Antibody and Complement in Transplant Vasculopathy

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Abstract—Advances in immunosuppression have decreased the incidence of acute rejection, but the development of vasculopathy in the coronary arteries of transplants continues to limit the survival of cardiac allografts. Transplant vasculopathy has also been referred to as accelerated graft arteriosclerosis because it has features of arteriosclerosis, but it is limited to the graft and develops over a period of months to years. Although the pathological features of transplant vasculopathy are well defined, the causative mechanisms are not completely understood. This review focuses on the mechanisms by which antibody and complement can cause or contribute to coronary vasculopathy in cardiac transplants. Antibodies and complement can have independent effects, but the combination of antibodies and complement with inflammatory cells has greater pathogenic potential for the endothelial and smooth muscle cells of the coronary arteries. For example, stimulation through receptors for IgG or complement split products can activate macrophages, but stimulation through combinations of these receptors generates synergistic results. Together, antibodies and complement efficiently integrate the activation of endothelial cells, platelets, and macrophages, which are 3 of the primary components in the pathogenesis of transplant vasculopathy. Recent findings indicate that antibodies and complement produced within the transplant may contribute to vascular pathology in some transplants. Acute rejection caused by antibodies and complement has been treated by combinations of plasmapheresis, intravenous γ-globulin and monoclonal antibodies to CD20 on B lymphocytes. The effect of these treatment modalities on the development of coronary vasculopathy is unknown. (Circ Res. 2007;100:191-203.)

Key Words: antibodies □ complement □ platelets □ macrophages □ coronary arteries

Transplant vasculopathy culminates in diffuse concentric intimal expansion and adventitial sclerosis of the coronary arteries (Figure 1A). Surveillance angiographic studies demonstrate narrowing of the lumen of the coronary arteries and their major branches by this process before pathological changes can be observed in the small arterioles that are captured in endomyocardial biopsies. Therefore, direct pathological observations of the initial stages of transplant vasculopathy are limited in human hearts, and data from experimental models have been a major source of current concepts of the pathogenesis of the vascular changes (Figure 1B). Although the progression of vasculopathy in transplants can be accelerated by nonimmunological risk factors, such as hypertension and hyperlipidemia (reviewed for this thematic series by McManus and colleagues1), alloimmune responses are thought to initiate the vascular pathology because these vascular lesions are much more readily produced in experimental allografts than isografts and in normal than immune-
deficient recipients. In these experimental models, immune-mediated injury of the endothelial cells results in infiltrates of mononuclear cells and proliferation of smooth muscle cells that expand the intima. There is experimental evidence that both T lymphocytes and antibodies can contribute to the initial vascular injury. Although the intimal and adventitial pathology contain mononuclear cell infiltrates, the mediators found in the intima and adventitia are different. This review focuses on the mechanisms by which antibody and complement can cause or contribute to myointimal expansion in the large arteries of cardiac transplants. Antibodies and complement are particularly relevant to transplant vasculopathy because receptors for antibodies and complement are expressed by all of the cells found in the lesion. Macrophages, neutrophils, eosinophils, natural killer (NK) cells, B lymphocytes, platelets, and endothelial cells express a range of receptors for the Fc tail of antibodies as well as for various complement components.

Antibody Responses to Transplants

Transplants are powerful stimulants of antibody production. The full impact of antibodies on transplants was manifested in the early years of renal transplantation. The most devastating effect of antibodies is hyperacute rejection, in which the transplant is rejected within minutes to hours after circulation is reestablished to the grafted organ. Hyperacute rejection is characterized by deposits of antibodies and complement components on the blood vessels associated with extensive disruption of the vasculature, as evidenced by fibrin deposition and hemorrhage. Before 1969, antibodies stimulated by a first transplant frequently led to hyperacute rejection of a second renal transplant. However, in 1969, Patel and Terasaki described a crossmatch test that detected antibodies to prospective donors in sera from potential transplant recipients. Since the introduction of crossmatch testing, hyperacute rejection has been almost eliminated.

The capacity of organ transplants to elicit antibody responses was more obvious when azathioprine and steroids were the mainstay of clinical immunosuppression. In that era, antibody responses were not controlled effectively and rejection was frequently characterized by immunoglobulin and complement deposits in blood vessels of transplants. As increasingly effective immunosuppressive protocols were developed, overt evidence of immunoglobulin deposits in transplant biopsies was diminished and attention to antibodies waned. However, more sensitive techniques for measuring circulating antibodies in the serum and complement deposition in biopsies have demonstrated that antibodies and complement continue to be associated with acute and chronic rejection. Antibody responses continue to present practical clinical problems because many patients have been exposed to histocompatibility antigens by previous blood transfusions, transplants, or pregnancy. Prior exposure to histocompatibility antigens results in memory B and T lymphocytes that are more easily stimulated by antigen, and, as a consequence, are less susceptible to immunosuppression. Moreover, some of the current protocols that attempt to reduce immunosuppression to a single agent are compromised by unexpectedly high incidences of antibody responses.

