Review paper

Pathologic diagnosis of antibody-mediated rejection in endomyocardial biopsy after heart transplantation based on renewed International Society for Heart and Lung Transplantation criteria

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Heart transplantation is a well-established life-saving treatment for patients presenting with end-stage cardiac failure. Despite improved efficacy of the procedure, allograft rejection continues to be a major cause of mortality and morbidity in cardiact allograft patients. Although acute cellular rejection (ACR) is quite unusual nowadays, acute antibody-mediated rejection (AMR) remains a significant problem. The role of pathologists in detection of AMR is very important, especially in sub-clinical cases. In 1990, histologic hallmarks of AMR were first stated by International Society for Heart and Lung Transplantation (ISHLT) and detailed histopathologic features and immunopathologic criteria were established in 2005. Recently (2013) ISHLT revised nomenclature and classification of AMR. Aim of this paper was to present practical changes coming from new criteria as well as to highlight difficulties concerning AMR assessment in endomyocardial biopsies (EMB).

Key words: antibody-mediated rejection, C4d, heart transplantation, immunohistochemistry.

Introduction

Heart transplantation (HT) is a well-established life-saving treatment for patients presenting with end-stage cardiac failure. According to the literature and own experiences, two major reasons for HT are cardiomyopathy (68%) and coronary heart disease (32%) [1]. Transplants cannot be performed neither in patients with active infection, cancer, or severe diabetes mellitus nor in patients who smoke or abuse alcohol [1]. The first heart transplantation in human being was performed in 1967 by surgeon Christiaan Barnard [2]. Although, the surgery was a success, patient died eighteen days latter due to pulmonary complications caused by medications. Since than, immunosuppressive therapies have become more effective and our knowledge concerning transplantations has improved. Organs transplantations, including HT, are nowadays performed all over the world and the number of these procedures has been increasing over years. Last year (2012) in Poland estimated number of HT was 79, which comprised 16% of patients from active waiting list [3]. The overall number of HT that have been performed in Poland since 1998 to 2012 was 1455 [3] and it was the third, after kidney and liver, procedure according to frequency. Patients considered for HT should be under 70 years of age with complete medical history and follow-up assessment carried out at least every 3 months. The patient's weight should be obtained at each visit and body mass index (BMI) should be calculated. Immunocompatibility testing should include ABO blood group typing, completed on two separate occasions. Donor hearts are not selected on the basis of human leukocyte

antigens (HLAs) because of time restrictions related to cardiac preservation, nonetheless, tissue type should be determined for retrospective analysis and may assist with determination of donor-specific antibodies (DSAs). Screening for humoral sensitization is accomplished to determine the presence of circulating anti-HLA antibodies [1]. Although donor-recipient compatibility tests performed before HT are more and more accurate, allograft rejection is believed to be a major cause of mortality and morbidity in cardiact allograft patients nowadays. Acute cellular rejection (ACR) has been precisely described and the advent of immunosuppressants significantly lowered its frequency. Acute antibody-mediated rejection (AMR) has been variably defined and is still considered a significant problem [4] thus proper diagnosis of allograft rejection is a major therapeutic target. Endomyocardial biopsy (EMB) remains the gold standard examination for detection of ACR and AMR [5, 6] with crucial role of pathologists in diagnostic process, especially in sub-clinical cases. The pathologic description of AMR and its association with unfavorable outcome after HT was first reported in 1989 [7]. In 1990 initial International Society for Heart and Lung Transplantation (ISHLT) stated histologic hallmarks of AMR defined as "positive immunofluorescence, vasculitis or severe edema with absence of cellular infiltrates" [8]. Detailed histopathologic features and immunopathologic criteria of AMR were established in 2005 and have been applied until recently [6]. Morphologic findings comprised: endothelial swelling, interstitial edema, intracapillary macrophages and leucocytes, myocyte injury, and necrosis, demonstrated on routine hematoxylin and eosin (HE) stain. Immunopathologic features were summarized as capillary deposition of immunoglobulin (IgG, IgM and/or IgA) and complement activation byproducts (C3d, C4d and/or C1q) by immunofluorescence or capillary deposition of C4d complement activation byproducts and the presence of CD68(+) intravascular macrophages by formalin-fixed paraffin-embedded immunochemistry of the myocardium. Presence of allograft dysfunction and/or circulating DSAs has been required to establish AMR diagnosis. In 2013 nomenclature and classification of AMR was updated as a result of 3 ISHLT workshops that focused on interpretation of EMB [7, 9]. Our aim was to present practical changes deriving from new criteria and to highlight difficulties concerning AMR assessment.

