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Complement-Fixing Elicited Antibodies Are a Major Component in the Pathogenesis of Xenograft Rejection

Tsukasa Miyatake,* Koichiro Sato,* Ko Takigami,* Nozomi Koyamada,* Wayne W. Hancock,* Herve Bazin,† Dominique Latinne,† Fritz H. Bach,* and Miguel P. Soares2*‡

Hamster to rat cardiac xenografts undergo delayed rejection as compared with the hyperacute rejection of discordant xenografts. Elicited xenoreactive Abs (EXA) are thought to initiate hamster to rat cardiac xenograft rejection. In this study, we demonstrate that following transplantation of a hamster heart, rats generated high levels of EXA. Adoptive transfer into naive recipients of purified IgM, IgG2b, or IgG2c, but not IgG1 or IgG2a EXA, induced xenograft rejection in a complement-dependent manner. Ability of EXA to cause rejection correlated with complement activation, platelet aggregation, and P-selectin expression in the xenograft endothelium. Cyclosporin A (CyA) administration, after transplantation, totally suppressed IgG1, IgG2a, IgG2b, and IgG2c EXA, and inhibited IgM EXA production, but failed to overcome rejection. Administration of cobra venom factor (CVF), 1 day before and at the time of transplantation, resulted in complement inhibition during 3 days after transplantation, which failed to overcome rejection. Combination of CyA and CVF, which we have previously shown to overcome rejection, resulted in suppression of IgG EXA production and in the return of IgM XNA to preimmunization serum levels, 3 to 7 days after xenotransplantation, while complement remained inhibited. Thus, under CyA/CVF treatment, complement activation by hamster cells was suppressed following xenotransplantation, and presumably for this reason xenograft rejection did not occur. In conclusion, our data demonstrate that EXA play a pivotal role in the pathogenesis of xenograft rejection and that CyA and CVF suppress xenograft rejection by preventing exposure of xenograft endothelial cells to complement activation by EXA. The Journal of Immunology, 1998, 160: 4114–4123.

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3 Abbreviations used in this paper: XNA, preformed xenoreactive natural Abs; CDC, complement-dependent cytotoxicity; CVF, cobra venom factor; CyA, cyclosporin A; EC, endothelial cell; EXA, elicited xenoreactive Abs; GVB, gelatin veronal buffer; HAR, hyperacute rejection; C$_4$H$_2$O$_5$N$_3$SBR; NF-sxB, nuclear factor-sxB.
switch and secretion of IgG2b (mouse IgG2a/IgG2b) and IgG2c (mouse IgG3) (15–17). Rat IgG2b activates complement and mediates complement-dependent cell-mediated cytotoxicity (CDC) as well as Ab-dependent cell-mediated cytotoxicity, while IgG2c binds C1q, but does not mediate CDC or Ab-dependent cell-mediated cytotoxicity (18). Most nucleated cells actively counteract the cytotoxic effects of complement activation and do not undergo necrosis (19). However, binding of C1q or generation of sublytic levels of C5b-9 activates NF-κB and up-regulates the expression of proinflammatory genes in EC (20, 21).

The generation of EXA following transplantation has been suggested previously to contribute to xenograft rejection (22). However, a detailed analysis of the role of EXA in the pathogenesis of xenograft rejection has not been formally tested. In the present study, we have analyzed the role of EXA in the pathogenesis of xenograft rejection and investigated whether CyA and CVF administration suppress xenograft rejection by inhibiting the generation of EXA. We demonstrate that upon immunization with a hamster cardiac xenograft, untreated rats generate high levels of anti-hamster IgM and IgG EXA, which upon adoptive transfer into naive recipients induce xenograft rejection through complement activation. Administration of CyA, after transplantation, significantly decreased IgM and suppressed IgG EXA production to undetectable levels. Combination of CyA with CVF resulted in inhibition of complement activation during the transient rise of IgM EXA, occurring during 10 to 15 days after transplantation. Once complement returned to pretreatment levels, IgM XNA had dropped to basal levels, suggesting that xenograft EC may never be exposed to complement in the presence of high levels of IgM XNA, and therefore xenograft rejection does not occur. The expression of the protective genes heme oxygenase-1, A20, bcl-2, and bcl-xL in EC of the graft may then contribute to suppression of xenograft rejection by inhibiting EC activation upon complement activation by IgM XNA.

Materials and Methods

Animals

Inbred male Lewis, ACI, and mu*/nu* rats (225–320 g) (Harlan Sprague-Dawley, Indianapolis, IN) were used as xenograft recipients, and male Golden Syrian hamsters (70–120 g) (Harlan Sprague-Dawley) were used as donors for cardiac xenografts. Lewis rats express the Igk allotype, and ACI rats express the Igκ light chain allotype (23). All animals were housed in accordance with guidelines from American Association for Laboratory Animal Care, and all research protocols were approved by Institutional Animal Care and Use Committees of both Israel Deaconess Medical Center (Boston, MA) and Deaconess Medical Center (Boston, MA).

