Inhibition of Terminal Complement Components in Presensitized Transplant Recipients Prevents Antibody-Mediated Rejection Leading to Long-Term Graft Survival and Accommodation

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Presensitization to potential donor MHC Ags remains a major risk factor for recipients awaiting solid organ transplantation (1). These individuals have elevated levels of broadly reactive alloantibodies, which may result from multiple blood transfusions, failed prior transplantation, or previous pregnancy (2). Transplantation of grafts into presensitized recipients in the absence of a “desensitization” therapy (3, 4) results in rapid Ab-mediated rejection (AMR), a process mediated either by preformed or induced anti-donor Abs produced as a result of an anamnestic response by memory B cells (5). AMR, also called accelerated humoral rejection, is characterized by markedly elevated circulating donor-reactive Abs, microvascular thrombosis, and C4d deposition in the graft (5). Currently available immunosuppressive agents, such as calcineurin inhibitors, fail to prevent AMR. These therapies target T cell immune responses and are less effective, inhibiting memory B cells and T-B cell interactions that result in an Ab response (6).

Some presensitized patients have benefited from immunoadsorption (7–9), plasmapheresis, i.v. Ig (10, 11), anti-CD20 mAb or splenectomy (12, 13) to temporarily eliminate pre-existing anti-donor Abs or to prevent the generation of new anti-donor Abs. Although effective in some patients, certain individuals are resistant to these desensitization therapies or must discontinue treatment because of unmanageable adverse side effects (9, 14–16). Therefore, development of a more effective strategy to prevent AMR in presensitized patients is warranted, based on a rational consideration of the pathogenesis of AMR.

Complement, a major component of natural immunity, is critically involved in anaphylatoxic activity, opsonization, and lysis of cells or foreign particles (17, 18). More recently, the role of complement in adaptive immune responses, including Ag processing and presentation, B cell activation, and T cell proliferation and differentiation, has been described (19–23). Furthermore, the complement system is an important effector mechanism in AMR (24, 25). C4d deposition in the peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific Abs (26, 27). In studies of acute allograft rejection, animals treated with complement inhibitors (28–30) or those genetically deficient...
FIGURE 1. Development of a murine model for AMR incorporating recipient presensitization. A, Evaluation of circulating anti-donor IgG and IgM Abs following skin presensitization. BALB/c mice were presensitized with C3H donor skin grafts. Serum was collected at the indicated time points, diluted 1/25 in PBS, reacted with donor strain splenocytes, and evaluated for Ab staining by immunofluorescence and flow cytometry. The results are expressed as mean fluorescence intensity of Ab-bound donor cells. B, Histology of heart allografts in presensitized BALB/c recipients (a) was evaluated at the time of rejection (POD3) by H&E staining of paraffin sections of cardiac allografts. The histology of a heart graft from a nonpresensitized BALB/c recipient similarly evaluated on POD3 is shown in b. Arrows indicate intravascular and/or interstitial changes in heart grafts; ×400 magnification was used for microscopy. C, Immunoperoxidase staining for intragraft IgG, IgM, C3, and C5 deposition among presensitized and nonpresensitized mice on POD3 was assessed by quantification of the percentage of positively stained areas within the sections evaluated using Empix Northern Eclipse imaging software and an Empix Imaging System. Statistical significance as compared with nonpresensitized recipients is indicated (*, p < 0.01). D, Immunoperoxidase staining for
in specific complement proteins (31, 32) exhibit significantly reduced Ab-mediated injury to renal and cardiac allografts. Currently, there is no approved therapeutic complement inhibitor to prevent AMR in clinical transplantation.

Activation of complement by anti-graft Abs is implicated in the pathogenesis of organ graft rejection (33). In some situations, however, a graft can survive in the presence of both anti-graft Abs and complement deposition—situation referred to as accommodation. Accommodation has been widely reported in experimental xenotransplantation models (34) as well as clinically in allotransplantation, leading to graft survival following transplantation across ABO barriers (35), in HLA-presensitized recipients (36), and in patients with both a positive cross-match and ABO incompatibility (37). The induction of accommodation in presensitized transplant recipients could therefore lead to graft acceptance, but the mechanisms required for accommodation are presently unknown.

Terminal complement components play an essential role in AMR. The C5 complement component is cleaved to form products such as C5a and C5b that exhibit multiple proinflammatory effects, thus representing an attractive target for complement inhibition within the immune-mediated inflammatory response. C5a is a powerful anaphylatoxin and chemotactic factor; cellular activation of C5a induces the release of multiple inflammatory mediators (38), and C5 cleavage results in the release of C5b, which combines with C6-C9 to form the cytolytic membrane attack complex C5b-9 (39). C5b-9, in turn, mediates direct tissue injury by cell lysis and, at sublytic doses, leads to cell activation (40, 41). Moreover, blockade of both C5a and C5b-9 generation may prevent complement-mediated graft damage during AMR without compromising the upstream C3b-driven processes of opsonization and clearance of pathogens and immune complexes (42).

We previously reported that a combination of C5 blockade and cyclosporine effectively prevented AMR and achieved indefinite graft survival in an MHC-mismatched murine cardiac transplant model (43). However, that study did not mimic the clinical setting in which patients who are highly sensitized to potential donor Ags reject their grafts through severe AMR. Therefore, the present study was undertaken to determine the efficacy of anti-C5 therapy in a model wherein AMR was induced by presensitizing recipient mice to donor MHC Ags before receiving a cardiac allograft. Accordingly, we show herein that anti-C5-based therapy effectively inhibits AMR and achieves indefinite graft survival in presensitized recipients. We also show that graft acceptance occurs as a consequence of accommodation. Finally, double transplant experiments demonstrate that immunological alterations in both the graft and the recipient are required for successful graft accommodation to occur.

Materials and Methods

Animals

Male adult C3H (H-2k) and BALB/c (H-2d) mice (The Jackson Laboratory) weighing 25–30 g were used as donors and recipients, respectively. Animals were housed under conventional conditions in the Animal Care Facility of the University of Western Ontario. All experiments were approved by the Animal Use Subcommittee and conducted according to the guidelines of the Canadian Council on Animal Care (44).