Antibody responses to transplants are not monitored routinely in transplant recipients, but one large multicenter study found that the frequency of antibodies to human leukocyte antigen (HLA) was 22.8% in 393 recipients of cardiac transplants, which was similar to the frequency of 20.9% found in renal transplant recipients. This estimate of antibody responses is likely to be low because the prevalence of antibodies in clinical transplant recipients is underestimated by routine serological techniques. Assays using isolated HLA or recombinant HLA for ELISA or flow-bead platforms can detect antibodies that are below the threshold routine serological assays. Even the application of these more sensitive assays for antibodies in serum measures only the unbound alloantibodies in the blood compartment and fails to detect antibodies bound to target antigens in the graft. Usually more antibodies can be eluted from rejecting transplants than can be found in the circulation. Elution studies are of more value in acute than chronic rejection because antibodies do not bind irreversibly to antigens. Antibodies either release or are shed from the surface of cells, depending on their avidity of binding. As a result, antibodies can initiate injury and then depart by the time transplant vasculopathy is diagnosed.

In experimental transplants to animals treated with minimal immunosuppression, antibodies and complement can be
demonstrated bound to endothelial cells in the large arteries as well as capillaries and veins early after transplantation. In contrast, the infiltrates of T lymphocytes are concentrated around capillaries and in periarterial adventitial spaces. These histological findings reflect the fact that antibodies are the product of a selection process that results in high-affinity binding. High-affinity antibodies are capable of binding to vascular endothelial cells in the high flow conditions of large arteries. Russell and colleagues have demonstrated the capacity of passively transferred alloantibodies to cause vasculopathy in arteries of hearts transplanted to immunosuppressed or immunodeficient mice. Together, these experimental models establish the capacity of antibodies to bind to the endothelium of large arteries and to induce pathological lesions. Obviously, these experiments do not exclude a role for cell-mediated immune responses, and many of the mechanisms of antibody-mediated injury are dependent on cells. Each subsequent section below highlights mechanisms by which antibody and complement activate vascular tissue as well as leukocytes.

Crosslinking of Major Histocompatibility Complex Antigens Antibodies Causes Rapid Exposure of Adhesion Molecules by Endothelial Cells

The antibodies that have been correlated most closely with vascular pathology in transplants are antibodies to antigens of the major histocompatibility complex (MHC). Arterial endothelial cells normally express high levels of MHC class I antigens in experimental animals and in humans. The expression of MHC class II antigens is less universal on endothelial cells. In humans, unlike rodents, capillary and venous endothelia express MHC class II antigens constitutively. MHC class II antigens are not expressed on most quiescent arterial endothelia in animals or humans, but human coronary arteries are exceptions. In addition, expression of MHC class II antigens can be upregulated by many inflammatory mediators, most notably interferon-γ (IFN-γ). Upregulation of MHC class II antigens is a recognized consequence of acute rejection episodes.

Antibodies to human MHC antigens can have multiple direct and indirect effects on endothelial cells. IgG antibodies are bivalent and can crosslink MHC antigens. As a result, IgG antibodies to HLA class I antigens stimulate a rapid release of preformed adhesion molecules, such as von Willebrand factor and P-selectin, from the Weibel–Palade storage granules in endothelial cells (Table 1). Crosslinking of HLA by antibody is required to activate exocytosis because monovalent Fab fragments do not cause exocytosis, but bivalent F(ab')2 fragments are effective in triggering exocytosis. The multimeric von Willebrand factor that is released from the Weibel–Palade granules is effective at aggregating platelets even in the high shear stress conditions of arteries. In contrast to von Willebrand factor, P-selectin has low affinity for ligands on leukocytes that results in transient interactions in the low-flow conditions of capillaries and venules. In the high-flow environment of arteries, P-selectin may act as a signaling molecule between endothelial cells or platelets and monocytes enmeshed in von Willebrand factor. As discussed below, platelet activation could be an important early stage in the development of vasculopathy because platelets release chemokines and express the costimulatory molecule CD154 for lymphocytes, macrophages, dendritic cells, and endothelial cells.

Protracted Synthetic and Proliferating Responses of Endothelial and Smooth Muscle Cells to Crosslinking of MHC Antigens by Antibodies

The rapid release of preformed molecules from Weibel–Palade storage granules in endothelial cells is followed by a more protracted synthetic response that includes upregulation of chemokines and growth receptors. Mouse endothelial cells produce monocyte chemotactant protein-1 (MCP-1; CCL2) after stimulation with antibodies to MHC class I antigens. This chemokine recruits monocytes and macrophages to vascular endothelium. Macrophages attracted by MCP-1 have receptors for the Fc tail of antibodies (FcR). Through these receptors, antibodies bound to endothelial cells can engage and activate neutrophils and macrophages.

Bian and Reed have demonstrated that crosslinking HLA by antibodies signals upregulation of receptors for fibroblast growth factor (FGF) by endothelial cells and smooth muscle cells. The expression of FGF receptor contributes to the proliferation of smooth muscle cells caused by antibodies to HLA.

It is important to note that these experiments on endothelial responses to antibodies have all been confirmed with purified monoclonal antibodies because Rose and colleagues demonstrated that interleukin-1 (IL-1) can contaminate antibodies prepared from human serum. IL-1 was found to be responsible for the upregulation of adhesion molecules on endothelial cells that had been attributed to alloantibodies.

As discussed in subsequent sections, these responses to antibodies are reinforced by activation of complement because isolated complement components can induce immedi-
ate release of the same preformed adhesion molecules from Weibel–Palade bodies followed by the synthesis of mediators and proliferation of endothelial cells (see Table 1).