Acute antibody-mediated rejection

Antibody-mediated rejection (also called humoral) is a condition diagnosed when host antibodies are directed against donor antigen which may lead to allograft injury. Different types of rejection varying in acuity and severity are distinguished. Hyperacute, acute and chronic humoral rejection are recognized. They differ in mechanism and time of presentation after transplantation. Hyperacute rejection occurs within minutes to hours of the blood flow being reestablished and is caused by preformed antibodies to ABO blood group antigens, HLA, or endothelial antigens. Antibodies in the blood of the recipient bind to the vascular endothelium of the transplant and activate complement, which results in neutrophil infiltration, vascular disruption, hemorrhage, fibrin deposition and platelet aggregation [10]. It is rare owing to tests for DSAs [11]. Acute humoral (also called vascular) rejection occurs days to weeks after HT. The alloantibodies are directed against donor HLA or endothelial cell antigens. It occurs in 6% of patients and its importance stems from its common association with severe ventricular dysfunction, presumably caused by diffuse ischemia secondary to a lack of coronary vasodilatory reserve [11]. Chronic rejection occurs months to years after transplantation and its mechanism is incompletely understood. The exact mechanism of AMR has not been completely understood, however, several *in vivo* experiments have been conducted upon this issue. According to published data macrophage, B-cell and T-cell responses contribute to transplant rejection [12-14]. Although, complement-activating alloantibodies have been connected with AMR, there are studies [8] that proved contribution of non-complement-activating alloantibody. In heart transplant recipients AMR is clinically associated with an increased expression of P-selectin and von Willebrand factor on the vascular endothelium [15], which lead to vascular inflammation and extensive aggregates of platelets that occlude the arteries, capillaries and veins [16, 17]. This mechanism explains why AMR in heart EMB is usually referred to coronary allograft vasculopathy (CAV) and manifests as diffuse atherosclerosis with myointimal proliferation in the coronary arteries [9, 11]. It was estimated that about 50% of heart transplant recipients have angiographically confirmed CAV by 5 years after transplantation, and severe CAV is a major cause of death in patients surviving the first post-transplantation year [18, 19]. Other studies [20, 21] suggested that cytokines/chemokines such as interleukin (IL)-1-α, IL-8, RANTES and monocyte chemotactic protein 1 (MCP-1) are upregulated in rejected hearts. These molecules recruit neutrophils and monocytes to the site of injury. Another protein that has been connected with AMR mechanism is mannose-binding lectin (MBL) which is known to bind to carbohydrates on microorganisms as well as IgM and IgG [22, 23]. According to published data [24, 25] it is most probable that MBL correlates positively with C4d deposition. Wasowska *et al.* [10] suggested combine mechanism of MBL depending