Skin presensitization

Full-thickness skin grafts from C3H donors were cut into pieces measuring 1 × 1 cm² and transplanted onto the backs of BALB/c recipient mice 1 wk before receiving C3H heart transplants.

Abdominal and cervical cardiac transplantation

Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomens of BALB/c recipients by anastomosis of the donor and recipient aortas and the donor pulmonary artery to recipient external jugular vein (end to end). The heart grafts were monitored daily by direct palpation until rejection (defined as complete cessation of pulsation), unless otherwise indicated. When cardiac impulses were no longer palpable, the graft was removed for routine histology and immunohistochemistry. Blood samples were collected from the inferior vena cavae at the indicated time points and the sera were isolated following centrifugation for flow cytometry or complement hemolytic assays.

Experimental groups

Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: group 1, untreated mice; group 2, mice treated with cyclosporine (CsA; 15 mg/kg/day, s.c., daily, starting from day 0); group 3, mice treated with cyclophosphamide (CyP; 40 mg/kg/day, i.v., on days 0 and 1); group 4, mice treated with CsA plus CyP according to the doses used in groups 2 and 3; group 5, mice treated with anti-C5 mAb (clone BB5.1, 40 mg/kg/day, i.p., days 0–2, followed by twice a week, days 3–60); group 6, mice treated with anti-C5 mAb plus CsA according to the respective doses used in groups 5 and 2; group 7, mice treated with anti-C5 mAb plus CyP according to the doses used in groups 5 and 3, respectively; group 8, mice treated with anti-C5 mAb in combination with CsA and CyP according to the doses used in groups 5, 2, and 3, respectively. When cardiac impulses were no longer palpable or at postoperative day (POD) 100, the grafts and sera were removed for in vitro studies. Five additional animals from groups 6 and 8 were euthanized on POD3 (equivalent to the mean survival time for groups 1–5 and 7) to allow comparisons at a uniform time point. Sera were also collected on POD11, 21, 28, and 60 in group 8 to detect sequential changes in anti-donor Ab levels and complement activity. Some triple therapy-treated presensitized recipients that carried a first heart graft for 100 days were retransplanted with either a second naive C3H heart or an accommodated 100-day surviving C3H heart from another presensitized BALB/c recipient. Three-to-five animals were included in each retransplant group.

Graft histology

At necropsy, heart tissue samples were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned for H&E staining (45). The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist (B. Garcia). Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage, and lymphocyte infiltration and were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change compared with normal tissues.

Immunohistochemistry

Four micrometer sections were cut from cardiac frozen tissue samples embedded in Tissue-Tek OCT gel (Sakura Finetek), mounted on gelatin-coated glass microscope slides, and stained by a standard indirect avidin-biotin immunoperoxidase method using an Elite Vectastain ABC kit (Vector Laboratories). Specimens were evaluated for the presence of CD4+ and CD8+ T cells using a biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.12; Cedara Laboratories) and a biotin-conjugated
rat anti-mouse CD8 mAb (clone 53-6-7; BD Biosciences), respectively. Intragraft monocyte/macrophage infiltration was detected with a biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories). Mouse IgG and IgM deposition was detected in grafts using biotin-conjugated goat anti-mouse IgG and goat anti-mouse IgM, respectively (Cedarlane Laboratories). For identification of complement deposition, tissue sections were sequentially incubated with polyclonal goat anti-mouse C3 or goat anti-mouse C5 sera (Quidel), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated streptavidin (Zymed Laboratories). Slides were washed with PBS between the Ab incubation steps and examined under light microscopy. Negative controls were performed by omitting the primary Abs. Ab reactivity was evaluated in five high-powered fields of each section using tissue samples from five animals per treatment group. Quantification of intragraft IgG, IgM, C3, and C5 deposition was determined as the percentage of positively stained areas within a given section. Intragraft infiltration of CD4+, CD8+, and Mac-1+ cells was quantified by counting the number of positively stained cells in the section and dividing by the area of the section using Empix Northern Eclipse imaging software (Empix Imaging).

**Flow cytometry**

Circulating donor-specific total IgG, IgG2a, IgG2b, and IgM Ab responses were evaluated in recipient sera by flow cytometry (BD Biosciences) following incubation with donor splenocytes as previously described (45). Briefly, recipient mouse sera were obtained at the indicated time points from the various treatment groups, diluted 1/25 in PBS (previously determined in titration studies to be the most appropriate dilution for Ag-Ab recognition while avoiding Ag-Ab saturation), and incubated with C5H mouse splenocytes at 37°C for 30 min. The cells were then washed and incubated with FITC-conjugated goat anti-mouse Ab specific for either total mouse IgG, mouse IgG2a, or mouse IgG2b (Caltag Laboratories) or with rhodamine red-conjugated affinity purified Fab goat anti-mouse IgM, μ-chain specific (Jackson Immunoresearch Laboratories). Following reactivity with the Abs for 1 h at 4°C, the cells were washed with PBS, resuspended at 5 × 10⁶/ml, and analyzed by flow cytometry. The results, expressed as mean channel fluorescence intensities, are a measure of each anti-donor Ab isotype level in the serum samples.