**Antibodies to Antigens Induced by Activation of Endothelial Cells**

Additional antigenic targets for antibodies can be generated on injured or activated endothelial cells. Autoantigens that are cryptic in normal endothelial cells can be exposed as the result of granzyme B released from lymphocytes. Rosen and colleagues have demonstrated that granzyme B cleaves autologous proteins to expose cryptic epitopes that become targets of autoantibodies. Antibodies to several autoantigens have been correlated with transplant vasculopathy. For example, antibodies to the protein filament vimentin have been demonstrated in nonhuman primate and human studies of cardiac transplantation. Vimentin, which is not normally expressed on endothelial cells, is present on apoptotic cells. Complement deposition (in the form of C4d) has been found in arteries of cardiac allografts to cynomolgus monkeys with high titers of IgM antibodies to vimentin, even in the absence of alloantibodies. Antibodies formed in cardiomyopathies before transplant may also play a role in graft rejection. High titers of preformed IgG antibodies to cardiac myosin have been correlated with decreased cardiac graft survival at 2 years. In mice, antibodies to myosin accompany acute and chronic rejection.

All of these studies suggest that an initial insult to the graft may lead to an increased range of antigens to expand the antibody responses.

**Activation of Leukocytes Through FcR by Antibodies**

Regardless of specificity, once bound to antigen, the Fc tail of antibodies can interact with FcR on cells. The responses of neutrophils and macrophages to antibodies depend on the type of FcR engaged and concurrent signals from other receptors, such as receptors for different complement split products (Figure 2). Macrophages express the high-affinity FcγRI (CD64), which is capable of binding monomeric IgG, as well as the low-affinity FcγRIIA (CD32), FcγRIII (CD16), and FcγRIV, which are engaged by clusters of IgG on antigen. These FcγR signal through immunoreceptor tyrosine-based activation motifs (ITAMs). Activation of macrophages through FcR upregulates many proinflammatory functions, including the production MCP-1 and IL-8 (Table I), and promotes their survival at sites of inflammation. FcγRIII is expressed by NK cells as well as macrophages, and this receptor signals through ITAM to initiate antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells. Because NK cells are rich sources of granzyme B, engagement of NK cells can diversify the antigenic stimulation to include autoantigens. Endothelial cells and platelets also express FcγRIIA, but the function of this receptor on these cells is less well understood. This is an area of great relevance to potential pathology in organ transplants.

Activating Fc receptors are counterbalanced by FcγRIIB on macrophages, which functions through an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIB is also expressed by B lymphocytes and functions as a feed back inhibitor of antibody production. The balance of activating and inhibitory FcR expression by macrophages is controlled by different mediators including cytokines and complement split products. For example, IFN-γ modulates the balance of receptor expression by upregulation of FcγRI and FcγRIII and downregulation of FcγRIIB.

Although antibodies can activate cells solely through FcR, the simultaneous stimulation through FcR and complement receptors can cause synergistic effects. Therefore, a consideration of complement is relevant before the effects of FcR are considered in further detail.

**Activation of Complement by Antibodies**

In addition to the direct effects that antibodies can have through crosslinking antigens and engaging FcR on leukocytes, certain subclasses of antibodies can alter endothelial cell and leukocyte function through activation of the complement system. Complement is a multifunctional system of receptors and regulators as well as effector molecules. The pathogenic power of complement is based on the capacity of the complement system to amplify both innate and adaptive immunity. This amplification is accomplished through 2 strategies. First, enzymatic reactions in the complement cascade generate multiple potent biological split products. Second, these split products stimulate leukocytes, platelets, and parenchymal cells through either specific receptors or receptor-independent pore formation (Figure 3).

Antibodies provide binding sites for C1 that initiates the enzymatic cascade of the classical pathway of complement. Bound C1 can enzymatically cleave C4 and C2, which then form an enzyme complex that is capable of cleaving C3. Amplification occurs because multiple C4 and C3 molecules are cleaved and each C4 and C3 produces 2 biologically active fragments: both C4 and C3 produce small soluble mediators of chemotaxis and activation (C4a and C3a), and large fragments that can bind covalently to proteins or carbohydrates (C4b and C3b). C3b has the potential to...
expand complement activation further because once it is bound to tissues, C3b can complex with factor B of the alternative pathway and initiate a self-perpetuating amplification loop that results in more C3b deposition (Figure 3B). In inflammation, these enzymatic reactions result in exponential increases in C4 and C3 split products. One study calculated that each C1 caused approximately 25 C4b and 250 C3b molecules to be bound to a cell surface. The amplification caused by C4b and C3b is not only quantitative; it is also kinetic. The ability of C4b and C3b to form covalent bonds with tissue results in a longer half-life for these complement components than for antibodies, C1 or C2, all of which can be shed rapidly. The amplification of complement in transplants is reflected by the fact that C4b and C3b split products are more readily detectable than antibodies in transplant biopsies even when relatively high titers of antibodies are present in the circulation.

Antibodies are the prime cause of complement activation in the high-flow conditions in the lumen of the coronary arteries, but 2 additional pathways of complement activation can also contribute to vascular pathology. The lectin pathway is initiated through mannose binding lectin (MBL). Similar to C1, bound MBL can activate the complement cascade by cleaving C4. MBL has globular lectin ends that can bind to terminal sugars on bacteria but also to injured or apoptotic endothelial cells. MBL participates in ischemia/reperfusion injury to hearts, and it may also be activated by cells undergoing apoptosis in transplants.

