4). However, it is important to distinguish the two conditions, because, if recurrent HCV is treated with augmentation of steroid, as for rejection, the disease could get worse and lead to allograft failure. Hence, a confirmatory method is needed to distinguish between the two conditions.

The lymphocytic infiltrates consist of CD4 and CD8 positive cells. These infiltrates have been studied by immuno-phenotyping in patients with primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis and chronic active hepatitis because of HCV infection (5–7). These studies have reported a predominance of CD4 positive lymphocytes in portal triads and CD8 positive lymphocytes in areas of necrosis (8, 9).

The role of humoral component in rejection has been studied in renal allografts with C4d deposits. C4d is the final inactive form of complement derived from classical pathway of the complement system, which gets deposited in peri-tubular capillaries of renal allograft during humoral rejection (10–12). Although it is believed that most ACR are mediated by T-lymphocytes, the association of a positive C4d stain with inferior allograft survival highlights the role of B-lymphocytes in ACR (13, 14). C4d deposits have also been reported in cardiac transplant recipients with humoral rejection (15, 16) and in lung transplant patients with ACR on lung biopsy and bronchoalveolar lavage (BAL) (17, 18). Similar observations have been made in live donor (19) and deceased donor liver transplantation (20, 21).

CD56 positive staining of bile ducts has been observed in patients with bile duct destruction after deceased donor liver transplants (22).

### Aim

Aim of the present study was to develop a model to distinguish ACR and recurrent HCV with a pilot project to identify the distribution of CD4, CD8, CD56 positive lymphocytes, and C4d deposits in lymphocytic infiltrates of patients with clinical ACR alone (HCV RNA negative, documented non-compliant patients with low or undetectable levels of immunosuppressive medication) or recurrent HCV alone (high HCV RNA load, hepatic dysfunction, medication compliant patients, and adequate baseline immunosuppression levels).

### Patients and methods

We retrospectively identified 10 patients (seven males and three females, mean age: 52.3 ± 6.9 yr), who underwent liver transplant between September 2000 and August 2004, five patients with unequivocal clinical ACR (HCV RNA negative, documented non-compliant patients with low or undetectable levels of immunosuppressive medication) and five with unequivocal recurrent HCV (high HCV RNA load, hepatic dysfunction, medication compliant patients, and adequate baseline immunosuppression levels). Demographic characteristics and liver biopsy findings with H&E stain are given in Table 1. Liver function tests (Total Bilirubin, AST, ALT, ALK, GGTP, INR, and Albumin) prior to liver biopsy are given in Table 1.

Liver biopsies from these patients were studied further. Immunocytochemical stain was performed on these formalin-fixed, paraffin embedded biopsies using monoclonal antibody for CD8 (Clone C8/144B, titer 1:100) (Dako, Carpinteria, CA, USA), CD56 (Clone 123C3.D5, titer 1:50) (Cell Marque, Hot Springs, AR, USA), CD4 (Clone 1F6, titer 1:25) (Novocastra/Vector Lab, CA, USA), and polyclonal C4d (titer 1:60) (Biogenesis, Kingston, NH, USA). A kidney biopsy with known humoral rejection was used as positive control (Fig. 1).

Paraffin embedded tissues were cut at 4–5 μm and floated on distilled water at 54°C. Sections were mounted on chemically charged slides and dried at room temperature until opaque, and then placed overnight in an oven at 57°C. Sections were de-paraffinized using standard procedures, treated with 3% hydrogen peroxide for six min and then cleared in running water followed by Tris Buffered Saline (TBS) (50 mM of Tris–HCL, 150 mM of NaCl, 0.05% Tween 20 at pH 7.6).