on both complement-activating and non-activating antibodies. Nonetheless, further studies are needed within this pathway. Because in clinical specimens, macrophages and monocytes have been recognized as a correlate of humoral rejection, it has been proposed that receptors for the Fc domain of IgG (FcγR) are involved in AMR mechanism [26, 27]. Engagement of stimulatory FcγRs, especially FcγRIII by IgG1 causes the accumulation of macrophages in sites of inflammation and blocks their apoptosis [25, 26]. All these mechanisms lead to specific features present in histologic specimens and thus EMB remains the gold standard test for detection of AMR [5, 6] with crucial role of pathologists in diagnostic process. To state histologic and immunohistologic criteria of AMR alone, has always been one of major ISHLT goals, and as a result, initial criteria comprising of characteristic morphologic features, defined immunofluorescence/immunochemistry stainings together with presence of DSAs, have been applied since 2005. In practice, stain for C4d deposits has been routinely used as a marker of AMR in EMB evaluation. However, AMR has recently been recognized as a continuum of 4 stages in the development of AMR, ranging from latent, silent, sub-clinical, to symptomatic stages. Only in stage III (sub-clinical) there are identifiable pathologic alterations in the graft in addition to deposition of complement split fragment C4d and circulating DSA even without symptoms of graft dysfunction [28]. It is well known, that patients with C4d positivity have worse outcome, thus, 3 following international consensus conferences under the auspices of ISHLT revised nomenclature and classification of AMR. New classification for AMR established in 2013, identifies a grading scheme for the pathologic diagnosis of AMR [9]. The categories for the reporting of pathologic AMR (pAMR) are presented in Table I. The most important outcome of new criteria is that clinical dysfunction or positive DSAs are no longer required for firm AMR diagnosis [9]. International Society for Heart and Lung Transplantation specially focused on intensity (weak or strong) and distribution (focal vs. diffuse) of both C4d and CD68(+) stains. The hallmarks of AMR are now strong positive multifocal/diffuse C4d staining and at least focal intravascular positive CD68(+) staining. Primary and secondary antibody panels for pathologic diagnosis of AMR (pAMR) were also settled.

C4d staining

C4d is a split product of C4 that is generated in the process of complement activation by classical pathway. Classical pathway of complement is most efficiently activated by antibodies of the IgM or IgG class, therefore C4d is used as a common marker of AMR [29]. In heart transplantation, immunopathologic features of AMR were summarized as capillary deposition of C4d by immunofluorescence (IF) or by formalin-fixed paraffin-embedded immunochemistry (IHC) of the myocardium [9]. The staining of venular, arterial, or arteriolar endothelial cells, arterial elastic lamina, the capillaries in Quilty effect are not considered to be indicative of AMR in either IF or IHC techniques (Fig. 1A,B) [9]. In paraffin IHC, C4d presents characteristic "donut" pattern in cross-sectional vessels (Fig. 2A,B) and an "elliptical" pattern in longitudinally cut vessels. Recently, ISHLT proposed C4d intensity and distribution scoring system. Referring to intensity of C4d, 3 categories are recognized: 0 – negative; 1 – faint positive staining and 2 – strong positive staining. Distribution of C4d is categorized as: < 10% capillaries, no staining; 10% to 50%, focal staining and > 50%, multifocal/diffuse; with > 50%, being considered as positive [9]. Strong C4d positivity in < 50% capillaries should be interpreted as negative result while diffuse weak C4d staining is classified as positive result. In both situations, it is recommended to correlate pathologic findings with clinical condition and DSA studies. Subsequent specimens should be stained for C4d until a negative result is obtained [29]. C4d positivity may not be seen in severe AMR due to loss of endothelial integrity but is very helpful in diagnosis of early-onset AMR [9].