Surgical procedures were conducted under anesthesia with pentobarbital (30–50 mg/kg i.p.) (Abbott, North Chicago, IL). Heterotopic hamster to rat cardiac transplantation was performed as described before (10).

Experimental protocols

Induction of accommodation was achieved by combination of CyA (Novartis Pharma, Basel, Switzerland) (15 mg/kg, i.m., daily from day 0 until the end of the experiments) and CVF administration (Quidel, San Diego, CA) (60 U/kg 1 day before xenotransplantation and 20 U/kg at the time of transplantation). To analyze the individual effect of CVF and CyA on xenograft rejection and investigated whether CyA and CVF administration suppress xenograft rejection has not been formally tested. In the present study, we have analyzed the role of EXA in the pathogenesis of xenograft rejection and investigated whether CyA and CVF administration suppress xenograft rejection by inhibiting the generation of EXA. We demonstrate that upon immunization with a hamster cardiac xenograft, untreated rats generate high levels of anti-hamster IgM and IgG EXA, which upon adoptive transfer into naive recipients induce xenograft rejection through complement activation. Administration of CyA, after transplantation, significantly decreased IgM and suppressed IgG EXA production to undetectable levels. Combination of CyA with CVF resulted in inhibition of complement activation during the transient rise of IgM EXA, occurring during 10 to 15 days after transplantation. Once complement returned to pretreatment levels, IgM XNA had dropped to basal levels, suggesting that xenograft EC may never be exposed to complement in the presence of high levels of IgM XNA, and therefore xenograft rejection does not occur. The expression of the protective genes heme oxygenase-1, A20, bcl-2, and bcl-xL in EC of the graft may then contribute to suppression of xenograft rejection by inhibiting EC activation upon complement activation by IgM XNA.

Serum levels of rat anti-hamster EXA were measured by cellular based indirect enzyme-linked assay (ELISA). The Syrian hamster kidney cell line HAK (ATCC, CCL-15; American Type Culture Collection, Rockville, MD) was used as an antigenic target. Briefly, HAK cells were cultured in DMEM (Life Technologies, Gaithersburg, MD), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies). Glutardialdehyde-fixed HAK cells were incubated (1 h, 37°C) in the presence of rat serum serially diluted in 0.05% PBS/Tween-20 (Sigma Chemical), and rat anti-hamster EXA were detected using mouse anti-rat IgG light chain (MARK-1, Igκ-1b light chain (RG11/15) (Phekang, San Diego, CA), IgM (MARM-4), IgG1 (MARG1-2), IgG2a (MARG2a-1), IgG2b (MARG2b-8), or IgG2c (MARG2c-5) (Zymed, San Francisco, CA) mAbs (1 μg/ml, 1 h, room temperature). Mouse anti-rat mAbs were detected using horse-radish peroxidase-labeled goat anti-mouse F(ab')2 fragments depleted from affinity-purified IgG (0.1 μg/ml, 1 h, room temperature (Pierce, Rockford, IL)). Rat anti-hamster EXA of the Igκ-1b allotype were detected using biotin-labeled rat anti-Igκ-1b (LORK1b) mAb (Experimental Immunology Unit, UCL). Biotin-labeled rat mAb was detected using horse-radish peroxidase-labeled streptavidin (0.2 μg/ml) (Sigma Chemical). Horseradish peroxidase was revealed using orthophenyldiamidine (OPD; Sigma Chemical) and H2O2 (0.03%) in citrate buffer (pH 4.9). Absorbance was measured at A = 490 nm. The relative amount of circulating EXA in the serum was expressed as OD (A = 490) taken from one serial dilution in the linear range of the assay (1/32–1/1024).

Binding of rat C3 to hamster cells was measured by a modified cellular ELISA using HAK cells as antigenic targets. Briefly, nonfixed HAK cells were incubated in presence of rat serum serially diluted in GVB2 buffer (1 h, 37°C). Cells were fixed in 37% PBS and 0.05% glutaraldehyde, and rat C3 deposition was detected using a mouse anti-rat C3 mAb (Serotec, Oxford, U.K.).

Complement-mediated cytotoxicity

Preconfluent HAK cells were incubated with purified rat anti-hamster EXA (1–2 h, 37°C) in DMEM, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were washed in PBS, incubated with 10% baby rabbit serum (Accurate Chemicals, Westbury, NY) in GVB2 buffer (1 h, 37°C), collected by trypsin digestion (Life Technologies), and resuspended in PBS, 3% FCS, and 10 μg/ml propidium iodide (15 min, room temperature) (Sigma Chemical). Cells were analyzed for viability by flow cytometry (1 × 10⁶ cells/sample) using a FACScan cytometer (Becton Dickinson, Mountain View, CA) equipped with Cell Quest software (Becton Dickinson). Alternatively, CDC was measured by a cytotoxicity assay based on cleavage of the tetrazolium salt MTT (Sigma Chemical). Briefly, preconfluent HAK cells were incubated in the presence of rat anti-hamster purified EXA (1–2 h, 37°C), washed in PBS, incubated with 10% baby rabbit serum (30–50 mg/kg i.p.) (Abbott, North Chicago, IL). Heterotopic hamster to rat cardiac transplantation was performed as described before (10).