#### Pre-treatment

Antigen unmasking was performed by one of the following methods: pre-heated (95–99°C) Dako antigen retrieval solution Citrate Buffer (pH 6.1) or in High pH (pH 9.9) antigen retrieval solution in a Black and Decker steamer (Model HS 800, Shelton, CT, USA) for 30 or 40 min followed by a 15-minute cool down. Slides were rinsed with TBS

Table 1. Demographic, biochemical and immuno-histochemical biopsy characteristics of patients

| No | Age | Sex | Diagnosis | Time | RAI | HAI | Fib | Fat | Bili | AST | ALT | ALK | GGT | INR | Alb | HCV RNA | CD4[a] | CD8[a] | CD56 | C4d | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acute rejection group | | | | | | | | | | | | | | | | | | | | | |
| 1 | 55 | Male | ETOH | 20.4 | 6 | 0 | 0 | 60 | 3.4 | 266 | 203 | 293 | 1081 | 1.1 | 3.6 | N.A. | 174 | 34.4 | 4.2 | 2+ | Non-compliant for 4 wk |
| 2 | 50 | Male | ETOH | 20.2 | 4 | 0 | 0 | 0 | 5.6 | 284 | 702 | 311 | 1312 | 1 | 3.8 | N.A. | 171.6 | 23 | 0 | 2+ | Non-compliant, undetectable tacrolimus levels |
| 3 | 63 | Male | ETOH | 7.4 | 5 | 0 | 1 | 0 | 6.3 | 252 | 348 | 1059 | | 0.8 | 3.1 | N.A. | 141.8 | 32.6 | 0.6 | 1+ | Non-compliant for 3 wk, received thymoglobulin |
| 4 | 40 | Male | ETOH | 33.8 | 5–6 | 0 | 0 | 0 | 7.4 | 279 | 383 | 1100 | | 1.1 | 3.2 | N.A. | 161.2 | 41 | 0 | 3+ | Non-compliant for 8 wk |
| 5 | 60 | Female | Cryptogenic, HCC | 7.7 | 7 | 0 | 0 | 0 | 19.6 | 2394 | 729 | 272 | | 1.6 | 2.3 | N.A. | 135 | 46 | 0.4 | 3+ | Non-compliant for 16 wk |
| Mean | | | | 17.9 | 5.5 | 0 | 0.2 | 12 | 8.5 | 695 | 473 | 607 | 1197 | 1.1 | 3.2 | | 156.7 | 35.4 | 1 | 2.2 | |
| HCV recurrence group | | | | | | | | | | | | | | | | | | | | | |
| 6 | 49 | Male | HCV | 4.4 | 3 | 7 | 1 | 5 | 3.5 | 214 | 165 | 1345 | 1350 | 1 | 4.1 | 46 200 000 | 47.6 | 4.6 | 0.2 | 1+ | Tacrolimus level: 3.3 |
| 7 | 57 | Female | HCV 1a/1b | 29.9 | 4 | 9 | 2 | 2 | 0.4 | 213 | 263 | 208 | | 1.1 | 4.2 | 8270 | 111 | 7 | 2.2 | 0 | Trough CsA level: 212 |
| 8 | 47 | Female | HCV 3a, ETOH | 4.6 | 0 | 7 | 3 | 50 | 0.6 | 206 | 285 | 189 | 451 | 1 | 3.6 | 3 820 000 | 43 | 6 | 0 | 0 | Tacrolimus level: 10.2 |
| 9 | 52 | Male | HCV 1a | 40 | 2 | 0 | 5 | 20 | 0.9 | 295 | 114 | 47 | 18 | 1.1 | 2.3 | 18 700 000 | 115 | 25 | 0 | 1+ | Tacrolimus level: 7.4, received interferon |
| 10 | 50 | Male | HCV 1 | 11.4 | 2 | 14 | 6 | 10 | 2.3 | 48 | 38 | 160 | | 1.9 | 2.4 | 35 600 | 132 | 59 | 0.8 | 1+ | Tacrolimus level: 6.2 |
| Mean | | | | 18.1 | 2.2 | 9 | 3.4 | 17.4 | 1.5 | 195 | 173 | 390 | 606 | 1.2 | 3.3 | | 89.7 | 20.3 | 0.6 | 0.6 | |
| Difference between the two groups (p-value) | | | | | | | | | | | | | | | | | 0.018 | 0.21 | 0.669 | 0.007 | |