Table I. Categories for reporting pAMR according to ISHLT 2013 criteria

pAMR	Interpretation
0	negative for pathologic AMR: histopathologic and immunopathologic studies are both negative
1 (H+)	histopathologic AMR alone: histopathologic findings present and immunopathologic findings negative
1 (I+)	immunopathologic AMR alone: histopathologic findings negative and immunopathologic findings positive; that is, CD68(+) and/or C4d(+) for IHC and C4d(+) with or without C3d(+) for IF
2	pathologic AMR: histopathologic and immunopathologic findings are both present
3	severe pathologic AMR: interstitial hemorrhage, capillary fragmentation, mixed inflammatory infiltrates, endothelial cell pyknosis, and/or karyorrhexis and marked edema and immunopathologic findings are present

2013 International Society for Heart and Lung Transplantation Working Formulation for standarization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J Heart Transplant 2013; 12: 1147-1161.
pAMR – pathologic antibody-mediated rejection, H+ – histopathologic +, I+ – immunopathologic +

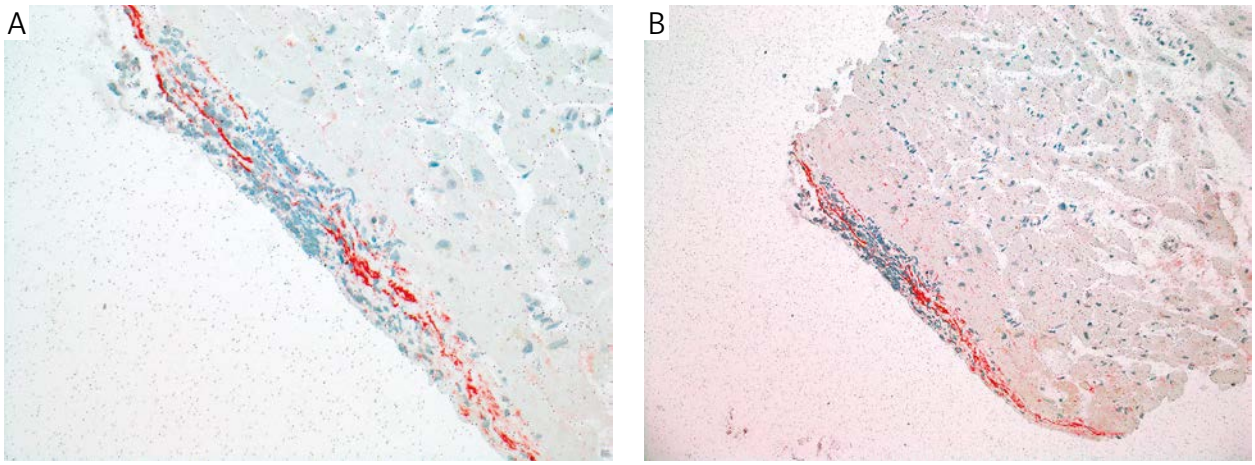

Fig. 1. Strong positive C4d staining in Quilty effect AMR 0, HE, original magnification 40×

CD68(+) staining

According to 2005 ISHLT revised Working formulation, presence of intravascular macrophages was recognized as one of the classic histopathologic features of AMR on HE stained sections, although increased number of interstitial macrophages can occur also in ACR, ischemic injury and infections. CD68(+) macrophages within capillaries have been required to diagnose AMR [6, 7, 30]. However, distinguishing between macrophages and other cell types, such as lymphocytes, is difficult, thus new ISHLT recommendations proposed term "activated mononuclear cells" instead of "intravascular macrophages" [9]. CD68 staining has remained one of the primary antibodies for pathologic diagnosis of AMR. The group agreed that at least ≥ 10% of the specimen should demonstrate the beading or cluster pattern of CD68 staining to be considered positive. The presence of CD68(+)

