Detection of Humoral Rejection in Human Cardiac Allografts by Assessing the Capillary Deposition of Complement Fragment C4d in Endomyocardial Biopsies

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Background: There are no well-established diagnostic criteria to detect humoral rejection in organ transplantation. The value of commonly used markers in immunohistochemistry, such as C1q, C3c, IgG, IgM and fibrinogen, is questioned by some groups. Complement fragment C4d is a more stable marker of complement activation as it is covalently bound to graft capillaries. C4d has been shown to identify clinically relevant, but otherwise undetectable humoral anti-graft reactions in human kidney transplants.

Methods: Immunohistochemical techniques were used to evaluate 155 endomyocardial biopsies from 56 heart transplant recipients less than 3 months post transplantation for the presence of capillary C4d staining. In a subset of patients, C4d staining was compared with C1q, C3c, IgM and fibrin staining and was correlated with clinical outcome.

Results: Within 3 months 9 of 56 patients died. Five of these nonsurvivors had prominent C4d staining (p < .05), whereas C1q, C3c and IgM showed no correlation with clinical outcome. Presence of fibrin correlated with clinical outcome and C4d staining (p < .05).

Conclusions: The capillary deposition of complement split product C4d in human endomyocardial biopsies was significantly associated with graft loss. Determination of fibrin deposition may yield additional information to establish a diagnosis of humoral rejection. The immunohistochemical assessment of capillary deposition of C4d and fibrin appears to be an appropriate tool for the identification of patients, who may require additional or alternative immunosuppressive therapy targeted against the humoral immune system. J Heart Lung Transplant 1999;18:904–912.

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Humoral rejection is a relatively rare but potentially fatal form of acute allograft rejection. Following the initial systematic reports characterizing this disease entity, numerous investigators have described in detail the occurrence, histopathological features and clinical outcome of this type of rejection. Allosensitization against graft determinants occurs either in the pre- or posttransplantation period. Preformed antibodies can lead to the phenomenon of hyperacute rejection. The de novo synthesis of antibodies directed against HLA antigens expressed on graft endothelium is also clearly associated with humoral rejection. In addition, there may be a complement activation solely by an activator surface via the alternative pathway, that is not yet proven for allotransplantation. The diagnosis of humoral rejection is made by histological criteria of endothelial swelling and vasculitis and by immunohistological demonstration of deposition of the complement split products C1q, C3 and the immunoglobulins IgG, IgM and IgA. In addition, fibrinogen and HLA-DR-staining have been suggested by some authors.

This diagnostic concept was questioned amongst others by Bonnaud et al, who found positive immunofluorescence with the suggested markers to be a nonspecific finding, detectable in normal myocardium as well as in cardiac allografts. Moreover, in this study positive immunofluorescence did not allow to identify patients with unexplained graft dysfunction of presumed immunologic cause. As a consequence, Bonnaud et al suggested that the usefulness of routine immunofluorescence for surveillance of acute humoral rejection might be questionable. Thus, there are no well established diagnostic criteria to diagnose humoral rejection in organ transplantation.

The detection of capillary deposition of immunoglobulins and complement split products, as in humoral rejection, depends on the rapidity of their turnover. Following a humoral attack, endothelial cells rapidly remove deposited immunoglobulins from the cell membrane by shedding or internalization. In addition, endothelial cells can inhibit activation of and remove complement through the action of surface (membrane cofactor protein; decay accelerating factor; CD59) and circulatory control proteins. Thus, antibodies binding to endothelial surface antigens and most complement components will be detectable only transiently in capillaries due to a high turnover of binding and removing despite a persistent and clinically relevant humoral attack.

Based on this concept, the diagnostic yield might improve with the use of more stable markers of humoral immune reactions. In contrast to most other complement fragments, complement C4d is covalently bound to endothelium. C4d fragment is generated via the classical, that is, antibody-induced pathway of complement activation. The human complement protein C4 consists of three polypeptide chains that are linked by disulfid bonds. After proteolysis by activated C1s, a small C4a fragment is released into the bloodstream whereas on the remaining large C4b-fragment, a now exposed highly reactive thioester-group reacts covalently either with the activating antibodies or the surface of the endothelial cells. C4b bound to endothelium is rapidly processed into two fragments, C4c, that goes into solution and C4d, that contains the covalently bound thioester group and resists shedding off endothelial cells.