The generation of EXA following transplantation has been suggested previously to contribute to xenograft rejection (22). However, a detailed analysis of the role of EXA in the pathogenesis of xenograft rejection has not been formally tested. In the present study, we have analyzed the role of EXA in the pathogenesis of xenograft rejection and investigated whether CyA and CVF administration suppress xenograft rejection by inhibiting the generation of EXA. We demonstrate that upon immunization with a hamster cardiac xenograft, untreated rats generate high levels of anti-hamster IgM and IgG EXA, which upon adoptive transfer into naive recipients induce xenograft rejection through complement activation. Administration of CyA, after transplantation, significantly decreased IgM and suppressed IgG EXA production to undetectable levels. Combination of CyA with CVF resulted in inhibition of complement activation during the transient rise of IgM EXA, occurring during 10 to 15 days after transplantation. Once complement returned to pretreatment levels, IgM XNA had dropped to basal levels, suggesting that xenograft EC may never be exposed to complement in the presence of high levels of IgM XNA, and therefore xenograft rejection does not occur. The expression of the protective genes heme oxygenase-1, A20, bcl-2, and bcl-xL in EC of the graft may then contribute to suppression of xenograft rejection by inhibiting EC activation upon complement activation by IgM XNA.
serum in GVB+ (1 h, 37°C), washed in PBS, and incubated (2 h, 37°C) with MTT (300 μg/ml). Cells were washed in PBS and lysed in 100% ethanol to dissolve MTT crystals. Absorbance was measured at λ = 570 nm and the percentage of cytotoxicity was calculated as follows: 100 – (100 × [ODsample– OD100% lysis]/ODtotal lysis– OD100% lysis). The spontaneous cytotoxicity by baby rabbit serum in the absence of rat EXA was subtracted from all experimental samples. One hundred percent cell lysis was calculated after lysis of HAK cells in lysis solution (Promega, Madison, WI) (10 μl, 1 h). Zero percent cell lysis was calculated from HAK cells in culture medium.

**Results**

**Rats have significant serum levels of preformed anti-hamster IgM XNA**

Rat IgM was immunopurified from the serum of naive or immunized rats, as described in Materials and Methods. Myeloma IgM was used as a negative control. IgM concentrations were measured by ELISA (data not shown). IgM purified from control or immunized rat sera recognized the hamster epithelial cell line HAK (Fig. 1A). Recognition of hamster cells was specific since myeloma IgM (IR202) recognize hamster HAK cells only weakly, due to nonspecific binding of rat IgM in this assay (Fig. 1A). In contrast to IgM purified from immunized serum, IgM purified from control serum or from myeloma ascites did not activate complement significantly and did not mediate CDC of hamster cells (Fig. 1B).

**Rats generate high levels of elicited anti-hamster xenoreactive Abs (EXA) after xenotransplantation**

Unlike IgM, circulating XNA of the IgG isotype remained at background levels before xenotransplantation (Fig. 2). Following xenotransplantation, rats generated high levels of anti-hamster IgM, IgG1, IgG2a, IgG2b, as well as IgG2c EXA that were directed against hamster endothelium and recognized HAK cells (Fig. 2). Anti-hamster IgM EXA reached a maximal serum level at day 6 after transplantation and decreased thereafter. The anti-hamster IgG EXA response was slightly delayed as compared with the IgM response, and once it reached a maximal level (approximately day 7–14) remained stable (Fig. 2). Increased serum levels of IgM and IgG EXA correlated with increased C3 deposition on the xenograft vascular endothelium (data not shown) and increased complement activation in vitro, as revealed by the detection of C3 deposition on the surface of HAK cells (data not shown), as well as by CDC of HAK cells (Fig. 2).

**Rejection of hamster cardiac xenografts is initiated by EXA of the IgM, IgG2b, and IgG2c isotypes in a complement-dependent manner**

To evaluate the role of EXA in the pathogenesis of xenograft rejection, IgM, IgG1, IgG2a, IgG2b, and IgG2c were purified from the serum of untreated and immunized ACI rats (expressing the κ-1b Ig light chain allotype) and transferred into naive Lewis rats (expressing the κ-1a Ig light chain allotype), 30 min after xenotransplantation. Immunization of ACI rats resulted in production of high levels of anti-hamster EXA, as detected by mAbs specific for the rat IgG-1b light chain allotype (LORK-1b) or the rat Igκ-1a and -1b light chain allotypes (MARK-1) (Fig. 3A).