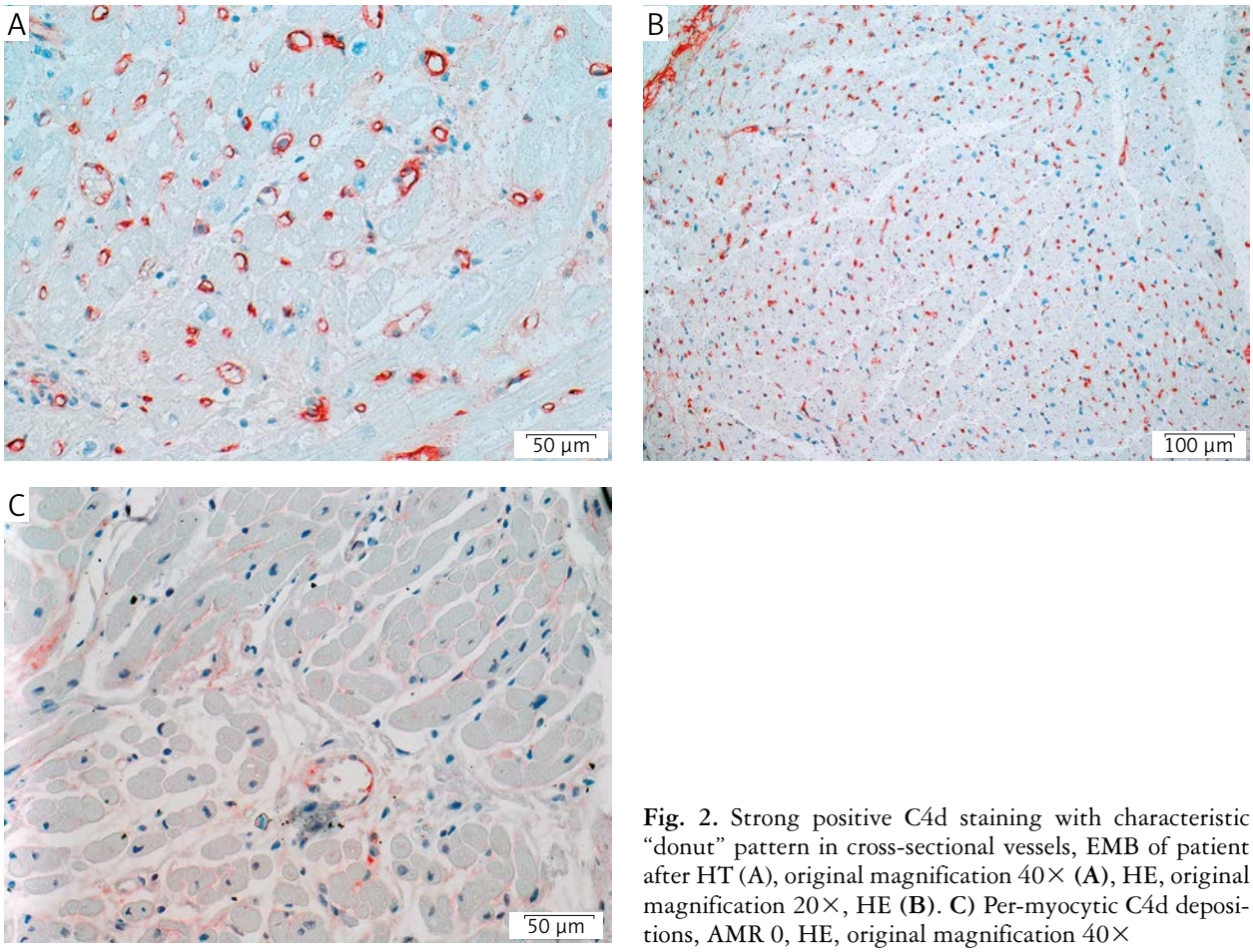

Fig. 2. Strong positive C4d staining with characteristic "donut" pattern in cross-sectional vessels, EMB of patient after HT (A), original magnification 40× **(A)**, HE, original magnification 20×, HE **(B)**. **C)** Per-myocytic C4d depositions, AMR 0, HE, original magnification 40×

intravascular macrophages should be initially recognized at scanning magnification and confirmed at high magnification to avoid over-interpretation of the results [9]. It is highly recommended, that when a definitive interpretation cannot be reached, the stain should be interpreted as negative for the purpose of grading and classification, the findings should be compared with patient's clinical condition and follow-up IHC in the subsequent biopsy specimen should be considered [9]. Pre-workshop validation study proposed 3-point score: 0-10% – negative; > 10% to < 50% – focal; > 50% – positive, respectively. Focal pattern was set a minimum for CD68 positivity.