**Complement hemolytic assays**

Terminal complement activity in recipient mouse sera was determined by standard methods to assess its ability to lyse chicken erythrocytes, which had been presensitized with erythrocyte-specific Abs as previously described (46). Briefly, purified anti-C5 mAb at 100, 2, and 0 μg/ml in gelatin Veronal-buffered saline (GVBS) containing 0.1% gelatin, 141 mM NaCl, 0.5 mM MgCl₂, 0.15 mM CaCl₂, and 1.8 mM sodium barbital was used as low, medium, and 100% lysis controls, respectively. Experimental samples were prepared by diluting the murine test serum 1/10 in GVBS. Control and experimental samples were added, in triplicate, to wells of a 96-well plate containing an equal volume of 10% normal BALB/c mouse serum and 10% human C5-deficient serum in GVBS. Two microliters of 500 mM EDTA was added to the third well of both the 100% lysis and experimental sample triplicates to generate “no hemolysis” color control standards for each condition (46). Chicken erythrocytes were washed in GVBS, sensitized by incubation with an anti-chicken RBC polyclonal Ab (Intercell Technologies; 0.1% v/v) at 4°C for 15 min, washed again, and resuspended in GVBS at a final concentration of ~7.5 × 10⁶ cells/ml. The sensitized chicken erythrocytes (~2.5 × 10⁶ cells) were added to the plate containing the controls and samples, mixed briefly on a plate shaker, and incubated at 37°C for 30 min. The plate was then mixed again, centrifuged at 3000 rpm for 3 min, and 80 μl of the supernatant was transferred to wells of a 96-well flat-bottom microtiter plate (BD Biosciences). The plate was read at OD₄₁₅ using a microplate reader and the percentage of hemolysis was determined using the following formula: % hemolysis = 100 × ((OD sample − OD sample color control)/(OD 100% lysis control − OD 100% lysis color control)).

**Western blot analysis**

Sonication of previously frozen heart samples was performed in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) at 4°C. Protein content of the clarified supernatants was measured (Bio-Rad) before electrophoretic separation (10 μg of protein/lane) on NuPAGE, downloaded from http://www.jimmunol.org/ by guest on May 3, 2017
4–12% gradient Bis-Tris gels, and the proteins transferred to polyvinylidene difluoride (Invitrogen Life Technologies) membranes. Intragraft expression of Bcl-2 and Bcl-xS/L was detected using the polyclonal rabbit antisera (N-19 and M-125, respectively; Santa Cruz Biotechnology). Anti-calsequestrin rabbit polyclonal sera (Calbiochem) was used to verify sample loading (47). Each membrane was cut and developed simultaneously with the appropriate test and internal control Abs to ensure equivalent sample detection of proteins having large molecular mass differences. Detection of primary Ab binding was detected by HRP-conjugated goat anti-rabbit IgG and ECL development (Roche) (48).

Statistical analysis

The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the log-rank test. Histological findings were analyzed using the ANOVA on rank test. Flow cytometric and immunohistological data were analyzed using one-way ANOVA. Differences with $p$ values <0.05 were considered significant.

**Results**

Presensitization with donor skin grafts induces AMR in BALB/c recipients receiving a C3H cardiac allograft

To develop a suitable animal model that mimics presensitized transplant recipients, BALB/c recipients were grafted with skin from C3H donors before C3H-to-BALB/c heart transplantation. Following presensitization, the circulating anti-donor IgG levels were markedly elevated, reaching peak levels 7–14 days after skin grafting (Fig. 1A). Recipient animals received their transplanted C3H allografts 7 days following skin graft presensitization. Heart

![FIGURE 3](image_url)

**FIGURE 3.** Immunohistological analysis of heart allografts in presensitized mouse recipients at necropsy. A, Immunoperoxidase staining for intragraft IgG, IgM, C3, and C5 deposition among untreated and treated presensitized mice at the time point of rejection or at study endpoint (POD100) was assessed by quantification of the percentage of positively stained areas within the sections evaluated using Empix Northern Eclipse imaging software and an Empix Imaging System. Statistical significance is indicated (+, $p < 0.01$, C5 deposition in triple therapy group on POD100 vs other groups at study endpoint (time of rejection); **, $p < 0.01$, IgG deposition in the triple therapy group on POD100 vs other groups at study endpoint (time of rejection)). B, Immunoperoxidase staining for intragraft infiltration of CD4$^+$, CD8$^+$, and Mac-1$^+$ cells among untreated and treated presensitized mice at the time point of rejection or at study endpoint (POD100) was analyzed by quantitatively all positively stained cells in the section, divided by the section area examined (cells/mm$^2$). Statistical significance is indicated (+, $p < 0.01$, triple therapy group on POD100 vs other groups at study endpoint (time of rejection)). C, Immunoperoxidase staining for intragraft IgG, IgM, C3, and C5 deposition at various time points among presensitized mice treated with anti-C5mAb/CsA/CyP triple therapy. The levels of intragraft Ab and complement components were evaluated as described in A. Statistical significance is indicated (+, $p < 0.01$, C5 deposition on POD100 vs other time points; **, $p < 0.01$, IgG deposition on POD60 and POD100 vs other time points). D, Immunoperoxidase staining for intragraft CD4$^+$, CD8$^+$, and Mac-1$^+$ cells at various time points among presensitized mice treated with anti-C5 mAb/CsA/CyP triple therapy. The levels of cellular infiltrate were evaluated as described in B. Statistical significance is indicated (+, $p < 0.01$, Mac-1$^+$ cell infiltration on POD60 and POD100 vs other time points; **, $p < 0.01$, CD4$^+$ and CD8$^+$ cell infiltration on POD100 vs other time points).
allografts were rapidly rejected in 3.1 ± 0.4 days by typical AMR characterized by severe thrombosis, hemorrhage, and infarction (Fig. 1Ba). In contrast, nonpresensitized BALB/c mice rejected C3H cardiac allografts in 8.2 ± 0.8 days (43, 45), demonstrating normal graft histology on POD3 (Fig. 1Bb). Immunohistological studies conducted at the time of rejection (POD3) in presensitized animals indicated massive IgG Ab, C3, and C5 deposition with modest CD4+ and CD8+ cell infiltration in the grafts relative to nonpresensitized animals evaluated on the same day (Fig. 1, C and D). Furthermore, circulating anti-donor IgG levels among presensitized recipients on POD3 were six times higher than those among nonpresensitized recipients evaluated on the same day (p < 0.01, Fig. 1E). The levels of circulating anti-donor IgM Abs were low but detectable in nonsensitized animals and did not appreciably increase with sensitization (Fig. 1E), which may indicate that IgG, but not IgM, is the predominant isotype of anti-donor Abs in this presensitized allotransplant model. As expected, skin presensitization did not affect serum complement activity as measured by an in vitro hemolytic assay (Fig. 1F). Taken together, these data suggest that the presensitized cardiac allograft model provides a stringent in vivo system in which to investigate the role of terminal complement in AMR among presensitized recipients.