All Ig isotype fractions retained reactivity against hamster cells after purification (Fig. 3B). The relative amount of EXA in each isotype fraction was evaluated (Fig. 3B), and the volume of purified Ig transferred was adjusted for each isotype to correspond to the relative amount of EXA of the same isotype present in 200 μl of the initial immunized serum (Fig. 3B), which reproducibly induced HAR of hamster cardiac xenografts (Table I). Purified Ig fractions revealed only minor cross-contamination with other Ig isotypes, as tested by cellular ELISA (Fig. 3C).

Upon binding to hamster HAK cells, IgM and IgG2b activated complement, as revealed by CDC of HAK cells (Fig. 4). The ability of IgM to activate complement was twofold higher than that of IgG2b. Low but detectable CDC was also observed upon binding of IgG1, and to a lesser extent IgG2c to HAK cells (Fig. 4). However, IgG1 and IgG2c mediated CDC 10- to 20-fold less efficiently than IgG2b or IgM, respectively (Fig. 4).

Shown in Table I are the results of administering unfraccionated serum or various purified Ig isotype fractions to rats having received a xenograft 30 min earlier. Immunized serum induced HAR of hamster xenografts in 7 to 15 min, while rats receiving control serum rejected xenografts in 72 h (Table I). Transfer of IgM purified from immunized serum induced xenograft rejection at 48 h, while rats receiving an equivalent amount of IgM purified from nonimmunized serum rejected xenografts in 72 h. Transfer of IgG1 or IgG2a purified from immunized serum had no significant effect in terms of xenograft survival. Transfer of IgG2b purified from
immunized serum induced xenograft rejection at 26 to 62 min, while transfer of IgG2b purified from nonimmunized serum had no significant effect in terms of xenograft survival. Transfer of IgG2c purified from immunized serum induced xenograft rejection in 3 to 24 h. This effect was, however, not observed in 2 of 10 animals analyzed. Transfer of equivalent amounts of control IgG2c had no significant effect in inducing xenograft rejection.

To evaluate the role of complement activation in the induction of xenograft rejection, IgM, IgG1, IgG2a, IgG2b, and IgG2c EXA were transferred into CVF-treated rats, 30 min after xenotransplantation. Transfer of EXA had no significant effect in inducing xenograft rejection, as compared with recipients treated with CVF alone (Table I). These data demonstrate that induction of xenograft rejection by IgM, IgG2a, or IgG2c purified from immunized serum. EXA were detected by cellular ELISA using mouse anti-rat isotype mAbs, as indicated in the figure and described in Materials and Methods. Values shown are the mean ± SD from three different samples. SD ranged between 1 and 2%, and thus these values are not visible at the scale used. C. Detection of anti-hamster EXA of the IgM, IgG1, IgG2a, IgG2b, IgG2c, IgA, and IgE isotypes in IgM, IgG1, IgG2a, IgG2b, and IgG2c purified from immunized serum. EXA were detected by cellular ELISA using mouse anti-rat isotype mAbs, as described in Materials and Methods. OD values (A = 490 nm × 1000) were measured from one serial dilution (1/64) in the linear range of the assay.

Immunopathology of xenograft rejection after EXA transfer

Analysis of hamster cardiac xenografts 3 h after transfer of purified Ig showed in each case deposition of EXA of the Ig κ-1b allotype on the xenograft endothelium (Fig. 6, a, d, g, j, and m) whereas the extent of concomitant deposition of C3 (Fig. 6, b, e, h, k, and n) and expression of P-selectin (Fig. 6, c, f, i, l, and o) lm l l p o varied considerably between the isotypes of Ig transferred. In recipients receiving purified IgM, IgG2b, or IgG2c EXA, indicating that distinct ability of these EXA isotypes to cause xenograft rejection was not related to variations in their serum concentration.

Detection of Igκ-1b EXA after transfer of purified IgM, IgG1, IgG2a, IgG2b, and IgG2c

Serum levels of Ig of the κ-1b allotype were evaluated 15 min after transfer of Ig of the κ-1b allotype, purified from immunized ACI rats, into Lewis rats expressing Ig of the κ-1a allotype. Ig of the κ-1b light chain allotype remained at undetectable levels before EXA transfer (Fig. 5, A and B). The levels of circulating EXA of the κ-1b allotype were similar in recipients receiving IgM, IgG1, IgG2a, IgG2b, or IgG2c EXA, indicating that distinct ability of these EXA isotypes to cause xenograft rejection was not related to variations in their serum concentration.