The third means of complement activation is the alternative pathway. This pathway has relevance to transplants both in the absence and presence of antibody. As mentioned above, factor B can bind to C3b that has been deposited on tissues as the result of antibody activating C1. In this capacity, the factors of the alternative pathway (factor B, factor D, and properdin) act as an autocrine amplification loop to generate more C3b. This mechanism of amplification can be responsible for the majority of C3b generated. The alternative pathway can also be initiated by spontaneous binding of small amounts of C3 to ischemic tissues. Unlike the classical and lectin pathways, which are triggered by particular substrates such as antibodies or phospholipids exposed on the plasma membrane, the alternative pathway is dependent on sporadic spontaneous conformational change in C3 at a rate of approximately 1% per hour. This mechanism is now thought to be of importance in the pathogenesis of ischemia/reperfusion as it occurs to the organ at the time of transplantation.

The amount of C3b bound to proteins is influenced by the properties of the available proteins and of the surrounding membrane structure. The available binding sites for C3b may be significantly increased in vessels of transplants when platelets become attached and activated on the endothelial cells because P-selectin on activated platelets has been found to bind C3b efficiently. This would result in a concentration of P-selectin and C3b deposits on endothelial cells to stimulate leukocytes.

Once C3b is deposited on tissues by any or a combination of these activation pathways, C3b can propagate the complement cascade when it forms an enzymatically active C5 convertase that can cleave C5. Cleavage of C5 results in 2 biologically active split products, C5a and C5b. C5b binds to C6 through C9 to form the MAC. Leukocytes have receptors for fluid phase (C4a, C3a, and C5a) and tissue-bound (eg, C4b and C3b) complement split products as well as the Fc portion of antibodies. B. Regardless of the mechanism by which C3b is deposited on a cell, factor B of the alternative complement pathway can bind to C3b. Cleavage of factor B by factor D produces a C3 convertase (C3bBb) that cleaves more C3. This autocrine activation of C3 is known as the amplification loop.

Figure 3. Phases of complement activation. A. Activation of complement progresses through initiation, amplification, regulation, and terminal complex formation. Antibodies bound to antigens on the transplant initiate the complement system via C1. Amplification occurs through a succession of enzymatic steps that split many C4 and even more C3 molecules. Continuous enzymatic cleavage is prevented by a number of RCA. Regulation of C4 and C3 limits amplification, but small amounts of C3b can perpetuate the complement cascade when it forms an enzymatically active C5 convertase that can cleave C5. Cleavage of C5 results in 2 biologically active split products, C5a and C5b. C5b binds to C6 through C9 to form the MAC. Leukocytes have receptors for fluid phase (C4a, C3a, and C5a) and tissue-bound (eg, C4b and C3b). B. Regardless of the mechanism by which C3b is deposited on a cell, factor B of the alternative complement pathway can bind to C3b. Cleavage of factor B by factor D produces a C3 convertase (C3bBb) that cleaves more C3. This autocrine activation of C3 is known as the amplification loop.
C5, and recently this mechanism has been demonstrated to be upregulated in C3 knockout mice. Gessner and colleagues have found that production and cleavage of C5 by macrophages is regulated by FcR. These researchers have demonstrated a feedback loop between FcR and C5a in several models of autoimmunity. In these models, C5 synthesis and cleavage is upregulated by antibodies engaging FcγRIII. Binding of the resultant C5a to receptors on macrophages in turn upregulates FcγRIII and downregulates the inhibitory FcγRIIB. This feedback is independent of the early components of complement because it occurs in C3 knockout mice. In the absence of C3, C5 is cleaved by an uncharacterized enzyme produced by macrophages. It is likely that this mechanism is even more significant in normal individuals because macrophages can synthesize all of the complement components, and therefore, macrophages could generate more C5a through multiple pathways including the alternative pathway. The large numbers of macrophages infiltrating arteries of transplants would provide a rich environment for complement activation through complement effector molecules and result in extensive tissue damage. These potentially deleterious effects of complement amplification are limited by multiple regulatory molecules.

Of particular relevance to vascular pathology is the group of regulators that has evolved to control the exponential generation of split products from the structurally related C4b and C3b components. This group of 6 regulators of complement activation is encoded in the RCA (Regulators of Complement Activation) gene cluster that includes decay accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), complement receptor type 1 (CR1; CD35), complement receptor type 2 (CR2; CD21), C4 binding protein (C4bp), and factor H. The first 4 regulators are expressed as membrane bound proteins, and the last 2 circulate in the plasma. DAF and MCP are present on a wide variety of cells including leukocytes and vascular endothelial cells, whereas CR1 and CR2 are expressed on selected leukocytes and antigen presenting cells.

Although these regulators are structurally related, they work by 2 different processes (Table 2). One set works by separation of associated complement components (eg, dissociation of C4b and C2a by DAF), and the second set (eg, MCP) function as cofactors for factor I, allowing it to cleave C4b and C3b into biologically active fragments: first iC4b or iC3b and then C4dg or C3dg. CR1 is remarkable because it regulates both as a dissociating factor and as a cofactor. These regulatory mechanisms truncate the expansion of the complement split products by disrupting the enzymatic activity, but a resulting succession of fragments remain attached to the membrane and serve as ligands. For example, C3b, iC3b, and C3d serve as ligands for cells with complement receptors CR1, CR3, and CR2, respectively (see Figure 2). As a result, complement-mediated damage in transplants is characterized by accumulation of neutrophils and macrophages, which possess CR1 and CR3.