RAI, rejection activity index; HAI, hepatic activity index; ETOH, alcohol-related end stage liver disease; HCC, hepatocellular carcinoma; CsA, cyclosporin A; Bili, total bilirubin; AST, aspartate amino transferase; ALT, amino alanine transferase; ALK, alkaline phosphatase; GGTP, gamma glutamyl transpeptidase; INR, international normalized ratio; Alb, albumin; Fib, fibrosis; Time, period (in months) of the biopsy from transplant; Fat, steatosis (%); CD, cluster differentiation; HCV, hepatitis C viral.
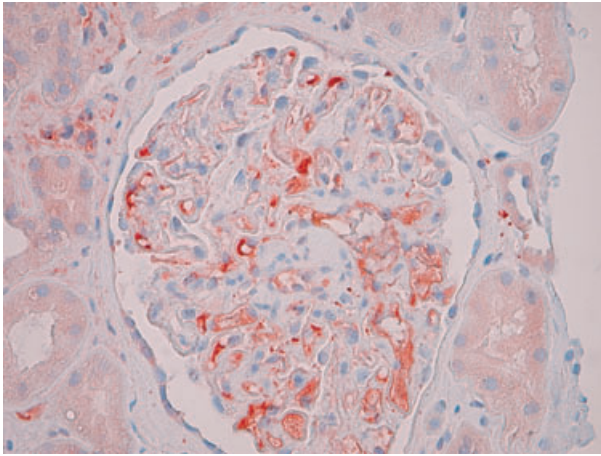[a]Mean count of five portal triads.

*Fig. 1.* Positive kidney control with known humoral rejection.

for five min. For C4d, slides were incubated overnight at 4°C in a humid chamber at the specified titer and then brought to room temperature for 20 min. C4d stain positive control in a renal allograft with known humoral rejection is shown in Fig. 1.

Staining

Sections were mounted in the DAKO Autostainer (Dako, CA, USA) covering with fresh TBS. Sections were stained for 15 (CD4) or 45 min (CD8 and CD56) at the specified titer followed by 30-minute incubation with the labeled polymer, HRP (Envision. Plus System, Dako, CA, USA) (either mouse or rabbit depending on species of antibody). Slides were developed with AEC + (Dako, CA, USA) (CD8, CD56, and C4d) or CSA Kit (Dako, CA, USA) for 10 min, rinsed in running distilled water and counterstained in modified Mayer's Hematoxylin Blue in 0.3% ammonia water followed by tap water. The slides were mounted using an aqueous media and viewed with a light microscope.

Number of CD4, CD8, and CD56 positive cells in one high power field (HPF: 40×) of 0.25 mm$^2$ for five randomly selected separate portal triads were counted for each patient by the same pathologist who was blinded to the clinical course of patients.

C4d stain in hepatocytes was graded semi-quantitatively, i.e. grade 0 or 1: none or minimal staining, grade 2: moderate staining, grade 3: strong staining.

**Results**

Their biochemical parameters for each patient are shown in Table 1 and individual patients' CD4, CD8, CD56, and C4d stains are shown in Figs. 2–5.

CD4 positive lymphocytes

Mean CD4 positive lymphocyte count per high power field was 156.7 ± 17.6/HPF (range 135–174) in ACR group compared with 89.7 ± 41.3/HPF in HCV group (range 43.0–132.0). This was not significant because of a wide range and small population. Individual patients' microscopic CD4 stain and CD4 lymphocyte counts are given in Fig. 2 and Table 1, respectively.

CD8 positive lymphocytes

Mean CD8 counts were, 35.4 ± 8.8/HPF (range 23.0–46.0) for patients experiencing acute rejection and 20.3 ± 23.2/HPF (range 4.6–59) for patients with recurrent HCV. Individual patients' microscopic CD8 stain and CD8 lymphocyte counts are given in Fig. 3 and Table 1, respectively.

C4d deposits

C4d did not stain the endothelium of small vessels in any case. The hepatic lobules showed moderate to strong staining in all patients except in Case 3 who received polyclonal anti-thymocyte antibody (Thymoglobulin, Genzyme, Cambridge, MA, USA) for previous incidence of acute rejection. Incidentally, Case 1 who was initially C4d positive became C4d negative nine months later, after he was treated with Thymoglobulin for an episode of rejection in the interim.

In all five patients who had recurrent HCV, none to minimal C4d deposits were observed. Individual patients' microscopic C4d deposits and C4d grading is given in Fig. 4 and Table 1, respectively.