Anti-C5 mAb in combination with CsA and CyP prevents AMR and achieves indefinite heart allograft survival in presensitized mouse recipients

We next evaluated whether anti-C5 mAb, administered as monotherapy or in combination with CsA and/or CyP, would prevent AMR in presensitized mouse cardiac allograft recipients. Untreated transplant recipients or those treated with CsA, CyP, or a combination of the two drugs rejected their grafts in 3.1 ± 0.4, 3.0 ± 0.0, 3.3 ± 0.5, and 3.5 ± 0.6 days, respectively (Table I). The rejected grafts among untreated and treated mice demonstrated similar pathological features of severe AMR (Fig. 2, A–D). Anti-C5 mAb, administered as monotherapy or in combination with CyP, was unable to improve graft survival; heart grafts among these mice were rejected in 3.5 ± 0.5 and 3.2 ± 0.4 days, respectively (Table I), again by AMR (Fig. 2, E and F). The combination of anti-C5 mAb and CsA substantially prolonged graft survival to 11.9 ± 1.8 days (Table I). Although these heart grafts still appear to be rejected through AMR (Fig. 2G), with high levels of intragraft IgG and C3 deposition (Fig. 3A), infiltrating CD4+ and CD8+ cells were also observed in the grafts among these animals (Fig. 3B), suggesting a cellular component to the mechanism of rejection as well. In contrast, triple therapy, consisting of anti-C5 mAb, CsA, and CyP, effectively prevented AMR and achieved indefinite heart graft survival for >100 days in presensitized recipients (Table I; p < 0.01, vs all other treatment regimens). We also performed additional heart transplants in some mice at later time points (12–16 days) following skin sensitization to ensure that 7 days was sufficient and optimal for the development of AMR. Heart grafts transplanted 12–16 days following skin sensitization were rejected by POD4 regardless of whether they were untreated or received monotherapy or two drugs in combination, while all animals (n = 3) that received anti-C5 mAb-based triple therapy survived >100 days (data not shown).

The cardiac allografts at POD100 in triple therapy-treated presensitized recipients had normal histology (Fig. 2H) with no detectable CD4+ and CD8+ cell infiltration (Fig. 3, B and D), despite evidence of C3, C5, and IgG and IgM accumulation in the grafts at this time point (note that treatment with anti-C5 mAb was terminated on POD60) (Fig. 3C). In addition, the moderate intragraft infiltration of Mac-1+ cells, including monocytes and macrophages, in untreated and CsA-, CyP-, or CsA plus CyP-treated animals was reduced in anti-C5 mAb-treated animals (Fig. 3B) and was absent in long-term surviving grafts (Fig. 3, B and D). These results indicate that administration of a functionally blocking anti-C5 mAb in combination with CsA and CyP effectively prevents AMR and achieves indefinite heart graft survival in presensitized recipients.

Treatment with anti-C5 mAb completely blocks terminal complement activity and local C5 deposition in presensitized recipients receiving a heart allograft

To determine whether anti-C5 mAb therapy effectively inhibits systemic terminal complement components in presensitized recipients, serum hemolytic activity in the different treatment groups was assessed at POD3. As expected, treatment of mice with either CsA or CyP alone or the two drugs in combination had no effect on serum complement activity as compared with naive or untreated controls (Fig. 4), whereas treatment with anti-C5 mAb alone or in combination with CsA and/or CyP completely inhibited complement activity at this time point (p < 0.01).

To confirm that blockade of terminal complement in the serum correlated with the absence of terminal complement deposition in the graft, C5 deposition in the allograft tissue was analyzed by immunohistochemistry. On POD3, 11, and 60, C5 deposition in heart grafts was completely prevented in presensitized recipients undergoing treatment with anti-C5 mAb, either alone or in combination with other immunosuppressants (Fig. 3, A and C) but was not affected in untreated, CsA-, CyP-, or CsA plus CyP-treated animals (Fig. 3A). Termination of anti-C5 mAb therapy at POD60 resulted in demonstrable C5 intragraft deposition by POD100 (Fig. 3, A and C) despite continuing graft survival. As predicted, blockade of terminal complement components with anti-C5 mAb did not prevent the deposition of the upstream complement protein C3 in the grafts at any evaluated time point (Fig. 3, A and C). These results indicate that chronic anti-C5 treatment consistently blocks systemic complement activity and terminal complement deposition in heart allografts transplanted into presensitized recipients.
Long-term surviving heart grafts are resistant to AMR despite systemic and intragraft anti-donor Abs and terminal complement

To investigate whether prevention of AMR with anti-C5 mAb occurs despite the presence of anti-donor Abs, levels of systemic and intragraft anti-donor IgG and IgM Abs were evaluated. Flow cytometric evaluation of the binding of circulating Abs to donor-strain splenocytes on POD3 demonstrated that untreated, presensitized BALB/c recipients had the highest levels of circulating anti-donor IgG Abs relative to all the other experimental groups (Fig. 5A). Levels of circulating anti-donor IgG Abs were reduced with CsA or CyP monotherapy relative to untreated controls, with a further decrease apparent in animals receiving a combination of the two drugs (p < 0.05 vs untreated, monotherapy, or other two-drug combination therapy groups). As predicted, treatment with anti-C5 mAb alone did not affect anti-donor Ab levels. Similarly, addition of anti-C5 mAb to CsA-, CyP-, or CsA plus CyP-treatment regimens did not further reduce anti-donor IgG Ab levels beyond that observed with these immunosuppressants alone or in combination. Importantly, the anti-donor IgG levels detected in the sera of animals with long-term surviving grafts decreased over the course of the experiment and stabilized at a relatively low level (Fig. 5B), albeit still elevated over that of naive controls (Fig. 5A). Circulating anti-donor IgM Abs remained at low levels following the presensitization procedure, and these levels did not appreciably change over the 100-day study period.

