Indirect Allorecognition of Donor Class I and II Major Histocompatibility Complex Peptides Promotes the Development of Transplant Vasculopathy

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Abstract. Recent clinical and experimental evidence suggests that indirect allorecognition may promote the development of chronic rejection, but definitive experimental studies are lacking. To study the contribution of indirect allorecognition to chronic rejection, naïve Lewis (RT11) rats were immunized with synthetic Wistar Furth (WF) class II-RT1u.D (HLA-DR–like) or -RT1u.B (HLA-DQ–like) or class I-RT1u.A (HLA-A–like) peptides emulsified in complete Freund’s adjuvant 7 d before transplantation (n = 5 to 7/group). Experimental and control animals then acted as recipients of fully mismatched WF vascularized cardiac allografts. Recipients received immunosuppression in the form of cyclosporine at a tapering dose that allows for long-term allograft survival. Animals were sacrificed at either 3 or 6 mo, with allograft arterial luminal occlusion scored on elastin stains by a blinded observer. At 3 mo, mean vessel scores were significantly higher in the RT1u.A-immunized versus class II–immunized and control groups (P < 0.05). By 6 mo, there was progression of chronic allograft vasculopathy and a significantly higher mean vessel score in the RT1u.A- and RT1u.D-immunized versus RT1u.B and control groups (P < 0.05). In vitro studies show evidence of shifting MHC allopeptide immunogenicity. It was concluded that T cells primed by specific donor class I and II MHC allopeptides promote the development of chronic vascularized allograft rejection. These novel observations provide definitive evidence of a link between indirect allorecognition and the development and progression of chronic rejection.

T cell recognition of alloantigen is the central event responsible for the rejection of allografts (1). It is generally accepted that there are at least two pathways of allorecognition. In the direct pathway, T cells recognize endogenous peptides in the context of intact donor MHC molecules on the surface of donor antigen-presenting cells. With the indirect pathway, donor MHC and minor antigens are shed from the graft and processed by recipient antigen-presenting cells, where they are presented to T cells in the context of self MHC (2). Early experimental work in our laboratory has focused on determining the immunogenicity of synthetic peptides that represent the polymorphic domains of Wistar Furth (WF) class I and class II MHC molecules in Lewis (LEW) responders (3–5). More recently, we have demonstrated that pretransplant immunization with one of these class II peptides, RT1u.D (residues 20 to 44), is sufficient to accelerate acute rejection in the WF → LEW acute cardiac allograft rejection model (6). Histopathologic analysis of these rejected allografts revealed marked vascular rejection in the accelerated but not control animals. The pattern of in vitro T cell proliferation to donor peptides in these acutely rejecting animals mirrored that seen in the previous studies. However, when acute rejection in this same model was delayed 30 d by the administration of cyclosporine, T cell proliferation displayed evidence of epitope spreading, with responses to previously nonimmunogenic peptides as well as loss of immunogenicity to previously immunogenic peptides (7). We hypothesized that, with time and under chronic immunosuppression, these shifting proliferative responses contribute to chronic vasculopathy, the sine qua non of chronic rejection. In support of this hypothesis, Valujskikh et al. (8) demonstrated that T cells reactive to a single self-restricted allopeptide (i.e., indirect allorecognition) could mediate a form of allogeneic skin graft rejection that exhibits characteristics of a chronic, fibrosing process in the absence of direct allorecognition. However, studies that link the indirect pathway of allorecognition with the pathogenesis of chronic rejection in vascularized organs are limited. In this study, we report on a clinically relevant experimental model...
of chronic vascularized allograft rejection designed to study the contribution of indirect allorecognition to this process.