CD56 positive staining

Very few positive CD56 lymphocytes were found in the portal triads in biopsies. The actual count per high power field varied from 0 to 4.2 in ACR group (mean = 1.04 ± 1.8) and 0–2.2 (mean = 0.6 ± 0.9) in HCV group (Table 1). CD56 stained the bile duct epithelium in many cases in both groups. More staining in bile ducts and ductules was observed in recurrent HCV patients. Representative patients' microscopic CD56 stain individual patients' CD56 lymphocyte counts are given in Fig. 5 and Table 1, respectively.

**Discussion**

End stage liver disease related to HCV infection is the leading cause of LTx in US and is expected to
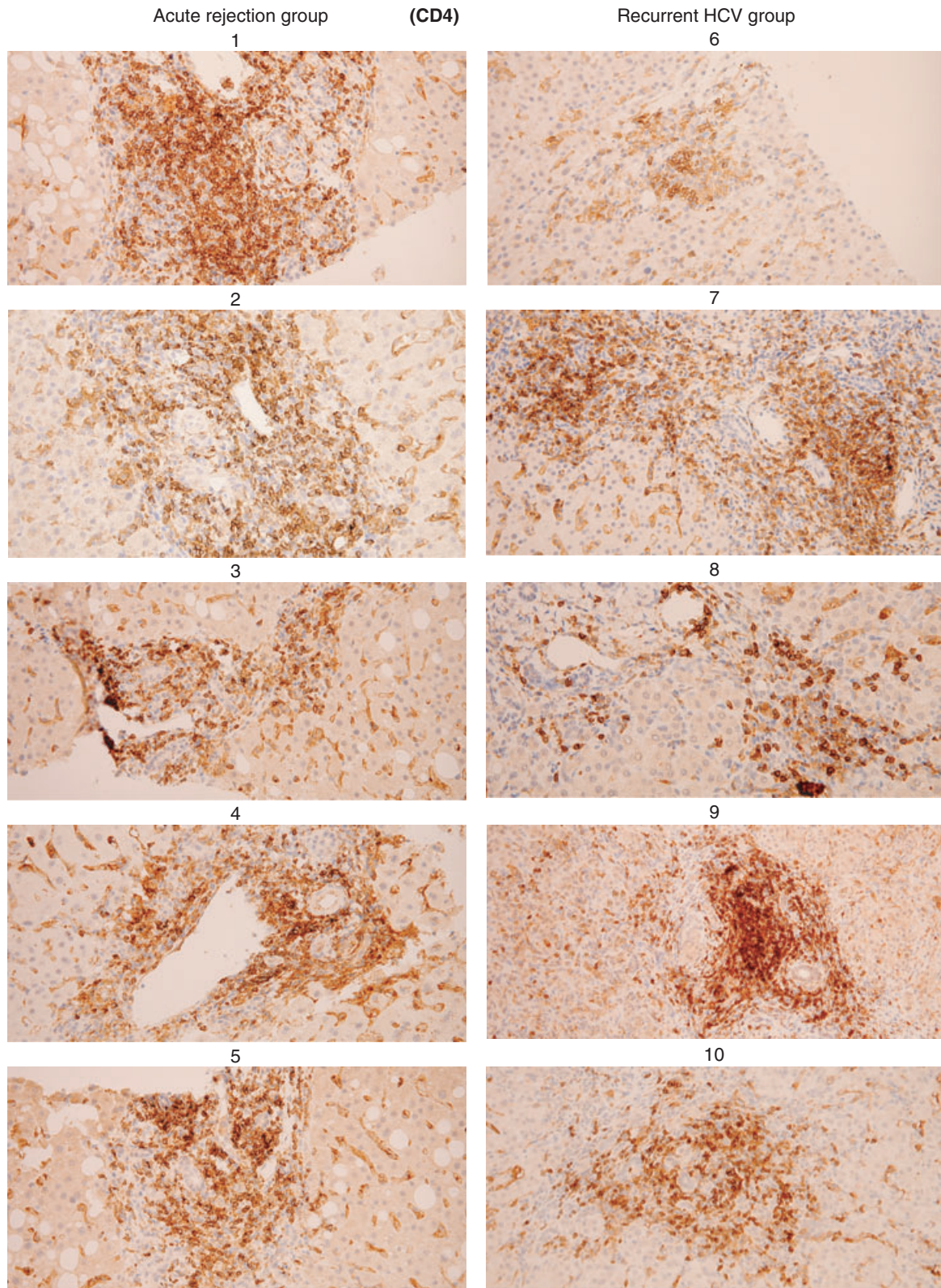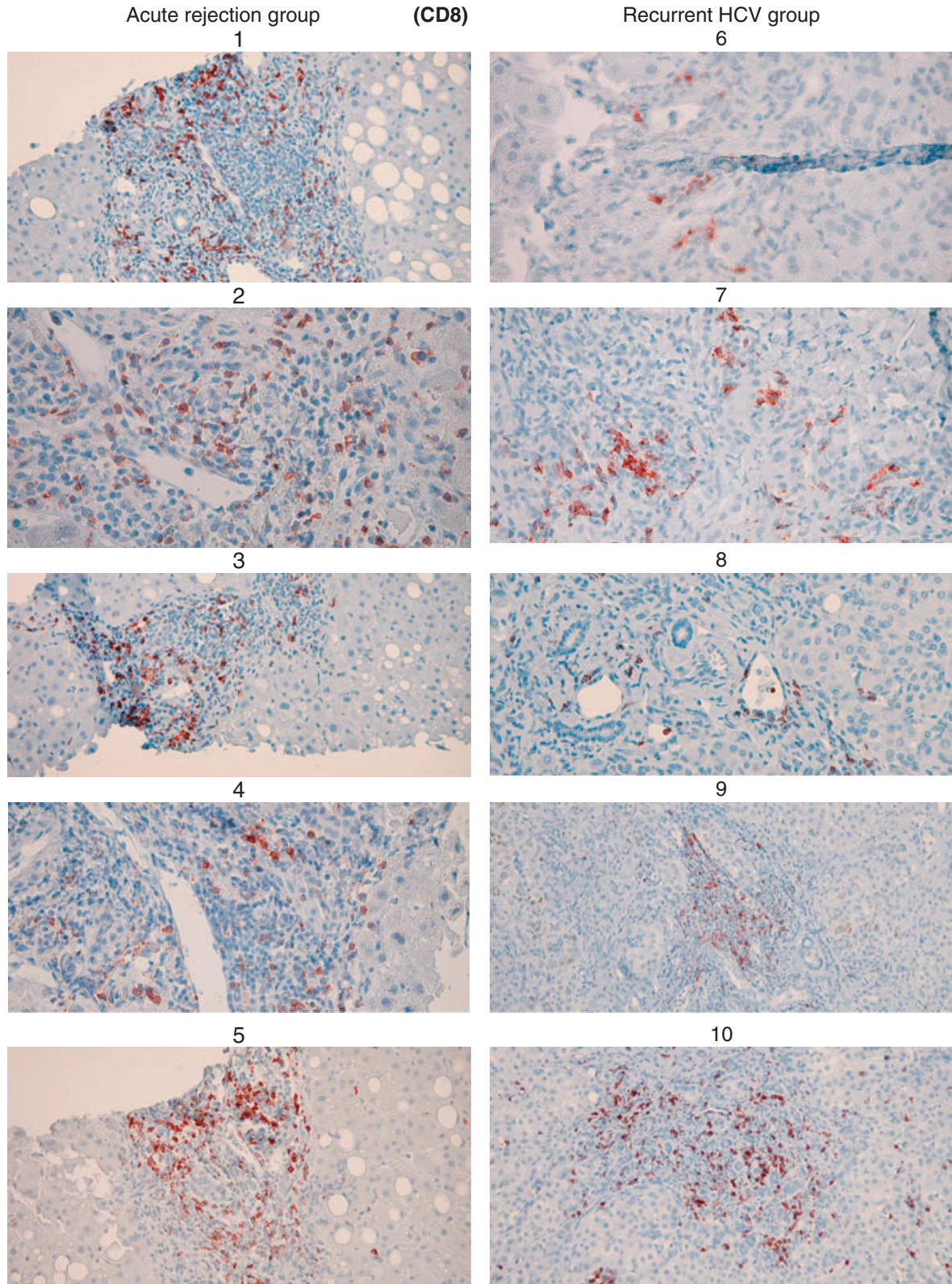
Acute rejection group  **(CD4)**  Recurrent HCV group



*Fig. 2.* CD4 immune-labeling (bright red indicates positivity) of lymphocytres in portal triads (400×). Numbers represent individual case numbers from Table 1. Cases 1–5; acute cellular rejection group on left. Cases 6–10, recurrent Hepatitis C viral group on right.

