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# Significance of complement split product C4d in ABO-compatible liver allograft: Diagnosing utility in acute antibody mediated rejection

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ABSTRACT

Diagnosis of liver allograft antibody-mediated rejection (AMR) is difficult and requires a constellation of clinical, laboratory and histologic features that support the disease and exclude other causes. Histologic features of AMR may intermix with those of biliary obstruction, preservation/reperfusion injury, and graft ischemia. Tissue examination for complement degradation product 4d (C4d) has been proved to support this diagnosis in other allografts. For this reason, we conducted a retrospective review of all ABO compatible/identical re-transplanted liver patients with primary focus on identifying AMR as a possible cause of graft failure and to investigate the utility of C4d in liver allograft specimens. We reviewed 193 liver samples obtained from 53 consecutive ABO-compatible re-transplant patients. 142 specimens were stained with C4d. Anti-donor antibody screening and identification was determined by Luminex100 flow cytometry. For the study analysis, patients were stratified into 3 groups according to time to graft failure: group A, patients with graft failure within 0–7 days ($n=7$), group B within 8–90 days ($n=13$) and C >90 days ($n=33$). Two patients (3.7%) met the diagnostic criteria of acute AMR. Both patients experienced rapid decline of graft function with presence of donor specific antibodies (DSA), morphologic evidence of humoral rejection and C4d deposition in liver specimens. C4d-positive staining was identified in different medical liver conditions i.e., acute cellular rejection (52%), chronic ductopenic rejection (50%), recurrent liver disease (48%), preservation injury (18%), and hepatic necrosis (54%). Univariate analysis showed no significant difference of C4d-positive staining among the 3 patients groups, or patients with DSA ($P>.05$). In conclusion, AMR after ABO-compatible liver transplantation is an uncommon cause of graft failure. Unlike other solid organ allografts, C4d-positive staining is not a rugged indicator of humoral rejection, thus, interpretation should be done with caution to avoid diagnostic dilemmas.



## 1. Introduction

Unlike renal, heart and lung allografts [1–3], HLA related antibody mediated rejection (AMR) does not cause a major concern after ABO compatible/identical liver transplantation [4,5]. Nevertheless, it is acceptable to transplant livers in crossmatch positive individuals. Liver allograft is currently considered a "unique immune graft" which not only resists humoral rejection caused by HLA related antibodies, but also creates an immune shield that protects other allografts when transplanted simultaneously (e.g. kidney [6,7]).

Over the past decade, few reports have described isolated incidences of AMR without cellular rejection occurring after ABO-compatible liver transplantion [8–11]. In these reports, patients experienced early graft failure, hemodynamic instability and coagulopathy. Liver pathology showed morphologic evidence supportive of AMR, C4d-positive staining and rising titers of anti-HLA donor specific antibody (DSA). Despite aggressive antihumoral rejection therapy, outcome was dismal and grafts failed within 3 months. Liver re-transplantation remained the only hope for these patients. For this reason, we conducted a retrospective review on re-transplanted liver patients with a narrow focus on early graft

*Abbreviations:* HLA, Human leukocyte antigens; PNF, primary non-functioning graft; EGF, Early graft failure; LGF, Late graft failure; PRA, Panel reactive antibody; AMR, Antibody mediated rejection; C4d, Complement split product 4d; POD, Postoperative day; DSA, Donor Specific Antibody; NIH, National Institutes of Health; IHC, immunohistochemistry; H&E, Hematoxylin & Eosin; AHG, Antihuman globulin.

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[1] I state that the material is original research, has not been previously published and has not been submitted for publication excluding abstract elsewhere while under consideration. An abstract form was presented at the United States and Canadian Academy of Pathologist.

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failure. Our main objective was to search for similar incidents that may have been missed and led to graft failure. Due to the lack of diagnostic consensus regarding AMR in ABO-compatible liver allografts, we analyzed cases adapting the constructed National Institutes of Health (NIH) working criteria for other solid organs [12] which have been subsequently incorporated in the Banff'09 report [13]. In the NIH consensus report, four criteria were described in AMR; (1) clinical evidence of graft dysfunction, (2) morphological evidence of tissue damage, (3) presence of DSA and (4) anatomical evidence for antibody complex formation, i.e. C4d staining. These criteria were also used by other authors in their studies [9]. Currently, two techniques are known for C4d staining: immunofluorescence from fresh tissue sample and immunohistochemical (IHC) stains from paraffin embedded tissue. The IHC technique is widely accepted as a marker for humoral rejection [14–16], with slight superiority to the fresh tissue preparation.

## 2. Patients, material and methods

### 2.1. Patients

After obtaining the Institutional Review Board approval (IRB app 6040), the liver transplant database was searched for patients who received multiple adult ABO-compatible orthotropic liver transplantation between January 2004 and May 2010. This may include patients first transplanted before this period but received a second transplant during the study period. A total of 62 patients were re-transplanted in our center. Patients were then stratified into three groups based upon time to first allograft failure: group (A) *primary non-function* graft *(PNF)* or initial poor functioning graft that required liver re-transplantation in the first week post-transplant; group (B), *early graft failure (EGF)* patients experienced graft failure and were re-transplanted within 8–90 days; and group (C), *late graft failure (LGF)* are patients that underwent re-transplantation after 91 days. The reasons for stratifying patients into 3 groups were because significant episodes of AMR were described in the first 3 months post-transplant, late graft failure was most likely due to recurrent disease and to facilitate statistical analysis.