Materials and Methods

Class I MHC Peptides

Seven peptides of 16 to 22 amino acids each, representing most of the α1 and α2 domains of the RT1u.A molecule (WF class I MHC), were synthesized (QCB, Inc., Hopkinton, MA) as described elsewhere (5,9). Four peptides were α1 domain-derived: peptide 1 (residues 1 to 19), peptide 2 (residues 18 to 38), peptide 3 (residues 40 to 60), and peptide 4 (residues 60 to 82). Three peptides were α2 domain-derived: peptide 5 (residues 93 to 109), peptide 6 (residues 144 to 162), and peptide 7 (residues 162 to 172). In the LEW recipient, the first four RT1.A peptide segments (residues 1 to 19, 18 to 38, 40 to 60, and 60 to 82) are immunogenic as determined by lymphocyte proliferation in vitro (9) (Table 1). All peptides were suspended in phosphate-buffered saline at a concentration of 1 mg/ml for later in vivo use.

Class II MHC Peptides

Eight overlapping peptides of 25 to 26 amino acids each, representing the full-length sequence of the β1 chain hypervariable domain of WF class II-RT1.B (HLA-DQ-like) and -RT1.D (HLA-DR-like) molecules, were synthesized (QCB Inc.) as described elsewhere (3,4,10). Immunogenicity of these peptides has previously been evaluated by testing for lymphocyte proliferation in vitro and delayed type hypersensitivity responses in vivo (10). In the LEW recipient, immunogenicity is confined to the first two RT1.B peptide segments (residues 1 to 25 and 20 to 44) and the first two RT1.D peptide segments (residues 1 to 25 and 20 to 44) (Table 1). All peptides were suspended in phosphate-buffered saline at a concentration of 1 mg/ml for later in vivo and in vitro use.

Table 1. WF class I and II MHC peptide sequences and their immunogenicity in LEW responders

<table>
<thead>
<tr>
<th>Peptide b</th>
<th>Residue</th>
<th>Immunogenicity c</th>
<th>Sequence d</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT1u.D1</td>
<td>(1 to 25)</td>
<td>+</td>
<td>RDPTPRFLGVLKFECHFYNGTQRVR</td>
</tr>
<tr>
<td>RT1u.D2</td>
<td>(20 to 44)</td>
<td>+</td>
<td>GTRQVRLLARLJYNREEYARFSDV</td>
</tr>
<tr>
<td>RT1u.D3</td>
<td>(39 to 64)</td>
<td>-</td>
<td>RFDSVGEYRAVTLEGRPSAEYRNKQ</td>
</tr>
<tr>
<td>RT1u.D4</td>
<td>(60 to 84)</td>
<td>-</td>
<td>YRNKQKFEMERRATVDTYCRHNYE</td>
</tr>
<tr>
<td>RT1u.B1</td>
<td>(1 to 25)</td>
<td>+</td>
<td>QRLRRDFLVRQFKPYCFTNTGQRIR</td>
</tr>
<tr>
<td>RT1u.B2</td>
<td>(20 to 44)</td>
<td>+</td>
<td>GTQRIRNVRYIYNREEYLRYSVDV</td>
</tr>
<tr>
<td>RT1u.B3</td>
<td>(39 to 64)</td>
<td>-</td>
<td>RYDSVGEYRAVTLEGRPSAEYFNKQ</td>
</tr>
<tr>
<td>RT1u.B4</td>
<td>(68 to 92)</td>
<td>-</td>
<td>LFRQTRAELDTVCRHNYKTEVPTSL</td>
</tr>
<tr>
<td>RT1u.A1</td>
<td>(1 to 19)</td>
<td>+</td>
<td>GSHSLRFLTAVSRGPLGE</td>
</tr>
<tr>
<td>RT1u.A2</td>
<td>(18 to 38)</td>
<td>+</td>
<td>GEPRFIAVGYVDTEFYRYS</td>
</tr>
<tr>
<td>RT1u.A3</td>
<td>(40 to 60)</td>
<td>+</td>
<td>AENPRYEPRAWMEREGPEYW</td>
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<tr>
<td>RT1u.A4</td>
<td>(60 to 82)</td>
<td>+</td>
<td>WERETOQAGKHEQVNRVLRTL</td>
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<tr>
<td>RT1u.A5</td>
<td>(93 to 109)</td>
<td>-</td>
<td>HTIQVMFCDVGTDSWL</td>
</tr>
<tr>
<td>RT1u.A6</td>
<td>(144 to 162)</td>
<td>-</td>
<td>RNKLERDGDDFYKAYLEG</td>
</tr>
<tr>
<td>RT1u.A7</td>
<td>(162 to 179)</td>
<td>-</td>
<td>GTCLESLRRYLELGKERL</td>
</tr>
</tbody>
</table>

