The Importance of Antibody Pathways for the Rejection of Xenografts: An Immunological Conundrum?

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The application of xenotransplantation as an effective medical therapy is limited by the vigorous humoral immune response mounted by the host against the foreign graft. This response occurs within a short period of time and is characterized by the production of a restricted population of antibodies directed against a small number of carbohydrate antigens. Important features of this response are consistent with the use of a primitive, nonspecific response to infectious agents to mediate the early response, while later the reaction becomes increasingly complex with time. Evidence for the involvement of both T-independent and T-dependent antibody production, as evidenced by the onset of a switch in IgM to IgG antibody production, and evidence for the influence of antigen-driven maturation in the response blur the distinctions that define the nature of the immune response pathways responsible for mediating the host response to the graft. This review focuses on a description of the current information relevant to the immune response pathways mediating the xenograft reaction and the potential approaches to the development of new therapeutic strategies to prevent the response.

Introduction

Antibody responses to xenografts dominate the early phases of the host immune responses to tissues from unrelated species. These humoral responses are responsible for hyperacute rejection of xenografts due to preformed antibodies present in normal individuals prior to graft placement and/or acute vascular rejection caused by the production of new xenoantibodies following transplantation. The importance of these host responses in the rejection of the graft is illustrated by the inability of established immunosuppressive regimens, highly effective for preventing the rejection of allografts, to allow for the prolonged survival of xenografts. While other pathways of immune responsiveness to xenografts have been identified, the failure to achieve long-term graft survival is most directly related to our inability to prevent humoral responses to the xenografts. This failure is in turn the product of a lack of a clear understanding of the importance of host antibody responses and the nature of the pathways responsible for their production.

Our current concepts of the pathways of antibody production would predict that 1 of 3 broad categories of antibody responses could potentially play a role in mediating the humoral response to xenografts. The first are the natural antibodies secreted by peritoneal B-1 cells, either spontaneously or in response to exposure to environmental antigens.[1,2] Circulating levels of antibodies to microbial agents in normal individuals provide the host with a rapid but nonspecific protective mechanism against infection. This primitive form of immune protection shares many of the characteristics of an antibody response to T cell-independent carbohydrate antigens and may be an evolutionarily conserved method to prevent infection associated with repeated exposure to bacterial and viral organisms in the environment.[3] Natural antibodies to infectious agents prevent dissemination of these agents

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to vital organs and improve immunogenicity by encouraging antigen distribution to secondary lymphoid organs.[4]

In addition to natural antibodies, 2 pathways of antibody production by specific B cells may also have a potential impact on xenograft rejection. The pressure to maintain a robust and effective barrier against immediate exposure to infectious agents has led to the conservation of a more primitive pattern of antibody production that involves the immediate production of antibodies by a subset of B cells (B-1 cells) that do not require T cell help to secrete antibodies.[2] Direct stimulation of B-1 cells by multivalent antigens, particularly carbohydrate antigens, leads to the production of antibodies encoded by Ig genes in their original germline configuration. B-1 cells can produce both T-independent (TI) and T-dependent (TD) antibody responses when stimulated by the same antigens presented on different carrier molecules.[5,6] The relative importance of TI versus TD production of antibodies produced in response to viral infection is a consequence of antigen dose and distribution in secondary lymphoid tissues.[7] Natural antibodies prevent dissemination of pathogens and provide an effective method to provide for antigen trapping in secondary organs.[4] T-independent responses predominate when antigen presentation, either because of high concentration or a highly repetitive structure, allows for cross-linking of B cell receptors. Efficient pooling of antigen by antibodies takes place in the splenic marginal zone, and this is, therefore, a key event associated with the induction of TI B cell responses. The ability of the host to mount a TI antibody response within a shorter period of time than seen with traditional T cell-dependent responses may provide an important selective advantage for protection against virulent high-dose systemic infections.

Alternatively, the second pathway consists of the more traditional T-dependent response to monovalent antigens, especially peptide antigens presented indirectly to T lymphocytes via professional antigen-presenting cells, and involves antibody production by a second subset (B-2) of B lymphocytes. This response is driven by T cells that induce proliferation of B-2 lymphocytes with the selection of antibody-producing cells that secrete antibodies highly specific for the target antigen and that bind to the antigen with high affinity. This antigen-driven maturation is the result of the introduction of new mutations that occur in a basic set of germline immunoglobulin genes that serve as the progenitor genes for the antibody response.[8]

HUMORAL IMMUNITY:

A form of immunity whereby B lymphocytes and plasma cells produce antibodies to foreign agents (antigens) and stimulate T lymphocytes to attack them (cellular immunity).