Acute rejection group    **(CD8)**    Recurrent HCV group



Fig. 3. CD8 Immune-labeling (bright red indicates positivity) of lymphocytes in portal triads (×250). Numbers represent individual case numbers from Table 1. Cases 1–5; acute cellular rejection group on left. Cases 6–10, recurrent Hepatitis C viral group on right. Magnification: Cases 1, 3, 5, 9, and 10: 250×, Cases 2, 4, 6, 7, and 8: 400×.

Acute rejection group     **(C4d)**     Recurrent HCV group

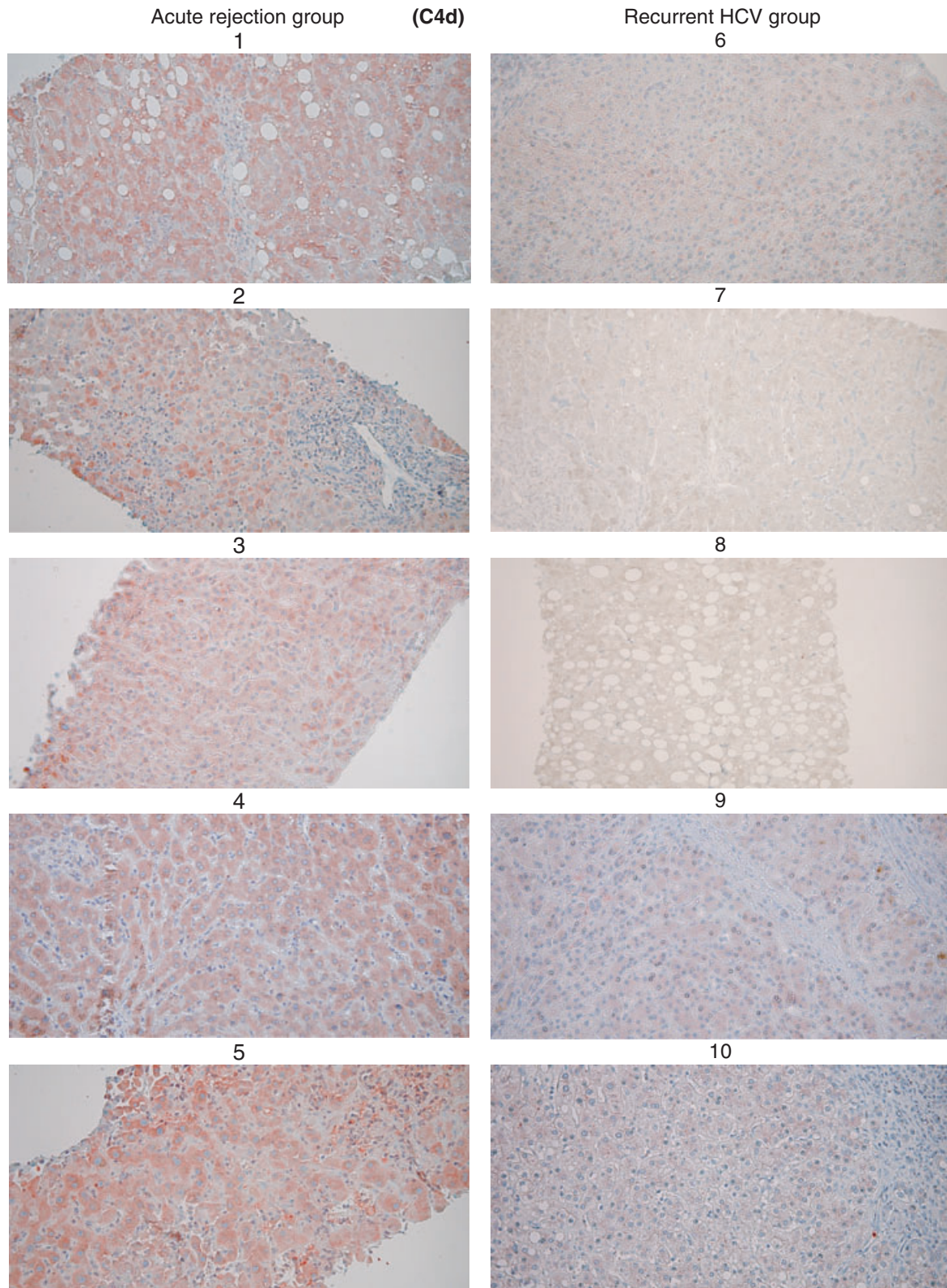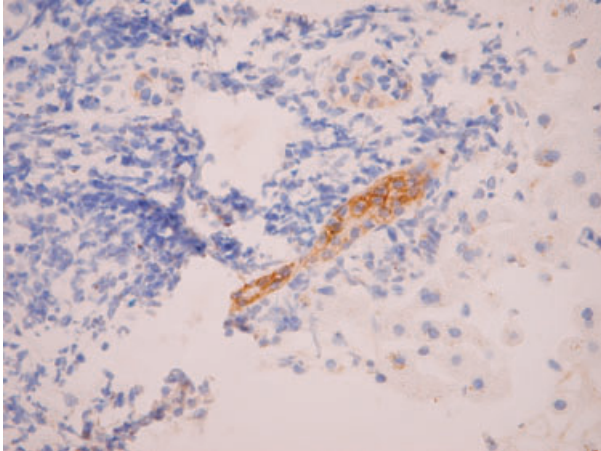*Fig. 4*. C4d immune-labeling of hepatic lobules. The intensity of red staining corresponds to the amount of C4d (×250). Numbers represent individual case numbers from Table 1. Cases 1–5; acute cellular rejection group on left. Cases 6–10, recurrent Hepatitis C viral group on right. Magnification: Cases 2, 3, 5, and 8: 250×, Cases 1, 4, 6, 7, 9, and 10: 400×.