Capillary deposition of C4d complement fragment was previously shown to identify clinically relevant but otherwise undetectable humoral antigen reactions in human kidney transplants. In these studies the authors demonstrated a stronger association of renal graft loss with complement C4d deposition than with other risk factors.

This study in human heart transplant recipients was designed to explore the diagnostic potential of C4d complement fragment in humoral cardiac allograft rejection. It was to be investigated whether C4d staining in capillaries is detectable in endomyocardial biopsies and whether it is, as a single criterion for the presumed presence of a humoral immune reaction, related to individual clinical outcome. Moreover, in a subset of patients, C4d and the conventional markers for humoral rejection were compared in their ability to predict clinical outcome.

**MATERIALS AND METHODS**

**Patients and endomyocardial biopsies**

A total of 243 right ventricular endomyocardial biopsies (EMB) of 56 patients less than 3 month posttransplantation were evaluated for this study. Only patients in whom serial EMB could be harvested, were eligible for this study. Four myocardial fragments were fixed in formalin; grading of acute cellular rejection was performed according to the modified criteria of the International Society for Heart and Lung Transplantation. One myocardial fragment was snap frozen in liquid nitrogen and used for this study. Of 243 EMB, 88 had to be excluded as they were not of...
sufficient quality for immunohistochemical evaluation. Thus, 155 EMB of 56 patients were included. Deposition of C4d was assessed in all specimens, by immunofluorescence staining without knowledge of histopathological diagnosis. In a subset of 92 EMB in 41 heart transplant recipients, C4d deposition was compared to deposition of IgM, C1q, C3c, and fibrin. Fibrin as the activated component of fibrinogen was assessed in this study in contrast to some authors who prefer fibrinogen.

The clinical status of the patients, defined as clinically suspected acute rejection or hemodynamic impairment as determined by echocardiography, was assessed at each visit an EMB was performed. Accordingly, patients were assigned stable or unstable and a correlation with the EMB was performed.

**Immunosuppressive therapy**

Patients were either treated with FK 506 (tacrolimus, \( n = 20 \)) or cyclosporine A (CsA, \( n = 36 \)). All patients received steroids and azathioprine. Episodes of rejection were treated with high-dose steroids for 3 days (\( n = 9 \)); OKT3 immunoglobulines were used as rescue therapy (\( n = 3 \)). CD4 chimeric antibodies (\( n = 8 \) patients) and ATG (\( n = 3 \) patients) induction therapy were given in a subset of patients with CsA. No induction therapy was given to the other 45 patients.

**Assessment of panel reactive antibodies**

Sera of patients with congestive heart failure waiting for HTX were screened for the presence of lymphocytotoxic antibodies. Positive serum samples were routinely investigated with a total of 150 typed lymphocyte samples in order to determine antibody specificity. A panel reactivity of greater than 30% was classified as “immunological risk,” and a reactivity greater than 84% as “highly immunized.”

**Monoclonal antibodies**

Commercially available antibodies were used in this study. The sources, specificities, and dilutions of antibodies are detailed in Table I. The anti-C4d antibody used has been purified from mouse ascites fluid and tested for purity by SDS-polyacrylamide gel electrophoresis by the manufacturer. The monoclonal antibodies M4d1, M4d2, M4d3 and the commercially available antibody used for this study react with the C4d (\( \alpha_2 \)) fragment of human C4, as determined by Western blot analysis. These antibodies have been characterized in detail elsewhere.

Each staining procedure included antibody, phosphate buffered saline (PBS) and controls. These were done at room temperature by exposure of cryostate sections to only PBS, isotype-matched antibody, or conjugate.

**Immunohistochemical staining**

Immunohistochemical staining was performed as previously described.

In brief, 6 \( \mu \)m air dried cryostat sections were fixed in cold acetone (Merck, Germany), rehydrated in PBS at pH 7.4 and incubated with appropriately diluted primary monoclonal antibody for one hour at room temperature. Directly fluorochrome-conjugated antibodies were incubated, 3 times washed in PBS for 10 minutes and coverslipped in permafluor (Immunotech). After the primary antibodies were washed three times in PBS for 10 minutes, the secondary anti-species antibody to the isotype of the primary antibody was incubated for one hour, washed again three times in PBS for 10 minutes and coverslipped in permafluor.
Microscopy and quantification of immunocytochemistry

Tissue preparations were studied by a Leica DMRBE, fluorescence microscope (Leica, Germany). A Wild MPS 52 camera that contained an ektachrome 64T colour reversal film was used for documentation. This was done the day after the staining procedure, and the sections were kept in a refrigerator overnight.