All patients in the PNF and EGF group were enrolled. Patients in the LGF group were chosen sequentially based upon the UNOS registration number. For example, each patient in the PNF or EGF group was compared with two cases (prior and consecutive case) in the LGF group, unless the prior/following patient was also a patient in group A or B. The reason that we went back to 2004 is because that was around the time we switched to the high-resolution Luminex100 flow cytometric based HLA antibody screening & identification.

### 2.2. Histological evaluation

Histopathological evaluation was performed by two histopathologists (AO, VS) and a pathologist in training (SA). Review was independent and without access to the clinical information. C4d staining evaluation and diagnosis of H&E stained specimens were done separately to avoid influence of H & E diagnosis on evaluation of C4d stain. Agreement was reached by discussion. Morphological criteria used for diagnosing humoral rejection were previously described in liver patients transplanted across the ABO barrier [17–19]. Despite the difference in alloantibodies versus isohemagglutinins, the histological changes remain similar. The 2004 NIH consensus criteria was used to establish the diagnosis of AMR [12]. All liver biopsies were performed due to some sort of liver dysfunction, or as part of a protocol biopsy, e.g., zero time biopsy. Cases were finally reclassified into three groups; *possible* for humoral insult when morphology, C4d staining and Luminex testing were supportive of AMR; *probable* for humoral rejection when two of the three previous parameters were convincingly present and *negative* when not enough evidence of AMR was present.

### 2.3. C4d immunostaining

We followed our standard method for C4d staining in kidney biopsies as [20]. All liver specimens were fixed in 10% neutral buffered formalin for 12 h and routinely processed. Paraffin embedded tissue blocks were cut into 4 um tissue sections and stained with Hematoxylin. Processing using deparaffinized tissue was performed. Antigen was retrieved using EnVision TM Flex antigen retrieval EDTA buffer (pH 9). Slides were then stained with Flex Peroxidase-Blocking reagent for 5 min to block the endogenous peroxidase. After washing, slides were incubated for 15 min with purified C4d anti-rabbit polyclonal IgG antibody (ARP, American Research Product, Inc. catalog #: 12–5000) (1:100 dilution). After washing, sections were incubated with DAKO's Envision Plus anti-rabbit primary antibody for 30 min. Then Envision Flex HRP was added to block the unbounded secondary antibody for 20 min. Finally, sections were stained with FLEX DAB + Chromogen (detection method, dark brown staining). Four sections from renal allograft explants diagnosed with AMR were used as positive controls. No frozen tissue was used for C4d immunofluorescence studies.

### 2.4. Interpretation of C4d immunostaining

C4d staining was considered positive when linear staining of the sinusoids and/or endothelium of the portal vessels (arteries and/or veins) was identified. This included more proximal large caliber blood vessels at the hilum of hepatectomy specimens. Hepatocyte staining, whether focal or diffuse, was not considered a true positive staining but was noted and discussed separately. Absence of detectable staining was considered negative. Other staining patterns such as portal stromal tissue, liver capsular, vessel elastic walls and intraluminal serum staining were considered non-specific and were ignored. We normally start reading the C4d slide using the 20× objective scanning power. Any positive staining was then semi quantified using a 40× objective into 1+, 2+ and 3+ as summarized in Table 1 [20]. To facilitate the statistical study we assigned an overall score to each positive case, which was basically the highest score of sinusoidal and/or endothelial staining.

### 2.5. HLA typing

All HLA typing was performed by molecular methods. The recipient's Class I (HLA-A, -B, -C) and Class II (HLA-DQB1 and -DRB1,3,4,5) low-resolution typing was determined using polymerase chain reaction reverse sequence-specific oligonucleotide probes (PCR-rSSOP) with commercially available reagents (One Lambda, Inc. Canoga Park, CA) at the Henry Ford Transplant Immunology Laboratory. The deceased donor HLA typing was performed at Gift of Life Michigan using polymerase chain reaction - sequence specific primer (PCR-SSP) with commercial typing reagents (Invitrogen, Carlsbad, CA).

### 2.6. Antibody screening

Recipient HLA Antibodies were identified using Luminex100 based Labscreen panel reactive antibody (PRA) and Single Antigen bead (SAB) (One Lambda) assays at the Henry Ford Transplant Immunology

**Table 1**
Semiquantitative assessment of C4d staining using ×40 objective (400 magnification).

| |
|---|
| 0— No staining identified |
| 1+ Positive staining detected in <10% of total vasculature in a single or multiple fields. |
| 2+ Positive staining in 10–50% of total vasculature of at least one single field |
| 3+ Positive staining for >50% of at least one single focus. |