Strong deposition of anti-donor IgG Abs was demonstrated in all grafts at POD3 or POD11, regardless of treatment regimen (Fig. 3, A and C). All therapies that included CyP appeared to reduce anti-donor IgG Ab graft deposition at POD3 and POD11 (Fig. 3, A and C). As demonstrated with circulating levels of anti-donor IgG Abs in triple therapy-treated animals with long-term surviving grafts, IgG deposition attenuated over time but was still detectable at the study endpoint (Figs. 3C and 5Ca). IgM levels remained

**FIGURE 5.** A, Serum levels of anti-donor Abs in presensitized BALB/c recipients evaluated on POD3. Serum was collected from untreated mice or mice treated with the indicated regimens, diluted 1/25 in PBS, and evaluated for levels of circulating anti-donor IgG and IgM by flow cytometry as described in the legend of Fig. 1A. The results are expressed as mean fluorescence intensity of Ab-bound cells. Statistical significance among presensitized recipients is indicated (*, p < 0.05, IgM level in groups with CyP treatment in either monotherapy, two-drug in combination, or triple therapy vs other groups; **, p < 0.05, IgG level in CsA+CyP-treated group vs untreated, monotherapy, or other two-drug combination therapy groups). B, Evaluation of circulating anti-donor Ab levels among presensitized mice treated with anti-C5 mAb, CsA, and CyP triple therapy at various time points after transplant. Serum was collected at the indicated time points, diluted 1/25 in PBS, and reacted with donor-strain splenocytes as described in the legend of Fig. 1A. Results are expressed as mean fluorescence intensity of Ab-bound cells. C, Deposition of IgG (a) and IgM (b) in long-term surviving grafts of presensitized BALB/c recipients treated with anti-C5 mAb, CsA, and CyP on POD100. Arrows indicate positive staining for IgG and IgM; ×400 magnification was used for microscopy. D, Serum complement hemolytic activity in triple therapy-treated mice at various time points following transplantation. Note that anti-C5 mAb treatment was halted on POD60.
low, but detectable, in transplanted heart grafts regardless of treatment (Figs. 3, A and C, and 5Cb).

Treatment with anti-C5 mAb eliminated serum complement activity to an undetectable level in animals with long-term surviving grafts (POD3 and POD100). Serum (diluted 1/25 in PBS) was reacted with donor-strain splenocytes as described in Materials and Methods and the isotypes of bound Abs was revealed by detection with anti-mouse IgG2a and anti-mouse IgG2b Abs followed by flow cytometry. The results are expressed as the mean fluorescence intensity of Ab-bound cells. The level of anti-donor IgG2a in the recipients with accommodated grafts was significantly lower than that in the recipients with rejected grafts (*, p < 0.01). Presensitized recipients carrying the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared with recipients carrying rejected grafts (**, p < 0.01).

FIGURE 6. Comparison of serum levels of anti-donor IgG subclasses between untreated recipients carrying rejected heart grafts (POD3) and anti-C5 mAb-based triple therapy-treated recipients carrying accommodated heart grafts (POD3 and POD100). Serum (diluted 1/25 in PBS) was reacted with donor-strain splenocytes as described in Materials and Methods and the isotypes of bound Abs was revealed by detection with anti-mouse IgG2a and anti-mouse IgG2b Abs followed by flow cytometry. The results are expressed as the mean fluorescence intensity of Ab-bound cells. The level of anti-donor IgG2a in the recipients with accommodated grafts was significantly lower than that in the recipients with rejected grafts (*, p < 0.01). Presensitized recipients carrying the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared with recipients carrying rejected grafts (**, p < 0.01).

Similarly, mild intragraft C5 deposition returned in these animals by POD100 (Fig. 3C). These results suggest that preventing AMR with anti-C5 mAb enables long-term graft survival, which is maintained despite the return of systemic complement activity and evidence of terminal complement and Ab deposition in the graft, a situation consistent with graft accommodation.

Anti-C5 mAb in combination with CsA and CyP leads to a shift from anti-donor IgG2a to IgG2b in presensitized recipients carrying accommodated grafts

Previous studies have implicated isotype switching in the process of accommodation (49). To determine whether anti-C5 mAb-based triple therapy induced a shift in IgG subclass that may be associated with accommodation, we compared the serum levels of anti-donor IgG2a and IgG2b subclasses among untreated presensitized recipients vs those carrying an accommodated heart graft. We found that sera obtained on POD3 from untreated presensitized recipients undergoing graft rejection contained anti-donor Abs of predominantly the IgG2a isotype (Fig. 6), while a significant and profound reduction of total IgG Abs, concomitant with a shift from IgG2a to IgG2b, was observed on both POD3 and POD100 among presensitized recipients carrying accommodated grafts (Fig. 6, p < 0.01). In addition, the recipients with accommodated grafts demonstrated increased levels of anti-donor IgG2b on POD3 and POD100 compared with presensitized recipients carrying rejected grafts on POD3 (Fig. 6, p < 0.01). The pattern of anti-donor IgG isotypes among presensitized recipients treated with either monotherapy or two-drug combination therapy was indistinguishable from that of untreated presensitized animals (data not shown). Taken together, these data suggest that this anti-donor IgG2a to

FIGURE 7. Expression of protective proteins in long-term surviving heart grafts of presensitized recipients. Heart grafts were harvested at the time points indicated. Western blot analysis was performed as described in Materials and Methods for protective proteins expression of Bcl-2 and Bcl-xL. The levels of calsequestrin are indicated as a control for sample loading. The data show one representative of five independent experiments in each group.