Immunofluorescence reactivities were graded for statistical purposes from coded photomicrographs of equivalent magnifications. These were projected from the same distance in a darkened room and scored by 2 investigators (TMB, KR) unaware of the origins of the biopsies, as previously reported by other groups. This quantification scheme was used for all antibodies. Routine surveillance of acute cellular rejection was performed independently by an experienced investigator.

Statistical analysis

Correlations of data were done using the two-tailed Fisher exact test. A p value of <.05 was considered to be significant.

RESULTS

Distribution of complement fragment C4d in capillaries of endomyocardial biopsies

The EMB were subdivided into 4 groups according to the degree of deposition of C4d in interstitial capillaries: biopsies were assigned C4d+++ when all capillaries showed marked deposition of C4d and C4d++, when segmental staining of capillaries was found; C4d+ denoted staining of only a few capillaries and C4d− absence of staining in capillaries. Patients were allocated to each group according to their maximal score in any biopsy investigated. Table II shows the numerical distribution of the EMB among the C4d subgroups.

Graft loss and clinical status in relation to capillary deposition of C4d

Within the first 3 months after transplantation, 9 of 56 (16%) patients died. As many as 5 fatalities within 3 months were recorded in the C4d+++ group compared with only 3 fatalities in the C4d− group. In the C4d+ group, 1 patient was lost. The association of capillary deposition of C4d+++ with subsequent fatalities vs C4d++, C4d+ and C4d− and fatality was found to be statistically significant (p < .0112). C4d+++ deposition in nonsurvivors occurred in the last EMB before death in 4 patients. Of these, 2 patients had 2 consecutive C4d+++ positive EMB before death. One patient had initially C4d+++ intensity and C4d++ in a subsequent EMB before he died.

The clinical status of the patients, as defined above, correlated with the intensity of C4d staining (p < .0017). Thus, in addition to the 5 fatalities, 3 patients of the C4d+++ group with no induction therapy showed a reduced myocardial function assessed by echocardiography. Although histology did not reveal cellular rejection, high dose glucocorticoid therapy was initiated. EMB were immunohisto logically graded less severe in a subsequent EMB.

Pathology in the lost grafts was available in 6 of 9 patients. Immunohistochemistry was not performed in the autopsy hearts. The time between the last

<table>
<thead>
<tr>
<th>Staining pattern of capillary C4d deposition</th>
<th>C4d+++</th>
<th>C4d++</th>
<th>C4d+</th>
<th>C4d−</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMB</td>
<td>14</td>
<td>26</td>
<td>46</td>
<td>69</td>
</tr>
<tr>
<td>Patients CsA</td>
<td>8</td>
<td>17</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Patients FK506</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Mean donor age years</td>
<td>36 ± 13</td>
<td>31 ± 12</td>
<td>35 ± 11</td>
<td>35 ± 14</td>
</tr>
<tr>
<td>Mean recipient age years</td>
<td>49 ± 11</td>
<td>50 ± 12</td>
<td>55 ± 5</td>
<td>44 ± 14</td>
</tr>
<tr>
<td>Cold ischemic time (minutes)</td>
<td>178 ± 57</td>
<td>190 ± 64</td>
<td>220 ± 69</td>
<td>209 ± 62</td>
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<td>Panel reactivity &gt;30%</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Induction therapy ATG</td>
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<td>1</td>
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</tr>
<tr>
<td>Induction therapy anti CD4</td>
<td>2</td>
<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>Acute rejection 1 A/B</td>
<td>3</td>
<td>11</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Acute rejection ≥3A/B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Graft losses (within 3 month)</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>3</td>
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</table>
EMB and death was 3.2 days (range 13 hours to 7 days). Patient 1 initially had a problem-free postoperative reconvalescence. Four weeks after the operation, he had repeated episodes of cellular rejection, once also Grade 3a/b that were treated with glucocorticosteroids and methotrexate. The last available biopsy showed acute cellular rejection Grade 1b. The patient died on the respirator in cardiovascular failure. Autopsy showed no cellular rejection. Immunohistochemically, the last 2 EMB presented with C4d+++ and fibrin++. Patient 2 had recurrent episodes of cellular rejection Grade 1a/b, but was discharged from hospital after 4 weeks. Her last EMB showed rejection Grade 1a. The same day she was found dead in her bed. Autopsy revealed Grade 3b rejection. Her last EMB showed C4d+++ and fibrin++. Patient 3 developed right heart failure early postoperatively. No cellular rejection was found in 2 consecutive EMB, but immunohistochemistry showed C4d+++ in his first and C4d++ in his second biopsy. Fibrin was not helpful. Pathology did not reveal further causes of death. Patient 4 developed rhythm problems 3 weeks after the operation and a DDDR-pacer was implanted. Despite flawless pacer function, 6 weeks postoperatively the patient was found dead in bed. Pathology showed cellular rejection Grade 1b. Immunohistochemistry showed C4d+++ and fibrin++. Patient 5 died 7 weeks postoperatively presenting a fresh myocardial infarction and pneumonia at autopsy. No cellular rejection was found and immunohistochemistry showed C4d+. Patient 6 showed repeated episodes of higher grade cellular rejection and died in multiorgan failure with acute cellular rejection Grade 3a. Immunohistochemistry was negative.