Indirect Allorecognition of Donor Class I and II MHC Peptides

Im bred 200 to 250 g male naïve LEW (RT1u) rats (Harlan Sprague-Dawley, Indianapolis, IN) were immunized with individual mixtures of either synthetic WF class II-RT1.D, -RT1.B, or class I-RT1.A peptide segments (100 μg of each) emulsified in complete Freund’s adjuvant (CFA) 7 d before heart transplantation (n = 6 to 7 per group for each time point). In addition, two control groups were set up: the first group was immunized with normal saline (NS) and CFA (n = 5 per group for each time point), and the second group was immunized with NS alone (n = 6 per group for each time point). Experimental and control animals then acted as recipients of fully mismatched WF vascularized cardiac allografts. Immunosuppression was administered in the form of cyclosporine (Bedford Laboratories, Bedford, OH) at a dose of 5.0 mg/d subcutaneously until posttransplant day 14, 1.0 mg/d until posttransplant day 45, and 0.5 mg/d thereafter. This immuno-suppressive protocol allows for long-term allograft survival and thus for the development of graft vasculopathy, the sine qua non of chronic rejection, in a clinically relevant small animal model.

Histology

Animals from each immunization group were sacrificed at 3 and 6 mo, with tissue obtained for histopathologic studies. Arterial luminal occlusion was scored on elastin stains by a blinded observer according to the following grading scheme: no luminal occlusion, 0; 1 to 20%, 1; 21 to 40%, 2; 41 to 60%, 3; 61 to 80%, 4; and 81 to 100%, 5 (Figure 1). The individual vessel scores for each animal were averaged. The mean value was then pooled with that of the other animals in each treatment group and expressed as the group mean vessel score ± SEM.

Peptide Proliferation Assays

The peptide proliferation assays have been described elsewhere (3,4,10). Briefly, popliteal, inguinal, and hypogastic lymph nodes were harvested at the time of death, and lymphocytes were isolated by
passing the tissue through a sterile 60-gauge stainless steel sieve. The cells were washed three times and resuspended in RPMI 1640 medium (Biowhitaker, Walkersville, MD) that contained 10% fetal calf serum, 100 U/ml penicillin, 100 μg/mL streptomycin, $2 \times 10^{-3}$M 2-mercaptoethanol, and 5 mM Hepes. Responder lymphocytes ($3 \times 10^5$) were cultured with 50 μg/mL peptide or Mycobacterium tuberculosis in 96-well flat-bottom plates (Costar, Cambridge, MA). All assays were set up in quadruplicate. Cells cultured in media without the addition of peptide acted as negative controls, and cells incubated in media and $M$. tuberculosis served as the positive control to confirm priming in vivo and cell viability/adequacy in vitro. The plates were incubated for 96 h at 37°C, with $^{3}$H-thymidine ($1 \mu$Ci/well; NEN Dupont, Boston, MA) added for the final 6 h of culture. After cell harvesting (Tomtec Harvester 96, Orange, CT), proliferation was assayed by measuring the incorporation of $^{3}$H-thymidine by lymphocyte DNA (Beta Plate; Wallac, Gaithersburg, MD). The stimulation index for each peptide was calculated according to the formula experimental cpm/control cpm (stimulation index $>2$ considered significant), as described elsewhere (11). Data are expressed as mean ± SEM.