Even when significant amounts of C4 and C3 fragments are deposited in normal autologous or allogeneic tissues, relatively little MAC is formed because of the density and diversity of regulators directed at the components of C4 and C3 (Figure 3). Lysis of autologous or allogeneic cells is further inhibited by regulatory proteins, such as CD59, that inhibit MAC formation. Although these regulators usually avert lysis of allogeneic cells, insertion of sublytic quantities of MAC into leukocytes or parenchymal cells can cause activation and promote inflammation.

Expression of protection of tissues by RCA does vary. Basal levels of RCA vary for different cells, and these levels can be modulated by inflammation and tissue injury. Many mediators that have been implicated in acute and chronic responses to transplants regulate DAF expression by endothelial cells in vitro. Mediators of acute inflammation, such as thrombin, tumor necrosis factor-α (TNF-α), and IFN-γ, increase DAF expression but not CD59 or MCP expression. The effects of cytokines on DAF expression are augmented in endothelial cells with MAC deposited on their surface. MAC upregulation of DAF expression may represent a feedback mechanism to protect vascular integrity in sites of complement activation. Mediators associated with chronic inflammation, such as basic FGF and vascular endothelial growth factor also upregulate the expression of DAF. The finding that cyclosporine A inhibits the upregulation of DAF expression suggests that some of the mechanisms that protect tissues from complement activation may be inhibited in transplant patients.

Therefore, the amount of complement injury in transplants is likely to be dependent on both the level of complement activation and the expression of complement regulators. Decreased or inhibited expression of complement regulators would promote vascular injury, whereas upregulation of complement regulators could protect transplants from continued antibody responses. There are in vitro and in vivo data that complement regulators can be upregulated to protect endothelial cells from acute antibody-mediated rejection as part of a process called accommodation. The expression of complement regulators is under investigation in endomyocardial

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<td><strong>Classical or MBL (C4bC2a)</strong></td>
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*Decay accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46) widely expressed on leukocytes and parenchymal cells. These molecules also modulate T-lymphocyte responses. †Complement receptor 1 (CR1; CD35) expressed on leukocytes.
dial biopsies and coronary arteries in transplants with transplant vasculopathy.

C-Reactive Protein Interactions With Complement
C-reactive protein (CRP) is another recognition pathway that has been implicated in activating complement. As an acute-phase reactant, large amounts of CRP are synthesized rapidly in response to tissue injury and inflammation.\(^6^0\) CRP binds to phosphatidylcholine and sphingomyelin that are exposed on plasma membranes of injured cells. After CRP complexes, it can engage the classical pathway of complement by binding C1q.\(^6^1,^6^2\) CRP simultaneously regulates as well as activates complement because CRP also binds factor H. Factor H limits the amplification loop and acts as a cofactor for factor I to cleave C3b to iC3b. Although iC3b is a ligand for CR3, stimulation through CR3 may deviate the immune response by downregulating IL-12 production by human monocytes.\(^6^3\) In addition to marking apoptotic cells with iC3b, CRP may interact directly with FCγRI and FCγRIIA on macrophages.\(^6^4\) On the whole, therefore, CRP may serve to clear injured tissue by noninflammatory means.

Complement Activation of Vascular Endothelial, Smooth Muscle Cells, and Fibroblasts
Endothelial cells are responsive to many components of complement activation. Human vascular endothelial cells and smooth muscle cells are stained by antibodies to C5a receptor.\(^6^5\) The potential for tissue activation through C5a receptors has been verified for endothelial cells in culture. Foreman et al\(^6^6\) found that C5a can activate endothelial cells to release von Willebrand factor and P-selectin. Interaction of C5a with its receptor, a 7-membrane spanning molecule, results in intracellular signaling. This receptor is sensitive to pertussis toxin and is coupled to G\(_i\) in both leukocytes and endothelial cells. However, the signal cascade that results from ligation of the C5a receptor on endothelial cells is distinct from that on leukocytes. Unlike C5a-mediated leukocyte chemotaxis, C5a mediated endothelial cell chemotaxis, as well as cell retraction and actin polymerization, depends on transactivation of the epithelial growth factor receptor.\(^6^7\) Activation of endothelial cells by C5a is augmented by proinflammatory cytokines, such as TNF-\(\alpha\). Leukocyte chemotaxis is augmented by C5a induction of IL-8 (CXCL8) and RANTES (CCL5) production by endothelial cells.\(^6^8\) IL-8 can have additional effects on endothelial cells including increased survival and proliferation.\(^6^9\)

Through data mining, Mastellos et al\(^7^0\) found that C5a has been linked to the following functions: chemotaxis, cell migration, cell invasion, endothelial cell activation, and cell proliferation, among many others. All of these functions could contribute to the stromal component of the lesion in chronic vasculopathy. C5a could potentially influence the accumulation of the poorly differentiated smooth muscle cells within the neointima, whether they are derived from the media or from adventitial fibroblasts or from circulating host derived precursors, smooth muscle cells, or mesenchymal stem cells. The possibility that C5a causes smooth muscle cells or their progenitors to migrate remains to be tested.