FIGURE 8. Experimental outline of secondary transplantation experiments. Presensitized BALB/c recipients carrying accommodated primary heart grafts for 100 days received additional transplanted heart grafts. Specifically, either naive hearts (group I) or 100-day accommodated hearts from other presensitized recipients (group III) were transplanted into the cervical region of presensitized recipients carrying accommodated primary heart grafts and receiving continuous CsA treatment. Accommodated heart grafts were also retransplanted into presensitized BALB/c recipients undergoing treatment with CsA and CyP (but without anti-C5 mAb) (group II).
IgG2b shift observed with triple therapy may contribute to preservation of the graft through accommodation.

Expression of the antiapoptotic proteins Bcl-2 and Bcl-xL: evidence of graft accommodation

Accommodated grafts can be distinguished from nonaccommodated grafts in experimental xenotransplantation models by the specific up-regulation of protective genes, such as heme oxygenase, A-20, Bcl-2, and Bcl-xL, in the graft vasculature (34). In the present study, we hypothesized that the resistance of long-term surviving grafts to the pathologic effects of anti-donor Abs and terminal complement may result from intragraft expression of protective antiapoptotic proteins. We therefore evaluated whether Bcl-2 and Bcl-xL were up-regulated in the surviving grafts of presensitized recipients receiving anti-C5 mAb-based triple therapy. We found that grafts at POD100 expressed high levels of Bcl-2 and Bcl-xL proteins (Fig. 7). Furthermore, these proteins were detected as early as 12 days following transplantation. In contrast, the expression of Bcl-2 and Bcl-xL proteins was undetectable in grafts of untreated presensitized animals (Fig. 7) or in grafts of presensitized animals treated with either monotherapy or two-drug combination therapy (data not shown).

FIGURE 9. Comparison of second heart graft survival, histological and immunohistological changes, and serum levels of anti-donor IgG subclasses in the retransplantation model. A, Presensitized BALB/c recipients with accommodated primary heart grafts received either second naive hearts (Fig. 8, group I) or second accommodated hearts (Fig. 8, group III) and were monitored for graft survival (postoperative days following the second transplant). In addition, presensitized BALB/c recipients transplanted with accommodated heart grafts (Fig. 8, group II) were also monitored for graft survival. B, Histological features of the second naive heart at time of necropsy (a) or second accommodated heart 90 days following retransplantation (b) into presensitized recipients with accommodated primary heart grafts. Arrows indicate intravascular and/or interstitial changes in heart grafts; ×400 magnification was used for microscopy. C, Histology of accommodated heart grafts before (a) and after (b) retransplantation into presensitized recipients that had received CsA+CYP treatment without anti-C5 mAb. Arrows indicate intravascular and/or interstitial changes in heart grafts; ×400 magnification was used for microscopy. D, Intragraft IgG, IgM, C3, and C5 deposition within second grafts at the time point of rejection (POD6 and POD4 for groups I and II, respectively) or at study endpoint (POD90 for group III) was assessed by immunoperoxidase staining according to the methods described in the legend to Fig. 3. Statistical significance is indicated (*, p < 0.01, intragraft IgG and C5 deposition in rejected heart grafts vs accepted heart grafts). E, Comparison of serum levels of anti-donor IgG subclasses. Serum was diluted 1/25 in PBS, reacted with donor-strain splenocytes as described in Materials and Methods, and the isotypes of bound Abs were revealed by detection with anti-mouse IgG2a and anti-mouse IgG2b Abs followed by flow cytometry. The results are expressed as the mean fluorescence intensity of Ab-bound cells. The level of anti-donor IgG2a in the recipients with rejected grafts was significantly higher than that in the recipients with accepted grafts (*, p < 0.01). Presensitized recipients carrying both primary and second accommodated heart grafts displayed a much higher level of anti-donor IgG2b than anti-donor IgG2a (**, p < 0.01).
These results suggest that the resistance of long-term surviving grafts to AMR in presensitized animals is associated with the up-regulation of Bcl-2 and Bcl-xL protein expression.

Accommodation requires changes in both the graft and the recipient

Graft accommodation may occur through molecular or cellular changes taking place in the transplanted graft, in the recipient, or in both graft and recipient (reviewed in Ref. 50). To distinguish among these possibilities, we performed second graft transplantations as outlined in Fig. 8. In the simplest interpretation, the acceptance of a naive donor graft by a presensitized recipient carrying a long-term accepted graft might indicate that changes in the recipient alone are sufficient for accommodation to occur (Fig. 8, group I), although it is also possible that the accepted graft might have undergone alterations that could then impact the recipient. By contrast, the acceptance of a previously long-term accepted graft by a presensitized, but naive, recipient would indicate that factors within the graft tissue alone are sufficient to induce accommodation (Fig. 8, group II). Finally, accommodation that occurs only if the transplant combination includes both a previously accepted, long-term graft and a presensitized recipient that is carrying a long-term accepted graft would suggest that factors from both the graft and the recipient are necessary for accommodation to occur (Fig. 8, group III).

In initial experiments, naive C3H hearts were transplanted into the necks of presensitized BALB/c recipients that still carried long-term accommodated heart grafts from a previous transplant, as they had received anti-C5 mAb/CsA/CyP-triple therapy (group I; n = 3). The accommodated recipients received only CsA monotherapy following transplantation with the naive second heart. The naive second C3H hearts were rejected at 6.6 ± 1.1 days (Fig. 9A) by severe AMR (Fig. 9Ba) with increased intragraft deposition of IgG and C5 (Fig. 9D) and a significant up-regulation of anti-donor IgG2a in the circulation (Fig. 9E) when compared with long-term surviving presensitized animals carrying accommodated heart grafts (p < 0.01). The first heart grafts continued to survive in these animals. These data indicate that changes in the recipient alone are not sufficient to induce accommodation in this model.

We then transplanted long-term surviving C3H hearts, removed from presensitized BALB/c recipients on POD100, into presensitized BALB/c mice treated with CsA and CyP, but not with anti-C5 mAb therapy (group II; n = 3). Accommodated grafts were rapidly rejected in 4.3 ± 0.6 days (Fig. 9A). Following retransplantation, the previously accommodated heart grafts that had shown normal histology (Fig. 9Ca) now revealed major histological changes with features of severe AMR (Fig. 9Cb). The rejected second hearts demonstrated significant deposition of IgG and C5 compared with accommodated hearts in long-term surviving presensitized animals (Fig. 9D, p < 0.01); in addition, these group II animals undergoing graft rejection showed high levels of circulating anti-donor IgG2a Abs (Fig. 9E, p < 0.01). These results suggest that changes solely within long-term surviving grafts are also not sufficient to explain accommodation in these presensitized recipients.