Statistical Analyses
Statistical analysis was performed by use of InStat Software (GraphPad Software Inc., San Diego, CA). Mean vessel scores and peptide proliferation stimulation indices were analyzed with the $t$ test. Group means were categorized as being significantly different when the two-tailed $P$ value was <0.05.

Results
Graft Vasculopathy
Our immunosuppressive regimen allows for long-term allograft survival in this model, defined as the presence of palpable cardiac contractions until the time of harvest. First, we evaluated the effects of priming with donor class I and class II MHC peptides on the development of graft arteriosclerosis, as a marker of chronic rejection in this model. Mean vessel scores for each immunization group were determined as outlined in the Materials and Methods section. At 3 mo, a significantly ($P < 0.05$) higher mean vessel score was seen in the animals immunized pretransplant with RT1.A peptides (1.3 ± 0.6) than in those immunized with the RT1.B peptides (0.3 ± 0.2), RT1.D peptides (0.5 ± 0.1), or NS alone (0.5 ± 0.2) (Figure 2A). At 6 mo, however, there was progression of graft arteriosclerosis and significantly higher mean vessel scores ($P < 0.05$) in animals immunized pretransplant with either RT1.A peptides (2.5 ± 0.7) or RT1.D peptides (2.1 ± 0.5), compared with those animals immunized with RT1.B peptides (0.6 ± 0.2) or NS alone (0.4 ± 0.2) (Figure 2B). There was no correlation between the in vitro response to MT and the development of transplant vasculopathy in any of the immunization groups, ruling out the efficiency as a possible reason for the differences in mean vessel scores. In addition, there was no significant difference in the mean vessel score between those control animals immunized with NS alone and those immunized with NS and CFA (0.8 ± 0.2), which rules out an adjuvant effect. These data indicate that priming with donor MHC peptides promotes the development of chronic allograft rejection. Recognition of class I and the HLA-DR–like class II MHC peptides appears to play the critical role in this process. Moreover, cyclosporine therapy, consistent with clinical observations (12), had no effect on the development of graft vasculopathy mediated by indirect allore cognition of donor MHC peptides.

Figure 1. Representative graft morphology (elastin stain, ×400). Vascular luminal occlusion was scored according to the following grading scheme: no luminal occlusion, 0; 1% to 20%, 1; 21% to 40%, 2; 41% to 60%, 3; 61% to 80%, 4; and 81% to 100%, 5.
Peptide proliferation assays were performed in selected animals, as outlined in the Materials and Methods section. At 3 mo, significant proliferative responses were seen in each immunization group (n = 5 to 6 per group) to the peptide mixture used for immunization. Except for the RT1.A-immunized group, significant responses were also seen to the RT1.D peptide mixture, presumably as a result of immunization by the allograft itself (Table 2). In all groups, significant proliferative responses were seen to the positive control, M. tuberculosis. No significant correlation could be demonstrated between the MT and donor MHC peptide responses to allow for any sort of normalization ratio. Proliferative responses to individual peptide segments were also tested at 3 mo in the RT1.A-immunized group (Table 3). Results showed a loss of proliferative responses to those peptide segments previously shown to be immunogenic (RT1.A1–3, RT1.B1–2, and RT1.D1–2) in immunized, nontransplanted LEW animals (3,4,9). In addition, significant proliferative responses were seen to segments previously found to be nonimmunogenic (RT1.A5, RT1.B3–4, and RT1.D3–4) in this same setting. These results are consistent with reported data that have indicated the presence of epitope shifting, which is characterized by intermolecular (between MHC molecules) as well as intramolecular (within the same MHC molecule) spreading of T cell responses to antigenic allopeptides over time (7,11,13). At 6 mo, significant proliferative responses were seen to the immunized peptide mixture in most, but not all, cases (data not shown).