A contribution of C5a to the adventitial fibrosis is also possible. C5a has been implicated in fibrocyte dedifferentiation and fibroblast proliferation in models of pulmonary fibrosis. In chronic bleomycin-induced pulmonary fibrosis, C5 is associated with expression of matrix metalloproteinase-3 and transforming growth factor-\(\beta_1\).\(^7^1\) Similarly, hepatic stellate cells differentiate into myofibroblasts and increase their expression of C5a receptor in response to liver injury. These observations suggest that it would be fruitful to explore the generation of C5a and the expression of C5aR within transplant vasculopathy lesions. Additionally, small molecule inhibitors of C5a could be useful as experimental and therapeutic tools in transplant vasculopathy. Hillebrandt et al\(^7^2\) found that small molecule inhibitors of C5a decreased production of collagen and hepatic fibrosis in genetically susceptible mice. The potential for inhibitors of C5a to decrease migration of vascular smooth muscle into the neointima is worth exploring.

Endothelial cells also express receptors for C1q and C3a. The responses of endothelial cells to these complement components need to be studied more completely. It is known that C1q stimulates human endothelial cells to produce the chemokines IL-8 and MCP-1 in vitro.\(^7^3\) C3a, like C5a, upregulates IL-1β in addition to the chemokines IL-8 and RANTES. The stimulation of this set of mediators (IL-8, MCP-1, and RANTES) is a shared pathway of multiple functions of antibody and complement activation (Table 1). Therefore, chemoattraction of neutrophils and macrophages would be one major effect of complement activation on arterial endothelium. Indeed, antibody activation of complement in cardiac transplants is associated with subendothelial accumulation of macrophages in capillaries and arteries.\(^1^5,^1^6,^7^4,^7^5\)

Chemotaxis and adhesion is intensified by insertion of sublytic amounts of MAC into the plasma membrane of endothelial cells and smooth muscle cells. The immediate effect of purified C5b-C9 on endothelial cell cultures is the translocation of P-selectin and von Willebrand factor from Weibel–Palade storage granules to the plasma membrane.\(^5^0\) von Willebrand factor ensnares platelets and monocytes against the endothelium, and P-selectin costimulates their production of inflammatory mediators.\(^2^1,^2^2\) Intravascular aggregation of P-selectin- and von Willebrand factor–expressing platelets is a prominent feature of experimental models of antibody-mediated rejection that is decreased in the absence of MAC deposition.\(^4^6,^7^6\)

In addition to releasing preformed mediators, MAC-activated endothelial cells synthesize IL-8, MCP-1, P-selectin, tissue factor, platelet-derived growth factor and basic FGF.\(^7^7,^7^8\) The secretion of basic FGF may result in autocrine stimulation when antibodies to HLA cause upregulation of FGF receptors on endothelial cells.\(^2^6\) In vitro studies have demonstrated that MAC can have direct proliferative effects on vascular smooth muscle cells, as well as decrease apoptosis of these cells.\(^7^9\) These effects would strengthen the proliferative and survival signals induced by antibodies to HLA. The relevance of the effects of MAC on transplant
vasculopathy has been verified in cardiac transplants to C6-deficient rats. The C6 deficiency prevents MAC formation and slows the progression of vasculopathy in cardiac transplants.80

**Platelets Can Potentiate Vasculopathy Through Complement Activation**

The localization of platelets on arterial endothelium through P-selectin and von Willebrand factor can augment innate and adaptive immune responses in the vessel. Activated platelets augment recruitment of leukocytes by both secreting chemokines and inducing endothelial cells to secrete chemokines. Platelets secrete IL-8, RANTES, MIP-1α (CCL3), and MCP-3 (CCL7), which recruit leukocytes. Endothelial cells stimulated by platelets secrete MCP-1 and IL-8. The finding that P-selectin on activated platelets can bind C3b and propagate the complement cascade provides more routes for recruitment and activation of leukocytes. C1q, MBL, and CRP can enter the equation when phospholipids are exposed on the plasma membranes of activated platelets. C1q, MBL, and CRP can bind to phospholipids, such as phosphatidylserine. Thus, a carpet of activated platelets would enrich and extend the area available for complement interactions.

Platelets not only potentiate the localization of leukocytes to arteries; they can also activate leukocytes of both the innate and adaptive immune system. The expression of P-selectin on platelets provides costimulation to monocytes for the production of inflammatory mediators including MCP-1 and TNF-α.21,22 This function of P-selectin may be greatly augmented by the activation and binding of C3b to P-selectin. In addition, platelets are a major source of soluble CD154, which is the ligand for CD40 on B lymphocytes, macrophages, dendritic cells, and endothelial cells.74 Platelet-derived CD154 has been demonstrated to initiate rejection of hearts transplanted to CD154-deficient mice.81 Finally, platelets secrete a variety of growth factors, such as platelet-derived growth factor, endothelial growth factor, and FGF, that promote proliferation of endothelial cells, smooth muscle cells, and fibroblasts.82

Even though the contribution of platelets to atherosclerosis has been studied extensively, immunologists have not considered the potential contributions of platelets to transplant vasculopathy.