Finally, long-term surviving C3H hearts, removed from presensitized BALB/c recipients on POD100, were transplanted into presensitized BALB/c mice that had also maintained heart allografts for over 100 days owing to treatment with anti-C5 mAb/CsA/CyP-triple therapy (group III; n = 5). These recipients continued to receive CsA monotherapy, but did not receive further anti-C5 mAb intervention. The second, previously accommodated grafts were fully accepted by the presensitized recipients carrying long-term accepted grafts (Fig. 9A), with no histological signs of rejection 90 days after the second transplant (Fig. 9Bb). Both first and second accommodated heart grafts in these animals expressed the protective proteins Bcl-2 and Bcl-xL, as detected by Western blot analysis (data not shown). The second accommodated heart grafts demonstrated only low levels of intragraft Ab and complement deposition (Fig. 9D). These group III recipients carrying second accommodated heart grafts demonstrated circulating anti-donor Abs of predominantly the IgG2b isotype at levels indistinguishable from anti-C5 mAb triple-therapy-treated mice carrying accommodated primary heart allografts (Fig. 9D). The results from these double transplant experiments clearly indicate that induction of graft accommodation in presensitized recipients requires immunologic and/or cellular alterations in both the graft and the recipient.

Discussion

The development of AMR represents a significant obstacle to performing organ transplantation in patients presensitized to donor Ags, as it is typically unresponsive to conventional antirejection therapies (12). Currently, therapeutic approaches designed to prevent AMR, such as splenectomy, plasmapheresis, or i.v. Ig or anti-CD20 mAb administration have focused on the clearance or inhibition of anti-donor Abs (7–13). Although achieving some success, these approaches are not always practical or effective (5, 37, 51, 52). Therefore, the presence of preformed anti-donor Abs often precludes transplantation in a significant proportion of potential recipients. Preformed Abs can activate complement (25), which further contributes to the development of tissue injury within the allograft (24). To our knowledge, the present study represents the first evaluation of the role of C5 and activated terminal complement components in Ab-mediated allograft rejection in a presensitized murine cardiac allotransplantation model. We demonstrate that C5 blockade, combined with a regimen of ongoing T cell immunosuppression and short-term B cell immunosuppression, provides an effective strategy to prevent both AMR and cellular rejection in highly presensitized graft recipients. Furthermore, our results suggest that inhibition of AMR through complement blockade may provide sufficient time for accommodation to occur via as yet poorly understood modifications to both the graft and the recipient.

We previously reported that the combination of anti-C5 mAb and CsA therapy effectively prevents AMR and achieves indefinite graft survival in an MHC-mismatched murine cardiac transplant model (43). Among presensitized patients, however, AMR is intensified by the presence of high-titer pre-existing anti-donor Abs, which can result from past blood transfusions, prior transplants, multiple pregnancies, or donor-recipient ABO incompatibility. To investigate the potential benefit of inhibiting terminal complement activity in highly presensitized recipients, skin from donor animals was grafted onto recipients to induce potent anti-donor Ab responses, at which time heart grafts were transplanted. In this model, skin presensitization resulted in the deposition of high levels of IgG, C3, and C5; occasional CD4+ and CD8+ cells, and also significant numbers of Mac-1+ cells, suggesting that this presensitization regimen elicits both innate and adaptive cellular immune responses as well as humoral immune responses. Despite the evidence of mixed cellular and humoral immunity, the predominant immunohistological features of the cardiac grafts at the time of rejection among untreated animals were consistent with severe AMR. Thus, a combination of conventional immunosuppressive agents CsA and CyP (which together inhibit both cellular and humoral immune responses) failed to prevent AMR despite a significant reduction in alloreactive Ab levels. This observation suggests that, in the presence of normal complement function, even moderate levels of anti-donor Abs can mediate early stage and vigorous
AMR following transplantation in presensitized recipients. In contrast, the addition of the terminal complement inhibitor anti-C5 mAb to the immunosuppressive regimen completely inhibited intragraft terminal complement deposition and prevented graft rejection in all animals, despite the presence of anti-donor Abs. These data demonstrate the importance of terminal complement in the process of AMR and suggest that blocking C5 may provide benefit for presensitized transplant patients.

The complement component C5 is an attractive target for potential therapies aimed at preventing AMR for the following reasons: 1) although the complement cascade can be initiated through three distinct pathways, all three converge, sharing the same terminal complement components (C5 through C9) which possess potent proinflammatory and cell lytic properties (38–41, 53, 54); 2) blockade of C5 cleavage prevents C5a generation, thereby reducing ischemia/reperfusion-mediated organ damage (55), neutrophil chemotaxis, and macrophage cytokine production (56); 3) complement inhibition at C5 also inhibits formation of the membrane attack complex C5b-9, thus preventing tissue injury through cell lysis or cell activation (57, 58). C5b-9 stimulates the secretion of mediators from storage granules and the translocation of P-selectin to the plasma membrane (59), initiating monocyte and platelet adhesion to the endothelium and contributing to the further production of inflammatory mediators (60). C5b-9-activated endothelial cells also synthesize IL-8, tissue factor, and monocyte chemotactic protein 1 (61), an important chemotactic factor in macrophage recruitment. In the current study, Mac-1+ macrophages were frequently seen in untreated and CsA plus CyP-treated allografts, whereas anti-C5 mAb significantly inhibited macrophage infiltration (Fig. 3, B and D).