Discussion
Early studies that documented the presence of soluble, intact donor MHC molecules at the site of the graft suggest that such circulating molecules could represent a source of donor proteins for presentation by recipient antigen-presenting cells (14). Although the indirect pathway of allorecognition is the physiologic mechanism of an immune response, it was not until the 1980s that this pathway was actually considered to contribute to the destruction of allografts (15). Lechler and Batchelor (16) noted that, although retransplantation of passenger leukocyte-depleted rat kidney allografts did not elicit significant alloresponses, with time posttransplantation, elevated serum urea levels and other signs of rejection were detected. These early findings, coupled with more recent evidence, suggest that indirect allorecognition is not only involved in chronic rejection but actually may be the dominant pathway that mediates the process (7,13,17–20). Studies from our lab have shown a strong statistical correlation between the detection of indirect allorecognition and donor MHC peptide responses to allow for any sort of normalization ratio.

Peptide Proliferation Assays
Peptide proliferation assays were performed in selected animals, as outlined in the Materials and Methods section. At 3 mo, significant proliferative responses were seen in each immunization group (n = 5 to 6 per group) to the peptide mixture used for immunization. Except for the RT1.A-immunized group, significant responses were also seen to the RT1.D peptide mixture, presumably as a result of immunization by the allograft itself (Table 2). In all groups, significant proliferative responses were seen to the positive control, M. tuberculosis. No significant correlation could be demonstrated between the MT and donor MHC peptide responses to allow for any sort of normalization ratio. Proliferative responses to individual peptide segments were also tested at 3 mo in the RT1.A-immunized group (Table 3). Results showed a loss of proliferative responses to those peptide segments previously shown to be immunogenic (RT1.A1–3, RT1.B1–2, and RT1.D1–2) in immunized, nontransplanted LEW animals (3,4,9). In addition, significant proliferative responses were seen to segments previously found to be nonimmunogenic (RT1.A5, RT1.B3–4, and RT1.D3–4) in this same setting. These results are consistent with reported data that have indicated the presence of epitope shifting, which is characterized by intermolecular (between MHC molecules) as well as intramolecular (within the same MHC molecule) spreading of T cell responses to antigenic allopeptides over time (7,11,13). At 6 mo, significant proliferative responses were seen to the immunized peptide mixture in most, but not all, cases (data not shown).

Table 2. Results of proliferation assays to peptide mixtures at 3 moa

<table>
<thead>
<tr>
<th>Imunization Group</th>
<th>RT1A.A Mix Response</th>
<th>RT1A.B Mix Response</th>
<th>RT1A.D Mix Response</th>
<th>MTb Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline alone</td>
<td>—</td>
<td>—</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>RT1A.A</td>
<td>10.5</td>
<td>—</td>
<td>—</td>
<td>58.6</td>
</tr>
<tr>
<td>RT1A.B</td>
<td>—</td>
<td>6.8</td>
<td>—</td>
<td>15.3</td>
</tr>
<tr>
<td>RT1A.D</td>
<td>—</td>
<td>—</td>
<td>3.6</td>
<td>5.8</td>
</tr>
</tbody>
</table>

a The numbers depict the mean stimulation indices (SI) for each group (n = 5 to 6 per group), as calculated by use of the formula cpm peptide/cpm media alone. Highlighted numbers represent significant proliferative responses (SI >2).

b MT, mycobacterium tuberculosis.
alloreactivity to synthetic donor-derived MHC class II allopeptides in vitro and the presence of chronic rejection in recipients of renal allografts (11). Similar findings have been made in recipients of both cardiac (13) and lung (21) transplants with chronic dysfunction. More recently, analyses of heart transplant patients have demonstrated elevated frequencies of T cells with indirect anti-donor specificity in recipients of cardiac transplants with chronic vasculopathy (22), which complements previous work by these investigators that demonstrated reduced anti-donor T cell frequencies with direct specificity in this same patient population (18). We now report for the first time definitive evidence of a link between indirect allorecognition and the development of chronic rejection in the WF → LEW rat model of cardiac allograft rejection.