Characteristic Features of Humoral Responses to Xenografts

The humoral immune response to xenografts displays features both T-independent and T-dependent pathways. Among different species combinations, the humoral responses to the grafts are directed at carbohydrate structures expressed by cell surface proteins or lipids, presumably organized in a repetitive pattern on cell surfaces in the graft. A large proportion of preformed antibodies found in the sera of normal individuals that react with the tissues of unrelated species are commonly antibodies that also react with carbohydrate antigens expressed by infectious agents. In mice, these antibodies are predominantly anti-phosphorylcholine antibodies,[9] while in humans, a large proportion of natural xenoantibodies bind to the αgal (1,3) gal carbohydrate epitope.[10] In humans and Old World primates, a defect in the expression of an αgalactosyltransferase gene results in the lack of αgal (1,3) gal carbohydrate antigens on cell surface–associated proteins and lipids. The absence of the αgal antigen allows for the non–self-recognition of the antigen and the production of antibodies to the αgal epitope in response to environmental exposure to viruses and bacteria that express this carbohydrate. The expression of αgal antigen by tissues of another species, such as the pig, then may serve as a primary target for the rejection reaction to the xenogeneic tissues.[11]

Early in the response to xenografts, IgM antibodies are the dominant isotype, and these antibodies are primarily responsible for the damage to the vascular endothelium of the grafts. The Ig V_H genes encoding their binding activity are expressed in their germline configuration, suggesting rapid production of antibodies in the absence of significant T cell–dependent stimulation of B cell proliferation.[12-14] This phase of the xenograft reaction is resistant to the effects of immunosuppression by agents, such as CsA and FK 506, that have been developed because of their influence on T cell function.

HYPERACUTE REJECTION:

A rejection that usually develops within minutes to hours following the implantation of a vascular graft; a form of antibody-mediated, usually irreversible damage to a transplanted organ, particularly the kidney, that is manifested predominantly by diffuse thrombotic lesions, usually confined to the organ itself.

The immunoglobulin heavy and light chain genes used to encode the binding activity of xenoantibodies display a conserved structural configuration. Immunoglobulin V_H and V_L genes used to encode antibody activity are restricted to the use of small groups of closely related genes to encode binding activity to graft antigens.[12,15] This use of a small number of Ig-variable region genes occurs despite the existence of a potentially large number of antigenic differences that could serve as targets for diverse donor/recipient species. Ig gene analysis in rats, humans, and mice all demonstrates a restricted use of similar Ig-variable region genes, suggesting that, even in diverse species combinations, selected carbohydrate configurations are the primary targets for the reaction.[16]

Following placement of a xenograft, the production of IgM xenoantibodies is followed by evidence for the maturation of the xenoantibody response. As the response progresses, the Ig V_H genes encoding antibody binding activity in the IgM antibodies undergo isotype switching, and the same V_H genes are used by IgG antibodies to encode a secondary antibody response directed at the xenograft target antigens. In rats and humans, approximately 50% of the Ig V_H genes continue to be expressed in a germline configuration, while the remainder display evidence for the appearance of somatic mutations within the variable regions.[13,15] At this point, it is not clear if the changes are the result of T-independent or T-dependent antibody responses. The appearance of these mutations, the use of isotype switching to involve IgG antibodies, and the reports that selected T cell subsets, particularly $CD4^+$ cells, are required for xenograft rejection reactions are consistent with a response dependent on T cells.[17,18] Alternatively, the persistent use of Ig V_H genes in their germline configuration, the apparent involvement of a specific subset of B cells (B-1b cells) in the production of xenoantibodies,[19] and the lack of precise information of the nature of the maturation of T-independent responses in general provide significant support for the involvement of non–T cell pathways in mediating the response. The amino acid substitutions that occur as a result of these mutations, for example, may not influence the binding of the antibody encoded by specific genes (see below).