**Chemoattraction and Activation of Macrophages by Complement**

Macrophages have a range of receptors for Fc and complement split products. C3a and C5a mediate chemotaxis and activation of macrophages through C3aR and C5aR. C5a is quantitatively much more potent as a chemotactic agent than C3a. Activation of monocytes through their C5a receptor causes upregulation of CR1 and CR3 and production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α.83,84 As discussed above, TNF-α from macrophages can augment C5a-induced activation of endothelial cells, as measured by surface expression of P-selectin. The larger split products C4b and C3b can bind covalently to cell membranes and target macrophage adhesion (Figure 2). After being bound covalently to cell membranes, C3b and its subsequent cleavage product iC3b serve as accessory adhesion molecules that strengthen the contact between target cells and macrophages that express CR1 and CR3. A strong correlation between antibody-mediated complement activation and margination of macrophages in vessels of transplants has been noted in clinical and experimental specimens.15,16,74,75 In animal models of transplant vasculopathy, macrophages are recruited to the arteries rapidly and persist as the neointima expands.80,85

Antibody and complement have additional stimulatory effects on macrophages through receptors for Fc and C1q. Among other functions, macrophage production of C3 and C5 is increased by stimulation through both the Fc44,45 and C1q receptor.86 This would increase local production of complement by macrophages in vessel walls. Comparisons of atherosclerotic plaques with adjacent normal-appearing artery have identified local production of many proinflammatory mediators including mRNA for complement components.47 Both smooth muscle and macrophages appear to be responsible for this response. We have found that local production of C6 by infiltrating inflammatory cells in the allograft is increased during rejection.46

Macrophages may be integral to transplant vasculopathy because macrophages continue to function in spite of current immunosuppressive treatment. In fact, in the first weeks after transplantation, when patients receive maximum immunosuppression, macrophages are found routinely in biopsies at sites of ischemia/reperfusion injury.

**Antiinflammatory Effects of Complement on Macrophages**

The interaction of complement split products with macrophages does not inevitably induce mediators of acute inflammation. Deficiencies of C1 and C4 result in decreased capacities to clear apoptotic cells and increased incidences of autoimmunity in both humans and mice. Several complement components have been demonstrated to aid in the noninflammatory clearance of apoptotic cells by macrophages.87 C1q, MBL, and CRP can initiate this clearance by binding to phosphatidylserine exposed on the flipped plasma membranes. Phagocytosis induced by C1q, C4b, and iC3b is accompanied by decreased production of IL-12 and IFN-γ.63 C5 activation is the tipping point between noninflammatory versus inflammatory responses to complement. As discussed in the previous section, C5a in particular causes macrophages to modulate Fc and complement receptors toward a heightened activated state.

**Localization of B Lymphocytes in Coronary Arteries With Chronic Vasculopathy**

Complement activation by antibodies is a powerful feedback loop for increased antibody production because C3d, the final end product of regulation of C3b by factor I, is the ligand for CR2 (CD21) on B lymphocytes. Fearon and colleagues88 have demonstrated that antigens bearing 2 and 3 copies of C3d are 1000- to 10 000-fold more immunogenic than antigen alone. This mechanism allows C3d to function as a molecular adjuvant for antibody production. For this mechanism to occur, the B lymphocyte is best stimulated by both
lymphocytes. Plasma cells were often found scattered on the vasculopathy showed nodules of compartmentalized B and T lymphocytes surrounded by T lymphocytes (Figure 5). Histology from 5 of the 8 patients with severe transplant vasculopathy showed nodules of compartmentalized B and T lymphocytes. Plasma cells were often found scattered on the adventitia, the elastic layers under the thickened neointima. Larger infiltrates of B cells (CD20+) and plasma cells (CD138+) as well as CR2 that are expressed by B lymphocytes were upregulated in coronaries with vasculopathy compared with native atherosclerosis or no lesions. Nine probes for κ and λ light chains were consistently increased in 11 of 12 cardiac allograft vasculopathy samples. In 5 of these samples, these probes were increased in the range of 5- to 25-fold (Figure 4).

The localization of B cells and plasma cells was investigated in coronary arteries from 8 of the patients with severe transplant vasculopathy. Immunohistology demonstrated that infiltrates of B cells (CD20+) and plasma cells (CD138+) were characteristic of coronary arteries with transplant vasculopathy. Linear arrays of these cells often infiltrated along the elastic layers under the thickened neointima. Larger numbers of lymphoid cells extended into the adventitia, where they often formed nodules with distinct inner zones of B lymphocytes surrounded by T lymphocytes (Figure 5). Histology from 5 of the 8 patients with severe transplant vasculopathy showed nodules of compartmentalized B and T lymphocytes. Plasma cells were often found scattered on the outside of the nodules. The presence of nodules was not correlated with the age or sex of the patient, length of graft survival, or number of HLA mismatches. Stains for κ and λ light chains and capillary gel electrophoresis of the immunoglobulin heavy chain demonstrated that the plasma cells were polyclonal, suggesting that the lymphoid nodules were not caused by posttransplant lymphoproliferative disease. Thaunat et al96 have reported similar findings in coronary arteries from 5 cardiac transplants.

To exclude the possibility that these nodules were merely the sequela of open heart surgery, coronary arteries from 6 control patients were examined. These patients had experienced open heart surgery 7 to 10 years before their hearts were explanted for a first cardiac transplant: 2 patients had valve replacements, 3 patients had coronary artery bypass grafts, and 1 patient had a coronary stent. Minimal inflammatory infiltrates and no lymphoid nodules were seen in these control coronary arteries. The control studies support the concept that the infiltrates and lymphoid nodules developed in response to the allogeneic transplant.