FIGURE 3. IgM, IgG1, IgG2a, IgG2b, and IgG2c purified from ACI rats expressing Ig of the κ-1b light chain allotype. Rat anti-hamster EXA were detected by a cellular-based ELISA, as described in Materials and Methods. A. Serial dilution of anti-hamster EXA in Lewis (circles) and ACI rat serum (squares) before (open symbols) and 14 days after xenotransplantation (black symbols). Anti-hamster EXA were detected using mouse anti-rat Igκ-1b (Igκ-1b) or anti-rat Igκ-1a and -1b light chain allotype (Igκ total) mAbs, as described in Materials and Methods. Values shown are the mean ± SD from three different samples. SD ranged between 1 and 2%, and thus these values are not visible at the scale used. B. Detection of rat IgM, IgG1, IgG2a, IgG2b, and IgG2c in total unfractionated immunized serum (black squares) and in purified IgM, IgG1, IgG2a, IgG2b, and IgG2c isotype fractions (white squares) was detected using mouse anti-rat isotype mAbs, as described in Materials and Methods. Values shown are the mean ± SD from three different samples. SD ranged between 1 and 2%, and thus these values are not visible at the scale used. C. Detection of anti-hamster EXA of the IgM, IgG1, IgG2a, IgG2b, IgG2c, IgA, and IgE isotypes in IgM, IgG1, IgG2a, IgG2b, and IgG2c purified from immunized serum. EXA were detected by cellular ELISA using mouse anti-rat isotype mAbs, as indicated in the figure and described in Materials and Methods. OD values (A = 490 nm × 1000) were measured from one serial dilution (1/64) in the linear range of the assay.
endothelium, IgG1 and IgG2a EXA do not activate complement efficiently (Fig. 6, e and h). P-selectin labeling was also absent upon transfer of EXA of the IgG1 or IgG2a isotypes (Fig. 6, f and I). Analyses of hamster xenografts 24 h after Ig transfer no longer showed deposition of EXA of the \( \kappa \)-1b allotype on cardiac endothelium, whereas endogenous EXA of the Ig\( \kappa \)-1a allotype were detected (data not shown). This data indicate that upon binding to the xenograft endothelium, the \( t_{1/2} \) of EXA is shorter than 12 h.

The combination of CyA and short-term CVF administration induces long-term survival of hamster to rat cardiac xenografts

We have demonstrated previously that rejection of hamster to rat cardiac xenografts is suppressed under the administration of the T cell immunosuppressant CyA and the complement inhibitor CVF (10). In our previous study, CVF was administered during 11 days, i.e., 1 day before xenotransplantation and during 10 days thereafter (10). To decrease the toxicity and immunogenicity associated with the administration of CVF, we have reduced the duration of the CVF treatment to 2 days, i.e., 1 day before xenotransplantation and at the time of xenotransplantation. Combination of CyA and CVF resulted in long-term survival of hamster to rat cardiac xenografts (>50 days), while xenograft survival was not prolonged significantly in recipients treated with CyA alone or CVF alone, as compared with untreated recipients (Table II). Xenograft survival was associated with the expression of the protective genes A20, bcl-2, bcl-x\(_L\), and heme oxygenase-1 in the xenograft endothelium, as well as with an ongoing Th2 cytokine response (data not shown). IgM and C3 were detected in the endothelium of xenografts undergoing long-term survival (data not shown).

Combined administration of CyA and CVF results in complement depletion during a period of transient elevation of elicited IgM EXA

Administration of CyA alone or in combination with CVF significantly decreased the generation of IgM EXA as compared with untreated or CVF-treated rats (Fig. 7). Administration of CyA alone or in combination with CyA had no significant inhibitory effect on the generation of IgM EXA as compared with controls or CyA-treated rats (Fig. 7). Administration of CVF led to depletion of complement hemolytic activity (CH50) to undetectable levels by the time of xenotransplantation and during 3 days thereafter.