Immune Response Pathways and the Xenograft Reaction

Similarities in the structure and function of antibodies directed at xenografts and those involved in natural protection against infectious agents suggest that the response to xenografts is a consequence of the inadvertent sharing of xenograft target antigens, with an important host survival strategy designed to prevent infection. Preformed xenoantibodies react to carbohydrate antigens commonly expressed by viruses and bacteria, including an antigen, the αgal (1,3) gal carbohydrate epitope, that serves as a primary target for the xenograft reaction in humans transplanted with tissues and organs from lower species. These "natural xenoantibodies" can cause rapid (hyperacute) loss of xenografts following their binding to cell surface–associated protein and lipid molecules expressing this epitope. Understanding the association between a primitive but powerful humoral mechanism for responding to microbial exposure and the rejection of xenografts may be of fundamental importance for understanding the basis of humoral immune responses to this type of immunological challenge. Is, for example, the restriction in Ig gene usage to xenografts a serendipitous consequence of the evolutionary pressure to maintain a group of Ig genes to mediate antibody responses to infectious agents, combined with the lack of the expression of the αgal (1,3) gal epitope on organs selected for human xenotransplantation, or is it the consequence of a sophisticated interaction of several immune response pathways working cooperatively to cause the rejection of the graft?

Ig Gene Control of the Antibody Function

The data accumulated to date regarding the nature of the xenograft response indicate that both T cell-independent and T cell-dependent pathways could be involved in mediating the humoral response. Traditional antibody responses involve the hypermutation of antibody-variable region genes, primarily in germinal centers in the spleen and lymph nodes. Mutations within immunoglobulin genes increase in frequency with time and may involve a shift in the repertoire of germline genes used to encode binding sites.[8] Hyperimmunization of $Gal^{-/-}$ knockout mice that lack expression of the αgal (1,3) gal carbohydrate epitope leads to the

IMMUNOGLOBULIN:

A specific protein substance that is produced by plasma cells to aid in fighting infection. Some immunoglobulins (gamma globulin) take part in various immune responses of the body to bacteria or foreign substances (allergens, tumor, or transplanted tissue). Examples include IgG, IgM, IgA, IgD, and IgE.

production of high levels of both IgM and IgG antibodies with the capacity to cause rejection of heart grafts expressing the target antigen. Examination of the binding affinity of these antibodies, however, demonstrates that the IgG antibodies may not always display evidence of increased affinity for the αgal epitope when compared to natural, preformed antibodies isolated from humans.[20] Kalinke and colleagues[21] demonstrated that germline and hypermutated antibodies to multivalent antigens, such as those directed at viral and bacterial pathogens, do not significantly differ in their binding affinity. In contrast, hypermutated antibodies against traditional monovalent antigens may display up to 1000-fold increases in their affinity maturation. These results suggest that antibodies directed at multivalent antigens may not display significant changes in binding characteristics despite the presence of multiple somatic mutations.

Examination of the structural configuration of xenoantibody binding sites demonstrates that there is an extensive conservation of the genes that encode xenoantibodies among species. Mouse and human Ig V_H genes that encode anti-gal xenoantibodies share overall amino acid similarities of 71%, including identity for 7 of the 8 amino acids at the important canonical binding sites in their variable regions. These sites are thought to be responsible for establishing the binding site configurations, and the preservation of identical amino acids at these sites for these 2 species is at a level that is higher than expected by chance alone ($P = 0.03$).[22] Computer imaging of the xenoantibody binding sites indicates that there are multiple potential contact sites for xenoantibodies and their target antigens.[23] A total of 22 antigen contact sites for single-chain antibody fragments can be identified for the human IGVH3-11 gene; approximately 13 heavy chain and 9 light chain residues may be in direct contact with the dimeric form of the αgal antigen.

Prospective changes at those sites may be expected to alter the strength of antigen binding. Preliminary examination of the effect of the changes at individual amino acid positions, however, has not to date demonstrated any preferential changes at these sites that result in increases in binding affinity to the gal antigen. Human Ig V_H genes involved in the antibody response to pig xenogeneic tissues display similar levels of binding affinity for the αgal epitope, irrespective of the number of amino acid replacement substitutions present in the genes encoding antibody binding. This would suggest that although somatic mutations have taken place, they may have a limited influence on antigen binding when the target is expressed as a multivalent antigen. Accordingly, future studies aimed at prospectively creating specific mutations within the binding site and establishing their influence on binding may provide important information relevant for understanding host responses to both infectious agents as well as xenografts.

In addition to establishing the fundamental importance of the structural changes on antibody function, prospective alterations of xenoantibody-variable regions may provide information on whether such changes may have value as a strategy for preventing xenograft rejection. Early studies of the use of soluble αgal carbohydrates to block xenoantibody functions in primates have been of minimal success,[24] presumably due to antigen/antibody binding qualities that were inadequate to prevent antibody damage to the graft endothelium. Antigen/antibody binding of high affinity is possible, so that the appropriate small molecule or high-affinity antibody generated by molecular engineering may have the potential to provide a long-term and relatively easy method to prolong xenograft survival. This type of molecular engineering has received substantial impetus in recent years as a function of the development of high-speed screening technologies for the binding of small molecules to their cognate receptors. A small hexapeptide molecule capable of binding to anti-αgal antibodies has been identified using this type of approach[25] but as yet has not been shown to be effective in preventing xenograft rejection.