Other studies of complement blockade in transplantation models have targeted early complement components such as C3 (28, 30, 62). Targeting the terminal complement protein C5, however, may have several advantages over blockade of C3. For example, by preserving early complement components, the main immunoprotective functions of the complement cascade remain intact, including microbial opsonization and immune complex clearance by C3b (63). In addition, inhibition of terminal complement will also preserve the generation of iC3b, a C3b product that plays a role in tolerance induction (64, 65) by modifying CD11b-expressing APCs such that they cannot initiate primary immune responses, but instead induce antigenic tolerance (64). Furthermore, genetic or therapeutic prevention of C3b generation restores full APC function (64). Whether iC3b is critical to the long-term acceptance of allografts in this model remains to be investigated. Finally, targeting C5 preserves the generation of C3a, which has recently been shown to down-regulate Th2 responses in a murine skin injury model (66). Down-regulation of Th2 cytokines may play an important role in the prevention of AMR (45). In the present study, inhibition of the complement cascade at C5 did not affect the generation of C3, as evidenced by intragraft deposition of C3.

Inhibition of C5a generation through anti-C5 mAb therapy may enhance Th1 polarization. Studies by Hawlisch et al. (67) indicate that C5a negatively regulates TLR4- and CD40-induced synthesis of IL-12 family cytokines, which in turn decreases Th1 responses. It is interesting to speculate that Th1 polarization through blockade of C5 cleavage may render presensitized recipients more susceptible to CsA therapy, which is more effective in the context of Th1-type immune responses (45). The precise role of terminal complement blockade in T cell immunity will be addressed in future studies.

We demonstrated that long-term surviving grafts in our model were resistant to AMR, despite the ready detection of anti-donor Abs, the return of intragraft terminal complement deposition, and the restoration of normal systemic hemolytic function following cessation of anti-C5 mAb therapy. These data suggest an acquired resistance of the graft to the recipient’s humoral immune system—a biological process known as accommodation (50). To determine whether accommodation indeed developed in our model and if it occurred through adaptations in the graft, the recipient, or both, we developed a retransplantation model that allowed animals to simultaneously carry two heart grafts. In the absence of further anti-C5 therapy, hearts transplanted from naive donors into recipients that had long-term surviving hearts from a previous transplant were rapidly rejected, suggesting that changes in the recipient are not sufficient to induce accommodation. Similarly, long-term surviving hearts that were transplanted into recipients that had received only conventional immunosuppressive drugs were also rapidly rejected, suggesting that changes in the graft alone cannot account for the process of accommodation. However, long-term surviving heart grafts from accommodated recipients that were transplanted into recipients already carrying long-term surviving hearts were fully accepted in the absence of anti-C5 mAb therapy. These experiments clearly indicate that accommodation occurred in our presensitized model and that the accommodation process requires cellular and/or molecular alterations in both the graft and the recipient.

To investigate potential mechanisms of accommodation specific to the graft, expression levels of the “protective” genes Bcl-2 and Bcl-xL were evaluated. These antiapoptotic genes, which protect against tissue damage by suppressing programmed cell death and inhibiting endothelial cell activation (68), have been shown to be up-regulated in the vasculature of accommodated grafts (34). In the present study, grafts from presensitized recipients treated with the combination of CsA, CyP, and anti-C5 mAb showed marked up-regulation of Bcl-2 and Bcl-xL protein expression from POD12 through POD100, while Bcl-2 or Bcl-xL protein expression was not evident in rejected grafts among other treated or untreated presensitized mice. Following retransplantation, second accommodated heart grafts also showed high levels of Bcl-2 and Bcl-xL protein expression that was not evident in second rejected heart grafts (data not shown). These data suggest that the up-regulation of these antiapoptotic proteins may be associated with, or at least contribute in part to, the accommodation process. Other possible modifications to the graft that may contribute to graft accommodation include the loss or antigenic alteration of immunodominant graft cell surface proteins, the up-regulation of cell surface complement inhibitors (e.g., decay accelerating factor or CD59), the regeneration of cell surface protective substances (e.g., heparan sulfate), or the acquired resistance of graft endothelial cells to humoral responses (reviewed in Ref. 50). Additional experiments are ongoing to better understand the nature of graft changes leading to accommodation.

In addition to changes in the graft, an altered immune response by the presensitized recipient may also contribute to the induction of accommodation. Indeed, others have shown that mice with accommodated grafts have demonstrated a preferential increase in levels of anti-donor Abs of the IgG2b subclass (49). To investigate potential changes in the immune responses of presensitized recipients that could contribute to accommodation in our model, iso-types of serum-derived anti-donor Abs were evaluated in recipients carrying accommodated grafts. Mice with long-term surviving grafts showed significant increases in levels of serum anti-donor IgG2b, but marked decreases in levels of anti-donor IgG2a compared with presensitized recipients undergoing graft rejection. This result could either reflect the favored production of anti-donor IgG2b in presensitized recipients receiving anti-C5 mAb-based triple therapy, or may simply reflect a situation in which preferential
binding of other Ab isotopes occurs within the surviving graft, leaving predominantly IgG2b Abs in the circulation to be detected. The precise role of the IgG2b isotype in the process of accommodation, if any, remains unclear.

A functionally blocking anti-human C5 mAb (eculizumab) has recently been evaluated for treating a rare form of hemolytic anemia called paroxysmal nocturnal hemoglobinuria (69, 70). Phase III studies have demonstrated long-term safety and efficacy and show that terminal complement can be effectively and consistently inhibited in these patients. Eculizumab has recently been approved for the treatment of paroxysmal nocturnal hemoglobinuria.

In summary, we have demonstrated that inhibition of terminal complement through specific targeting of C5, in combination with conventional immunosuppressive therapy, prevents AMR in highly presensitized recipients and allows indefinite graft survival in all animals tested. Long-term surviving grafts appear to undergo accommodation, suggesting that an immunosuppressive regimen that includes anti-C5 therapy may translate into a clinically applicable strategy to prevent AMR in sensitized transplant patients.

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Disclosures

S.J.F., D.R.G., and R.P.R. are employed by Alexion Pharmaceuticals, Inc., whose potential commercial product or related products were evaluated in this study.

References