Table I. Effect of adoptive transfer of purified EXA in the survival of hamster cardiac xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Xenograft Survival Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunized serum</td>
<td>3</td>
<td>7 m; 9 m; 15 m</td>
</tr>
<tr>
<td>Control serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>IgM from immunized serum</td>
<td>3</td>
<td>48 h (n = 3)</td>
</tr>
<tr>
<td>IgM from control serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>IgG1 from immunized serum</td>
<td>5</td>
<td>24 h; 48 h; 72 h (n = 3)</td>
</tr>
<tr>
<td>IgG1 from control serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>IgG2a from immunized serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>IgG2a from control serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>IgG2b from immunized serum</td>
<td>3</td>
<td>33 m; 26 m; 1 h</td>
</tr>
<tr>
<td>IgG2b from control serum</td>
<td>4</td>
<td>72 h (n = 3); 96 h</td>
</tr>
<tr>
<td>IgG2c from immunized serum</td>
<td>12</td>
<td>1 h; 2 h; 3 h (n = 3) =24 h (n = 5); 72 h (n = 2)</td>
</tr>
<tr>
<td>IgG2c from control serum</td>
<td>3</td>
<td>72 h (n = 3)</td>
</tr>
<tr>
<td>CVF-treated rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Ig transfer</td>
<td>7</td>
<td>48 h (n = 3) 72 h (n = 3); 120 h</td>
</tr>
<tr>
<td>IgM from immunized serum</td>
<td>5</td>
<td>96 h (n = 5)</td>
</tr>
<tr>
<td>IgG2b from immunized serum</td>
<td>3</td>
<td>96 h (n = 3)</td>
</tr>
<tr>
<td>IgG2c from immunized serum</td>
<td>5</td>
<td>96 h (n = 5)</td>
</tr>
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* Rat IgM, IgG1, IgG2a, IgG2b, and IgG2c were purified as described in Material and Methods and transferred into untreated or CVF-treated rats 30 min after xenotransplantation. The volume of purified Ig was adjusted for each isotype fraction to correspond to the relative amount of EXA of the same isotype in 200 \( \mu l \) of the initial immunized serum as described in Material and Methods. Taking this into consideration, 200 \( \mu l \) of IgM (896 \( \mu g/ml \)), 200 \( \mu l \) of IgG1 (217 \( \mu g/ml \)), 800 \( \mu l \) of IgG2a (640 \( \mu g/ml \)), 400 \( \mu l \) of IgG2b (3157 \( \mu g/ml \)), and 600 \( \mu l \) of IgG2c (1919 \( \mu g/ml \)) were administered after xenotransplantation. The same amount of Ig purified from control serum was transferred in control groups. Transfer of unfractionated control serum was used as a negative control. Cardiac xenograft survival time was analyzed by ventricular palpation every hour, during the first 10 h following transplantation and every 24 h thereafter. Xenografts were considered rejected once ventricular function was no longer detectable. Rejection was confirmed by histological examination in all cases. Survival corresponding to \( \leq 24 \) h indicates that the xenograft was rejected between 10 and 24 h after transplantation. m, minutes; n, number of animals analyzed.
regaining pretreatment levels 14 to 17 days after transplantation (Fig. 7). In rats treated with the combination of CyA and CVF, IgM XNA decreased to preimmunization serum levels between days 3 and 7, at which time the CH50 was still suppressed by 40 to 60% of pretreatment levels (Fig. 7).

Combined administration of CyA and CVF after transplantation results in suppression of complement activation by hamster cells To measure rat complement activation by hamster cells, we developed a semiquantitative cellular ELISA for the detection of rat C3 deposition on the surface of hamster HAK cells. Consistent with the observation that serum from naive rats has preformed anti-hamster IgM XNA (Fig. 1), naive rat serum exhibits low but detectable levels of complement activation in the presence of hamster cells (Fig. 8). In untreated rats, a significant increase in complement activation was detected 3 to 7 days after transplantation (Fig. 8), which was correlated with detection of C3 deposition on the xenograft vascular endothelium (data not shown) and xenograft rejection (Table II). In CyA-treated rats, a modest but detectable increase in complement activation was observed 3 to 7 days after transplantation (Fig. 8), which correlated with C3 deposition in the xenograft endothelium (data not shown) as well as with xenograft

FIGURE 5. Serum levels of EXA of the κ-1b light chain allotype after transfer of purified Ig. Rat anti-hamster Ig from the κ-1b light chain allotype were detected by a cell-based ELISA using the RG1/15 mAb, as described in Materials and Methods. Purified IgM, IgG1, IgG2a, IgG2b, and IgG2c expressing the κ-1b light chain were transferred into untreated (A) or CVF-treated (B) Lewis rats (n = 3), 30 min after xenotransplantation of a hamster heart. Circulating EXA of the κ-1b light chain allotype were detected before (gray bars) and 15 min (black bars) after Ig transfer. OD (λ = 490 × 1000) were taken from one serial dilution (1/4) in the linear range of the assay. Background values (in absence of serum) were subtracted from all values. Values shown are the mean ± SD from three treated animals.

FIGURE 6. Immunopathology of hamster to rat cardiac xenografts following transfer of purified Ig of the κ-1b light chain allotype. Ig of the κ-1b light chain allotype were purified from the serum of ACI rats, 14 days after immunization with a hamster cardiac xenograft. Purified Ig were transferred into naive xenograft recipients, 30 min after xenotransplantation of a hamster heart. Binding of EXA and C3 to the xenograft endothelium as well as P-selectin expression were detected 3 h after administration of purified IgM (a–c), IgG1 (d–f), IgG2a (g–i), IgG2b (j–l), and IgG2c (m–o). Binding of EXA to the xenograft endothelium (a, d, g, j, and n) was detected using a mAb recognizing the Igκ-1b light chain allotype, as described in Materials and Methods. Serial sections show endothelial deposition of EXA of the Igκ-1b allotype (a, d, g, j, and n), C3 (b, e, h, k, and n), and P-selectin expression probably associated with platelet activation and aggregation (c, f, I, i, l, and o). Positive labeling is indicated by black arrows. Notice positive staining for C3 and P-selectin labeling upon transfer of IgM (b and c), IgG2b (k and l), or IgG2c (n and o), but not IgG1 (e and f) or IgG2a (h and i) EXA. Cryostat sections, hematoxylin counter stain, ×100 magnifications.
The role of ELICITED ANTIBODIES IN XENOGRAFT REJECTION