Alternatively, the use germline genes and/or T cell-independent pathways to encode antibody activity may limit the plasticity of the response by preventing isotype switching and the introduction of somatic mutations as features of the antibody responses to xenografts. T cell-independent responses have generally (although not exclusively) been considered to lack these features of antibody maturation following antigen exposure. If new antibody configurations cannot be rapidly established once

the primary response has been compromised, it may be possible to disrupt the response by targeting specific Ig genes or antibodies responsible for graft rejection. We see evidence for isotype switching and somatic mutation in response to xenotransplantation. For influenza viral infections in mice (see below), the response can be divided into 2 independent components, one mediated by B-1 cells and a secondary response by B-2 cells. For xenografts, the issue has not been addressed adequately, and it is not clear whether somatic mutations and isotype switching reflect the following: (1) the expression of a T cell-independent response that undergoes these events as a normal series of changes is associated with maturation of the response, or (2) the appearance of an initial T cell-independent antibody response is followed by a nonoverlapping T-dependent response that is indirectly related to the xenograft reaction. This indirect response may occur as a result of damage to the graft with subsequent exposure and dissemination of new tissue antigens.

HYPERIMMUNIZATION:

The induction of a heightened state of immunity by the administration of repeated doses of antigen, often used in allergy desensitization, or passively acquired immunity by the injection of hyperimmune gamma globulin.

Cellular Control of the Xenoantibody Response

The production of antibodies is the principal function of B lymphocytes and their progeny. Among mature B cells, there are important differences in the nature of the stimulus for antibody production and the type of antibodies produced. Although a number of generalizations provide a rough guideline to the nature of these differences, there are many individual exceptions to each of these generalizations.[26] In the case of the rejection of xenografts, the information we have available is clearly insufficient to provide the basis for a comprehensive and accurate analysis of the cellular pathways used to provide the humoral responses to xenografts. Despite these shortcomings, however, several interesting observations provide for useful comparisons to recognized general patterns of antibody production.

In general, B-1 and B-2 antibody responses are thought to differ with regard to their developmental origins, phenotype, and function.[26,27] Functionally, B-1 and B-2 cells differ in their requirement for T cell help to mediate antibody production. B-1 cells are derived from the fetal liver cells, and they are found in predominant numbers in the peritoneal and pleural cavities, as well as in small numbers in the spleen and secondary lymphoid organs. They are responsible for the production of most natural antibodies present in the serum and make a significant contribution to the IgA-producing plasma cells of the lamina propria of the gut. They make relatively few antigen-stimulated antibody responses, primarily, although not exclusively, to T-independent antigens.[5,28] B-1 cells can, however, make antibodies to both T-independent and T-dependent antigens under selected conditions.[5,6] B-1 cells can make responses to the same antigenic epitope when presented in either a T-independent or T-dependent format, suggesting that the form in which the antigen epitope (in this case, phosphorylcholine [PC]) is presented is a critical factor in shaping the response. Antigens that present repetitive structures, especially when configured with a 2-dimensional spacing of 5 to 10 nm, do not require second signals produced by helper T cells to stimulate B cells.[29,30] T cell-independent antigens are commonly expressed by viruses and bacteria and can frequently stimulate strong antibody responses in the absence of helper cell activity.[31] In contrast, B-2 cells mediate most antigen-driven antibody responses, primarily to T-dependent antigens. The stimulation of antibody production by T-dependent antigens requires 2 signals: antigen recognition by the B cell receptor and a second signal provided by T helper cells.

Natural antibodies to infectious agents or autoantigens are generally considered to be produced by B-1 lymphocytes. These are antibodies that are present in the serum of normal individuals without clear evidence of antigen stimulation and whose production remains constant despite antigen stimulation. B-1 cells, as an example, produce most if not all natural antibodies to the influenza virus in mice despite the lack of natural exposure of mice to the virus. Infection results in the production of high titers of antibodies, all of which are now produced by B-2 cells.[32] The levels of innate antibody remain constant, while the primary response rises and then falls as expected. The importance of the natural antibodies to the flu virus is the role that these antibodies play in protection from infection. Natural antibodies are critical for preventing pathogen dissemination and acting to concentrate

the infectious agents in secondary lymphoid tissues by antigen trapping.[4] In knockout mice, B-1 and B-2 responses to the flu virus infection are nonoverlapping and equally critical for preventing infection. The inability to mount either response results in a high morbidity in infected animals.