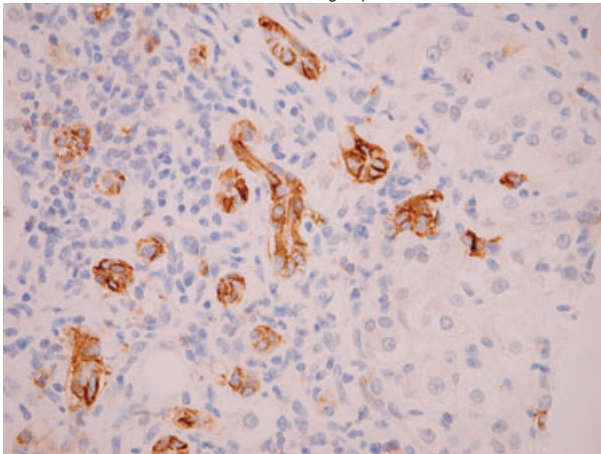**(CD56)**

Acute rejection group: 4



Recurrent HCV group: 10



*Fig. 5.* Representative cases stained with CD56 immune-stain. Red color indicates bile duct positivity (×250). Numbers represent individual case numbers from Table 1.

increase exponentially in coming years. Virological recurrence of disease after successful LTx is almost universal (1–3). A significant number of these patients develop progressive fibrosis and allograft failure 3–5 yr after LTx. A small number of these patients develop fibrosing cholestatic hepatitis which is a rapidly progressive disease leading to allograft failure. Hepatitis C viral recurrence is seen in both live donor and deceased donor LTx patients. The currently available anti-HCV therapy has approximately 20% success rate in achieving sustained viral response and also has considerable side effects resulting in premature termination of therapy. Also, renal insufficiency secondary to calcineurin-based immunosuppression limits the use of Ribavirin to a great extent (23), which further decreases the response rate.

Accurate diagnosis of recurrent HCV on liver biopsy in liver allografts and its distinction from ACR is essential. It is often difficult to definitively

distinguish recurrent HCV from ACR on H&E staining, even by experienced pathologists (4). It is very important to differentiate between the two conditions, since ACR is most commonly treated with augmentation of steroids and immunosuppression, which would allow permissive environment for multiplication of HCV and worsening of recurrence. The common feature of both these conditions is the presence of lymphocytic infiltrates on liver biopsy. It is also a well-known fact that recurrent HCV infection is associated with an increased risk of chronic rejection (24).