Table II. Combination of CyA and short-term CVF administration induces long-term survival of hamster to rat cardiac xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>CVF</th>
<th>CyA</th>
<th>n</th>
<th>Survival Time (days)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
<td>7</td>
<td>2 (n = 3), 3 (n = 3), 5 (n = 1)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>−</td>
<td>12</td>
<td>3 (n = 7), 4 (n = 5)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>4 (n = 3), 5 (n = 1), 6 (n = 1)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>18</td>
<td>2*; &gt;50 (n = 17)</td>
</tr>
</tbody>
</table>

*Rats were treated as described in Materials and Methods and received a hamster cardiac xenograft at day 0. Group 1, untreated; group 2, CyA (15 mg/kg, daily); group 3, CVF (60 U/kg at day −1 and 20 U/kg at day 0); group 4, combination of CyA (15 mg/kg, daily) and CVF (60 U/kg at day −1 and 20 U/kg at day 0). Survival time is indicated in days. n, Number of animals analyzed. Xenograft survival time was analyzed by ventricular palpation every hour, during the first 10 h following transplantation and every 24 h thereafter. Xenografts were considered rejected once ventricular function was no longer detectable. Rejection was confirmed by histological examination in all cases. Survival corresponding to >50 days was considered as long-term survival. * Indicates that another rat died at the expected rate.

Discussion

The role of EXA in xenograft rejection has dominated our view of the pathogenesis of discordant xenograft rejection as well as delayed rejection of discordant xenografts. Generation of EXA has been previously suggested to contribute to pathogenesis of rat to mouse cardiac xenograft rejection (22). However, the contribution of EXA in the pathogenesis of rejection has never been formally tested. In the present study, we tested whether the generation of EXA that occurs rapidly after transplantation of hamster hearts into naive rats contributes to the pathogenesis of rejection of xenograft rejection.

Our results show that rats have low but detectable levels of circulating preformed anti-hamster XNA of the IgM isotype, which activate complement weakly (Fig. 1) and presumably do not initiate HAR of hamster cardiac xenografts because of the weakness of complement activation (Fig. 2). Following xenotransplantation, rats produce high levels of circulating EXA of the IgM, IgG1, IgG2a, IgG2b, and IgG2c isotypes, which activate complement efficiently and thus are likely to be involved in xenograft rejection (Fig. 2). To analyze the ability of different EXA isotypes to initiate xenograft rejection, we adoptively transferred allotype-specific purified EXA into naive recipients that received a cardiac xenograft 30 min earlier. Transfer of EXA of the IgM, IgG1, IgG2a, IgG2b, and IgG2c isotypes, which activate complement efficiently and thus are likely to be involved in xenograft rejection.

To assess whether CyA administration inhibited EXA production through blockade of T cell help, T cell-deficient rnu/rnu rats were immunized with cardiac hamster xenografts, and the effect of CyA treatment was analyzed. Anti-hamster IgG1, IgG2a, IgG2b, and IgG2c EXA remained at background levels in rnu/rnu rats, suggesting that IgG EXA are generated in a T cell-dependent manner and that the administration of CyA in euthymic rats suppresses IgG EXA through blockade of T cell help. Untreated as well as rnu/rnu rats generated low but detectable levels of anti-hamster IgM EXA, which were not suppressed by CyA, suggesting that anti-hamster IgM EXA are partially T cell independent.

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are the mean ± SD. SD ranged from 1 to 2%, and thus these values are not always visible at the scale used.

An alternative explanation may be that IgG2c does not mediate CDC in vitro because of a species incompatibility between rat IgG2c and rabbit complement, used as a source of complement in the CDC assay. Therefore, it is possible that IgG2c may activate rat complement in vivo, and thus could initiate xenograft rejection through complement activation.

Taken together, our present data support the following model for the rejection of hamster to rat cardiac xenografts. Following xenotransplantation, rats generate high levels of EXA, among which IgM, IgG2b, and IgG2c activate complement (Fig. 4) to mediate EC activation, platelet aggregation, P-selectin expression, and microvascular congestion (Fig. 6). These events result in rapid generation of microvascular hypoxia and provide a nidus for recruitment and activation of host leukocytes, which will presumably contribute to xenograft rejection (30, 31).

Our present data may also be relevant for the mechanism of pathogenesis of acute vascular rejection of allografts. Generation of T cell-dependent complement-fixing alloantibodies, mainly directed against MHC, may also contribute to the pathogenesis of allograft acute vascular rejection. However, in contrast to xenografts, complement inhibitor proteins expressed in allograft endothelium (CD46, CD55, and CD59) may, at least to some extent, inhibit complement activation by cytotoxic alloantibodies. This may not occur as efficiently in xenografts because the interaction of complement inhibitors with components of the complement cascade is species specific. Therefore, the threshold level of complement activation needed to initiate acute vascular rejection of allografts may be higher than that necessary to induce xenograft rejection.