Preformed xenoantibodies share many functional and structural characteristics with natural antibodies to infectious agents.[3] They are primarily IgM antibodies directed against carbohydrate antigens and encoded by V_H genes expressed in a germline configuration. These xenoantibodies are present in the sera of normal individuals who have no known exposure to the tissues from unrelated species. Once the graft has been placed, there is a rapid production of antibodies, primarily IgM, directed at carbohydrate target antigens expressed by the graft. In rodents and humans, this antibody production is associated with an isotypic switch to the use of the IgG antibodies, especially of the IgG1 isotype, which results in the accumulation of approximately equal numbers of Ig V_H genes expressing either germline or somatically mutated variable region genes. In $Gal^{-/-}$ mice, the cells that produce these antibodies are present primarily in the spleen, albeit in small numbers, and are phenotypically characteristic of a unique B-1b subset of B cells.[19] Early in the response, the primary antibody production is germline IgM antibodies; this is when the response is highly resistant to heavy immunosuppression, presumably due to the stimulation of B-1 cells by a T-independent form of xenogeneic antigen.[33] As the response matures and the switch to IgG antibodies takes place, the response is now sensitive to immunosuppressive agents, such as CsA, that have the ability to prevent secondary IgG antibody responses.

The dual T-dependent and T-independent nature of the humoral responses to xenografts could reflect the use of 1 or more immune pathways to mediate the response, none of which need be exclusive. B-1 cells make T-independent antibody responses, and these responses may or may not exhibit T-dependent components, including isotype switching and somatic mutations. Conceptually, the B-1 Ig variable regions encoding antigen recognition could be T cell independent or subject to T helper activity, depending on the nature of the presentation of the antigen as described above for the anti-PC response and independent carriers in mice. Initial antigen presentation and tissue antigens released from the damaged grafts could have major effects on the pattern of the response stimulated. Alternatively, nonoverlapping B-1 and B-2 responses may also exist, once again as a function of the nature and presentation of graft target antigens, as has been observed for the antibody responses to influenza.[4,34] The involvement of B-1 versus B-2 cell pathways in the xenograft response is complicated by the existence of a B-1-like population of cells in the marginal zones of lymphoid germinal centers in the spleen and lymph nodes.[35] These MZ lymphocytes share many phenotypic and functional characteristics with B-1 cells yet may also share common developmental pathways with B-2 cells.[36] These cells, along with B-1 cells, are early participants in anti-carbohydrate and T-independent antibody responses.[37] Their presence in the spleen may explain the prominent role of IgM^+ cells in the spleen seen as a component of xenograft rejection reactions.[38]

ISOTYPE SWITCHING:

The switch of immunoglobulin isotype that occurs, for example, as the immune response progresses (IgM to IgG).

Potential Therapeutic Approaches

The rejection of xenografts represents a host response that is primarily shaped by humoral responses to the foreign graft. This response shares many features with primitive humoral responses that provide protection from infectious agents for the host. The response is directed against carbohydrate antigens expressed by the grafts that share structural similarities with important carbohydrate components of viral and bacterial protective capsids or cell walls. The response to xenografts involves, most important, IgM antibodies that mediate the primary damage to the graft, while subsequent switching to IgG isotype antibodies and their role in the reaction are less clearly defined. Of particular interest are the observations that the immunoglobulin genes/target antigens that serve as the basis for the response represent only a limited portion of the repertoire available that might be expected to participate in the rejection reaction. It is possible that unique therapeutic approaches might include the development of small molecule blocking of antibody-antigen interactions, modification of antibody binding characteristics, inactivation or deletion of specific B cell subsets, gene therapeutic targeting of Ig V_H/V_L gene function, and

SOMATIC MUTATION:

Mutation that occurs in the somatic tissues of an organism and that will not, therefore, be heritable since it is not present in the germline.

anti-idiotype blocking of specific xenoantibodies. Each of these would take advantage of unique features of the response and provide an opportunity to more specifically target components of the reaction to prevent rejection. In each of these strategies resides the potential to specifically prevent graft rejection and create a basis for establishing long-term xenograft survival without the complications of generalized immunosuppression.

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