Histopathologic results from our experimental model of chronic rejection clearly indicate that pretransplant immunization with synthetic donor MHC class I and II peptides promotes the development of transplant arteriopathy. At 3 mo, higher mean vessel scores were demonstrated in the group immunized pretransplant with the donor MHC class I peptides, compared with all other immunization groups, including NS controls. These findings were interesting, given that the class I peptide mixture did not result in acceleration of acute rejection in this same strain combination in our previous studies (6). At 6 mo, higher mean vessel scores were seen in those animals immunized with the class I peptide mixture as well as the class II peptide mixture, RT1.D (HLA-DR–like), compared with all other immunization groups, including NS controls. Although the class II peptide mixture, RT1.B, is immunogenic in immunized LEW responders (3,4), this immunogenicity is not sufficient to promote the development of transplant arteriopathy, consistent with our previous findings. For example, we have previously demonstrated that pretransplant immunization with only the RT1.D mix results in acceleration of acute rejection in the WF → LEW cardiac allograft model, despite significant in vitro responses to the RT1.A and RT1.B mixture of peptides (6). Likewise, work on T cell clones from rejecting LEW recipients of WF hearts has shown that not all Th1 clones proliferating to donor peptide are able to produce delayed-type hypersensitivity responses to peptide when transferred into naïve LEW animals, suggesting that not all immunogenic peptides are pathogenic (23). These data also suggest that a functional difference exists between individual T cell responses to the various donor MHC loci, which may change over the course of allograft rejection. Although the exact mechanisms remain unknown, these putative differences may explain why not all T cell responses promote the development of chronic rejection. The fact that progression of arteriopathy was seen between 3 and 6 mo only in those animals immunized with donor MHC peptides and not those immunized with NS alone lends credence to the argument that alloantigen-dependent factors represent the predominant mechanism responsible for the pathogenesis of chronic rejection (19). These findings also suggest that true transplantation tolerance to the antigens of the donor may be, in fact, sufficient to prevent the histopathologic findings characteristic of this process (24,25).

Our peptide proliferation results provide in vitro evidence
that the indirect pathway of allorecognition is active and exhibits the phenomenon of epitope shifting during chronic rejection. These findings complement our previous studies in the delayed acute rejection model (7) and indicate that T cell memory responses to the indirect pathway are active late during the course of rejection. These responses are also present despite immunosuppression, because all animals received cyclosporine until the day of harvest. In addition, in vitro studies with the individual peptide segments showed proliferative responses to segments found to be nonimmunogenic in our previous studies that used immunized, nontransplanted animals and a loss of responsiveness to previously immunogenic segments. Indeed, although indirect T cell responses appear to be restricted to a limited repertoire of peptides (immunodominant epitopes) during initial acute rejection episodes (4,26,27), a change in the pattern of T cell responses, termed “epitope shifting” or “spreading,” has been demonstrated to occur in human recipients of kidney and heart transplants with evidence of chronic dysfunction (11,13). These data suggest that diversification of the immune response against the graft contributes to chronic rejection, which supports the hypothesis that chronic allograft vasculopathy results from continual injury initiated by the allopeptide-reactive T cells (20,28). These T cells provide help to monocytes/macrophages to induce delayed type hypersensitivity responses and to B cells for donor specific alloantibody production (7). In addition, they secrete the necessary cytokines and chemokines to activate endothelial and smooth-muscle cells as well as to promote trafficking of mononuclear cells into the graft, thus perpetuating chronic injury. (7,19,28).

We conclude that T cells primed indirectly by specific donor class I and II MHC allopeptides promote the development of chronic vascularized allograft rejection. These novel observations provide definitive evidence of a link between indirect allorecognition and the process of chronic rejection.

Acknowledgments

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References