**Distribution of conventional immunohistochemical markers compared to C4d**

The distribution of capillary deposition of IgM, C1q, C3c and fibrin was assessed as suggested previously by Hammond et al. in a subgroup of patients and subdivided into 4 categories as applied for C4d. As IgG is commonly found in EMB in our experience, it was not considered a helpful marker for detection of humoral rejection and omitted. Figure illustrates typical staining results for the different antigens. Compared to C4d, staining intensity of the conventional markers was usually less prominent with the exception of fibrin. Table III gives the numerical distribution of the EMB, patients and fatalities among the different subgroups. The association of capillary deposition and fatal clinical outcome was not found to be statistically significant for IgM, C1q, and C3c ($p > .05$). There was also no correlation between clinical status, defined as suspected acute rejection or hemodynamic impairment, and intensity of staining of conventional markers ($p > .05$). In Table IV the intensity of C4d staining vs the traditional markers for humoral rejection is given for each EMB. There was no statistically significant association between more prominent C4d staining (C4d++; C4d++) and the intensity for IgM, C3c and C1q.

Also in this subgroup, C4d+++ was significantly associated with fatality ($p < .0073$). The deposition of fibrin in capillaries was associated with C4d deposition ($p < .0421$) and correlated also with graft loss ($p < .0296$).

**Distribution of donor and recipient risk factors**

The distribution of several factors with possible impact on patient outcome is depicted in Table II. There were no apparent differences among the subgroups with respect to donor age, recipient age and panel reactivity. Induction therapy with ATG or anti CD4 was significantly associated with complement deposition but not with graft loss ($p < .043$). The histological grade of acute cellular rejection did not correlate with the C4d staining intensity.

Complement staining patterns, immunological risk factors and histological diagnosis in 9 non-survivors are summarized in Table V. Panel reactivity of more than 10% was not seen in any patient. Patients treated either with CsA or FK506 but no induction therapy did not show significant differences with regard to severe humoral rejection and clinical outcome ($p > .05$). In the CsA group, there was a trend towards more complement activation as compared to the FK506 group.

**DISCUSSION**

Capillary deposition of C4d complement split product revealed a substantial number of EMB with complement activation. As this complement split product was not assessed previously in EMB of heart transplant patients, we compared the staining pattern of C4d with the commonly used split products C1q and C3c. Furthermore, deposition of IgM and fibrin was assessed. C4d and fibrin proved to be more sensitive markers for early graft loss than C1q, C3c and IgM.

**C4d deposition and patient outcome**

The deposition of the “classical” complement split product C4d in capillaries of cardiac allografts was significantly associated with the clinical status and
fatal patient outcome early after transplantation. Donor and recipient risk factors with possible impact on patient outcome were not related to complement activation. There was a timely fashion of high C4d expression and fatal outcome, either the last EMB was C4d$$^{+++}$$ ($n = 4$) or C4d$$^{++}$$ and the preceding EMB was C4d$$^{+++}$$ ($n = 1$). Thus, C4d deposition may be a clinically relevant indicator for this presumed humoral immune reaction rather than an epiphenomenon.