Having established that EXA play a central role in the pathogenesis of xenograft rejection, we asked whether the mechanism by which administration of CVF and CyA led to long-term xenograft survival and accommodation involved the inhibition of EXA (Table II). Our findings show that in rats treated with CVF for 2 days, complement is suppressed to undetectable levels during 3 days after transplantation and does not return to normal levels until 14 days after transplantation (Fig. 7). In rats treated with CyA and CVF, it is during this period of complement depletion that there is an increase in the serum levels of IgM XNA (Fig. 7). Once IgM XNA returned to preimmunization levels, by day 14 after transplantation, rats generate high levels of EXA, among which IgG2b, IgG2c, and IgG2c EXA did not cause xenograft rejection when transferred into complement-depleted rats (Table I).

It should be noticed that IgG2c EXA mediated xenograft rejection through complement activation, while this IgG subclass did not mediate CDC of hamster cells in vitro (Figs. 4 and 6) (18). One possible explanation as to why this occurs may result from the fact that IgG2c binds C1q as efficiently as other IgG subclasses that mediate CDC, i.e., IgG2b, without activating the entire complement cascade (29). Therefore, IgG2c may initiate xenograft rejection by promoting endothelial cell activation through C1q, which has been shown to occur in vitro (21).

An alternative explanation may be that IgG2c does not mediate CDC in vitro because of a species incompatibility between rat IgG2c and rabbit complement, used as a source of complement in vitro (18).

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lead to a different interpretation. We find that in the absence of circulating IgM, rats receiving only CyA do not reject hamster cardiac xenografts that undergo long-term survival (>100 days) (Sato et al., manuscript in preparation). Similar results were obtained in \textit{rnu/rnu} rats treated with anti-\(\mu\) mAb, indicating that IgM EXA play an essential role in the pathogenesis of xenograft rejection (Takigami et al., article in preparation). Furthermore, these data also indicate that under T cell immunosuppression, xenograft rejection does not occur in absence of IgM XNA. The difference between our results and those reported by Lin et al. (32) may result from the fact that in the study of Lin et al., preexisting IgM XNA were not depleted. These Abs may have a cumulative effect with time, which we suggest would lead to low levels of complement activation responsible for EC activation and xenograft infiltration by host macrophages and NK cells, leading to rejection.

Our data also indicate that a significant portion of the anti-hamster IgM response is T cell dependent, which contrasts with previous reports suggesting that anti-hamster IgM EXA are generated exclusively in a T cell-independent manner (35, 36). This conclusion is based on two observations: 1) In euthymic rats, but not in \textit{rnu/rnu} rats, the T cell immunosuppressant CyA significantly decreased the production of IgM EXA after transplantation (Fig. 7), and 2) the levels of IgM EXA produced after transplantation of a hamster heart in \textit{rnu/rnu} rats were significantly lower than those detected in euthymic rats (Figs. 7 and 10).

The effects of CyA and CVF in inhibiting IgM EXA and complement may contribute to avoid rejection in the first 3 to 6 days after transplantation, but cannot account for long-term xenograft survival (>50 days). However, CyA has additional effects that explain long-term xenograft survival. Administration of CyA suppressed the generation of all IgG EXA subclasses to undetectable levels, including those we have shown to be involved in xenograft rejection, i.e., IgG2b and IgG2c (Table I; Figs. 6 and 9). An additional effect of CyA that potentially contributes to suppress xenograft rejection is to deviate T cell activation toward a Th2 cytokine response (data not shown) (10). Th2-driven cytokines inhibit the production of proinflammatory Th1-driven cytokines such as IL-12 and IFN-\(\gamma\) (37), and may therefore account for suppression of IgG2b and IgG2c EXA, since production of these Ig isotypes is accentuated on binding to the xenograft endothelium. Given these findings, we tested how CyA and CVF were implicated in leading to long-term survival (>50 days). To allow xenograft survival for the first week, CVF had to be given only twice, on day 1 and on the day of transplantation. This treatment suppressed complement for a sufficiently long time that the increased levels of IgM EXA were not accompanied by a competent complement system capable of initiating rejection. By the time complement recovered to pretreatment levels, the IgM EXA titers had decreased to background, and thus complement activation on the surface of hamster cells did not occur. With regard to the long-term survival (>50 days) of the xenograft in the presence of normal IgM XNA levels and normal levels of complement, we found that CyA blocked all EXA IgG to undetectable levels, including those subclasses involved in xenograft rejection. Finally, under CyA administration, EC in the xenograft expressed a series of protective genes, such as heme oxygenase-1, A20, bcl-2, and bcl-x\(_{-}\), which might explain the lack of EC response to the sublytic effects of IgM and complement.

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