Monoclonal antibodies are used to characterize the types of cells in lymphocytic infiltrates. The distribution of CD4 and CD8 positive cells in lymphocytic infiltrates of biopsies has been described in patients with PBC, PSC, and autoimmune hepatitis (5). A predominance of CD4 positive cells in the portal triads and CD8 positive cells in areas of necrosis has been reported by Hashimoto et al. (5). Similar observation has been reported in chronic hepatitis patients by Banner et al. (25). It has also been hypothesized that defective CD4 response against HCV is responsible for chronic active hepatitis (26). Imada et al. have shown decrease in CD8+ memory T lymphocyte infiltrates with interferon therapy in HCV patients (27). Leroy et al. have shown the role of CD8+ lymphocytes in hepatic damage during chronic HCV infection with their inability to control viral replication (28).

Role of CD56 staining in bile ducts and ductules has been described by Torbenson et al. (29). CD56 positive cells are seen in the bile duct and ductules of patients with extrahepatic biliary atresia and in patients with dead or regenerative cells after damage to the bile duct (22).

In kidney transplantation, although majority of acute rejections are cellular in nature, mediated by T-lymphocytes, the role of humoral component in ACR, mediated by B-lymphocytes has been recognized recently (30). Kidney biopsies of patients with humoral rejection exhibit C4d deposits in the peri-tubular capillaries (16, 31–35). It is also known that renal allograft with C4d deposits have poorer outcome (13, 14). Such observations have also been reported in cardiac allografts with rejection and lung biopsies and BAL of lung transplant patients with acute rejection (15–18).

Sawada et al. (19) and Dankof et al. (20) have independently described C4d deposits in portal capillaries and sinusoidal areas of liver allografts with acute rejection.

We stained CD4, CD8, and CD56 positive cells using monoclonal antibodies to characterize the

infiltrates and studied the pattern of C4d deposition in post-liver transplant biopsies. We chose post-LTx HCV negative patients who were clinically strongly suspected to have acute rejection based on documented non-compliance with immunosuppressive medications for several days to several weeks, undetectable levels of calcineurin inhibitors and elevated biochemical parameters indicative of hepatic injury. Similarly, we also identified another group of five patients with recurrent HCV infection with high viral load who were known to be compliant with immunosuppressive medication and had therapeutic trough concentrations of calcineurin inhibitors.

As expected, mean CD4 counts in recurrent HCV was lower compared with patients experiencing acute rejection. However, it did not reach statistical significance. There were no differences in CD8 counts or CD56 counts in both groups.

However, the most striking difference was moderate to strong C4d immunohistochemistry staining in hepatocytes of four of five patients with acute rejection, while all patients with recurrent HCV had none to mild C4d deposits. One patient in the ACR group who had mild C4d stain (Case 3) had received polyclonal anti-rabbit antibody treatment ('Thymoglobulin', which is also known to have anti-B cell activity). Another patient (Case 1), who was initially C4d positive, experienced another episode of rejection and was treated with thymoglobulin had negative C4d staining after nine months. In kidney transplantation, C4d deposits are known to disappear after successful anti-rejection therapy (36).

Our pilot project suggests that C4d deposits in hepatocytes may be an important diagnostic tool and may be employed to differentiate between ACR and recurrent HCV and perhaps can be used when in doubt. Other studies using C4d stain in liver allografts have reported staining of sinusoids, portal veins and hepatic arteries but not in the cytoplasm of hepatocytes. More prospective studies in larger populations would be helpful to confirm our observations.

## Conclusion

Semi-quantitative counts of CD4, CD8 or CD56 monoclonal stains failed to distinguish between ACR and recurrent HCV. Semi-quantitative polyclonal complement C4d stains showed moderate to strong deposits in hepatocytes of 80% of patients with ACR and none to minimal staining in hepatocytes of all patients with recurrent HCV. More prospective studies are required to confirm our findings, which may provide a useful tool to distinguish between ACR and recurrent HCV.

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