There was no significant correlation between the deposition of IgM, C1q, and C3c in capillaries and the clinical status and outcome of the patients. The deposition of IgM in capillaries tended to be higher in EMB with a low score for C4d that is in line with earlier studies suggesting that IgM is not related to graft failure$^{26}$ and may even have a protective effect.$^{27}$ In contrast fibrin showed an association between deposition and outcome. Clearly, this appears to be in conflict with a vast number of reports confirming the usefulness of the markers IgM, C1q, and C3c. There is also a report in which the authors could not demonstrate the usefulness of these conventional markers.$^{12}$ As outlined above, endothelial cells in vivo rapidly remove deposited immunoglobulins after a humoral attack by shedding or

FIGURE  Example for an acute humoral rejection 35 days post transplantation. A marked C4d$$^{+++}$$ (a) staining in capillaries and veins corresponds to a prominent C1q$$^{+++}$$ (b) and fibrin$$^{+++}$$ (c) staining. C3c (d) as well as IgM (not shown) were not detectable (immunofluorescence staining; magnification $\times 250$).
Furthermore, endothelial cells are able to inhibit the activation of complement at early and late stages through the action surface proteins in concert with control proteins from the circulation. If an EMB is not performed within days of the onset of an acute humoral rejection, the immunohistological markers C1q, C3c and IgM may well be removed, whereas C4d, covalently bound, is still in situ. We found 9 EMB positive for C3c and 14 EMB positive for C1q out of 40 EMB negative for C4d. The intensity was low in the majority of EMB, however it supports the finding by Bonnaud et al. that deposition of these traditional markers may not always reflect humoral activation.

The fact that fibrin deposition was associated with C4d deposition in capillaries may indicate an impaired ability of endothelial cells for fibrinolysis due to the humoral attack. In addition, it is well known that complement split products interfere with the coagulation system. Fibrin deposition early after transplantation was associated with subsequent coronary artery disease and graft failure in a recent publication.

Complement deposition, immunological risk factors and immunosuppression

An induction therapy was applied in 11 of 56 patients of whom all received CsA. Eight patients received CD4 chimeric antibodies. Two of these patients were grouped C4d++, 6 were judged C4d+. ATG was given to 2 patients, one grouped as C4d++, the other one as C4d+. None of these patients died. This is in line with other groups that also report a higher incidence of complement activation in patients who received induction therapy. Immunosuppression with CsA vs FK 506 did not show a significant difference in severe complement activation and graft outcome if patients with induction therapy were excluded from analysis. CsA patients without induction showed a trend towards more frequent complement deposition.

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Deposition of IgM, C1q, C3c, fibrin and C4d in 92 EMB from 41 heart transplant recipients</th>
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<tbody>
<tr>
<td></td>
<td>IgM+++</td>
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<tr>
<td>EMB</td>
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<td>Patients</td>
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<td>Fatalities</td>
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<table>
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<tr>
<th>TABLE IV</th>
<th>Intensity of C4d deposition versus traditional markers of humoral rejection in 92 EMB from 41 heart transplant recipients</th>
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<tr>
<td></td>
<td>C4d+++</td>
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<td>IgM+++</td>
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<tr>
<td>Fibrin-</td>
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</table>

*Diagnosis was made at autopsy.
ing needs to be verified with a larger patient cohort than investigated here.

Pathology was not available in all fatalities due to various reasons. The findings in the 6 of 9 cases, that could be evaluated, were heterogeneous. Probably due to the small number of patients, we did not find a higher incidence of high-grade cellular rejection in combination with humoral rejection. The coexistence of these 2 entities of acute cellular rejection was observed earlier and was also found in this study. In the light of recent publications a mutual pathway leading to both forms of acute rejection may be the existence of preformed antibodies directed against HLA class II antigens. C4d staining may help to identify patients with a combination of acute cellular and humoral rejection, who may even have a higher risk.

**Limitations**

There are several limitations to this study. First, EMB are nowadays less often obtained per patient at our transplant unit for various reasons, resulting in smaller numbers of biopsies for investigation. Second, no screening for the de novo appearance of serum alloantibodies was done in parallel for correlation with immunohistochemical findings.

**Conclusion**

The capillary deposition of the complement split product C4d in human EMB is significantly associated with graft loss. The other markers C1q, C3c and IgM did not show the same correlation in the subgroup of patients investigated. Fibrin, however, may yield additional information for the diagnosis of humoral rejection. The interpretation of C4d staining in patients with anti-CD4 or ATG induction therapy was not feasible and knowledge of the patients immunosuppressive regimen is mandatory. In conclusion, the immunohistochemical assessment of C4d and fibrin deposition may represent a useful tool to identify patients, who may require additional or alternative immunosuppressive therapy targeted against the humoral immune system.

The authors thank Dr. Max Weiss for providing most of the acute cellular rejection analysis.

**REFERENCES**