The potential role of these lymphoid nodules in antigen presentation and antibody production has been discussed, but few data are available. Thaunat et al97 used segmental aortic grafts in rats to demonstrate that lymphoid nodules formed adjacent to these large arteries were associated with the production of antibodies to histocompatibility antigens.

Ectopic lymphoid nodules with distinct B and T lymphocyte zones are frequently formed in tissues affected by chronic autoimmune diseases, such as thyroiditis, diabetes, and rheumatoid arthritis. This has been referred to as lymphoid neogenesis. Experimentally, transgenic expression of certain chemokines has been shown to initiate the recruitment and organization of lymphocytes into functional appearing lymphoid structures.98 Ectopic expression of CXCL13 (B-lymphocyte chemoattractant), CCL21 (secondary lymphoid tissue chemokine), and CCL19 (Epstein–Barr virus–induced chemokine) induce the development of lymph node–like structures through stimulation of lymphotixin. All of these

The transplants were removed 3 to 14 years (average 6.9) after transplantation. Genes for immunoglobulins (IgG3, λ, and κ) as well as CR2 that are expressed by B lymphocytes were upregulated in coronaries with vasculopathy compared with native atherosclerosis or no lesions. Nine probes for κ and λ light chains were consistently increased in 11 of 12 cardiac allograft vasculopathy samples. In 5 of these samples, these probes were increased in the range of 5- to 25-fold (Figure 4).

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structures contain B- and T-cell zones, but those elicited by CXCL13 and CCL21 are larger and form more rapidly. CXCL12 (stromal cell–derived factor 1) induces small lymphoid nodules with a preponderance of B lymphocytes and plasma cells. Steinmetz et al. described 4 renal biopsies obtained in the first 4 to 9 days after transplantation that contained nodules of B cells that stained with CXCL13 and the corresponding CXCR5 receptor.

In experimental models, lymphoid neogenesis has been described in 2 models of transplantation. Lakkis and colleagues described organized nodules of lymphocytes with T- and B-lymphocyte compartments in heterotopic mouse hearts undergoing chronic rejection. These experimental heart grafts have a couple of differences with human heart transplants. First, the heterotopic transplants are located in the peritoneal cavity. Second, mouse coronary arteries penetrate into the myocardium closer to their origin. As a result, these lymphoid nodules were on the epicardium of the transplanted mouse heart but not adjacent to the coronary arteries.

**Future Investigations**

The potential for antibodies and complement to contribute to the development of transplant vasculopathy needs to be explored more fully. Our review of the current information concerning transplant vasculopathy indicates that the interactions of antibody and complement with platelets and macrophages may be central to injury of arterial intima. The high-flow conditions of the artery only permit high-affinity interactions to occur. Antibodies and complement have been demonstrated to bind arterial endothelium and release von Willebrand factor, which can ensnare platelets and macrophages on the endothelial surface. Complement split products can pivotally modulate the mediators secreted by macrophages. These interactions need to be investigated more completely.

Clinically and experimentally, improved methods of testing for antibodies and complement will allow more accurate assessment of these mediators in transplant recipients with vasculopathy. In addition, more patients with demonstrable alloantibodies are being transplanted. In the field of cardiac transplantation, patients who are supported with left ventricular assist devices have an increased incidence of antibodies to HLA. Acute rejection caused by antibodies and complement has been treated by combinations of plasmapheresis, intravenous γ-globulin and monoclonal antibodies to CD20 on B lymphocytes. The effect of these treatment modalities on the development of coronary vasculopathy is unknown. This cohort of patients with documented antibody in their circulation and complement deposition their transplants will provide a large database to correlate the effects of antibodies and complement with the development of vasculopathy.

The increased clinical interest in antibodies and complement has been followed by an increase in experimental studies. The development of experimental models has been aided by a variety of animals with useful genetic deficiencies of selective antibody and complement components, regulators, and receptors. Potent inhibitors for complement fragments and receptors have also been synthesized.

These advances will permit a fuller understanding of the contributions of different antibodies and complement components to transplant vasculopathy.

**Summary**

Antibodies and complement can induce vasculopathy in experimental cardiac transplants in the absence of T or B lymphocytes. In the clinical setting, it is likely that antibodies augment platelet and macrophage function through activation of complement. The initial mechanisms stimulated by antibodies and complement are the expression of chemotaxis and adhesion molecules by endothelial cells and platelets. P-selectin promotes interactions among macrophages, plate-
lets, and endothelial cells. The central role of macrophages is enhanced by signaling through receptors for Fc and complement split products. Complement split products also modulate T- and B-lymphocyte immunity. Identification of tertiary lymphoid structures formed within transplants has raised the possibility that local immune responses contribute to the antibody responses in chronic graft rejection. Further experimental animal studies will be needed to define the importance of individual Fc and complement receptors to transplant vasculopathy.

Acknowledgments

We thank Karen Fox-Talbot for expert help with the immunohistochemistry preparations.

Sources of Funding

Supported by NIH grants K08HL074945, R01-AI42387, P01HL070295, and P01HL056091.

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_Circ Res._ 2007;100:191-203
doi: 10.1161/01.RES.0000255032.33661.88
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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