Secondary stains in paraffin section IHC, frozen section IF

International Society for Heart and Lung Transplantation proposed secondary/optional antibody panel for pathologic diagnosis of AMR. According to recent recommendation paraffin section IHC includes: pan-T-cell CD3, pan-B-cell CD20, complement C3d, endothelial cell CD31 or CD34, complement regulatory proteins and others depending on individual centers' preferences. Primary antibody panel for frozen section IF comprises C4d and, C3d and anti-HLA-DR while optional panel includes fibrin, immunoglobulin G and M and others depending on individual centers' preferences [9]. Long-lasting complement split product C3d has been well reported in frozen IF studies [31, 32] but experience in paraffin IHC has been limited and further studies are needed. Therefore, it was proposed as optional staining in paraffin section IHC and primary staining in frozen section IF. The distribution and intensity of complement staining is graded similarly to paraffin section IHC with distribution > 50% considered a positive result and intensity minimum moderately intense on medium and high magnifications.

Practical considerations and difficulties concerning antibody-mediated rejection diagnosis in endomyocardial biopsies

Detection of histopathologic changes in EMB may depend on a number of factors, including the quality of the tissue preservation, adequate fixation and processing, section thickness, and staining quality. Each of them may render AMR difficult to be diagnosed. Time after HT is also important. The normal biopsy schedule is: weekly for the first month, every 2 weeks for the next month, once for the next 4 weeks, once for the next 6 weeks, then every 3 months for the next two years, and afterwards every 6 months for the next years [33]. The interpretation of C4d and CD68 should be cautiously assessed in first 2 weeks after HT due to a number of per-operative issues that can confound staining and diagnosis. Histologic findings related to per-operative ischemic injury and prolonged high-dose inotrope administration include formation of coagulative necrosis with vacuolization of myocyte fibers, and fat necrosis [34], thus the confusion with acute rejection may be likely. Obscurities attributed to C4d staining concern primarily the antibody location. Sub-endocardial connective tissue and per-myocytic (Fig. 2C) deposition are occasionally observed and their significance is currently unknown thus the subject warrants future multicenter studies. Because both C4d and CD68 staining are proposed as mandatory, the problem may occur in apparent discrepancy of results, when, in the presence of circulating DSAs, specimens suggesting AMR showed CD68 positivity without C4d positivity or C4d positivity without CD68 positivity. However, Takemoto *et al.* [35] proved that such equivocal findings will be rare and the combination of C4d and CD68 is useful. Another problem may be related to biopsy technique itself, as myocardial samples are taken from the right ventricular and in patients with long-lasting allograft it is highly possible that the tissue would be composed entirely of scar tissue (from previous biopsies) or of thrombus (fresh, organizing or organized), from previous biopsy sites, instead of endomyocardium. To consider EMB as diagnostic, it should comprise at least three, preferably more pieces of endomyocardium. In our everyday practice, we often face certain difficulties such as too meager material (eg. one diagnostic piece of tissue, too little fragments). Hence, clinical data is of significance here.

Summary

Antibody-mediated rejection occurs in about 6% of patients after HT. Its mechanism has been variably defined, but the association between AMR (even without cardiac disfunction) and greater mortality has been well documented, thus, it remains a significant problem. New ISHLT criteria (2013) for pathologic assessment of AMR stated that clinical dysfunction or positive DSAs are no longer obligatory for firm humoral rejection diagnosis. Although great progress has been made since first description of AMR, numerous challenges and unresolved problems concerning its clinical, pathologic and immunologic aspects remained.

The authors declare no conflict of interest.

This paper is supported by scientific project number 4968/B/P01/2011/40, financed by The National Scientific Center.

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