Long-Term Survival of Hamster Hearts in Presensitized Rats

Yuan Lin, Miguel P. Soares, Koichiro Sato, Eva Csizmadia, Simon C. Robson, Neal Smith and Fritz H. Bach

*J Immunol* 2000; 164:4883-4892; doi: 10.4049/jimmunol.164.9.4883

http://www.jimmunol.org/content/164/9/4883

**References**

This article cites 61 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/164/9/4883.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Long-Term Survival of Hamster Hearts in Presensitized Rats

Yuan Lin,†* Miguel P. Soares,* Koichiro Sato,* Eva Cszizmadia,* Simon C. Robson,* Neal Smith,† and Fritz H. Bach*

We transplanted hamster hearts into rats that had been sensitized to hamster cardiac grafts 5 days earlier as a model for discordant xenotransplantation. Sensitized rats had high serum levels of elicited anti-donor IgM and IgG that caused hyperacute rejection. Transient complement inhibition with cobra venom factor (CVF) plus daily and continuing cyclosporin A (CyA) prevented hyperacute rejection. However, grafts underwent delayed xenograft rejection (DXR). DXR involved IgG and associated Ab-dependent cell-mediated rejection, because depletion of IgG or Ab-dependent cell-mediated rejection-associated effector cells prolonged graft survival and the serum-mediated Ab-dependent cell-mediated cytotoxicity in vitro. Blood exchange in combination with CVF/CyA treatment dramatically decreased the level of preexisting Abs, but DXR still occurred in association with the return of Abs. Splenectomy and cyclophosphamide acted synergistically to delay Ab return, and when combined with blood exchange/CVF/CyA facilitated long-term survival of grafts. These grafts survived in the presence of anti-donor IgM, IgG, and complement that precipitated rejection of naive hearts, indicating that accommodation (survival in the presence of anti-graft Abs and complement) had occurred. We attribute the long-term survival to the removal of preexisting anti-donor Abs and therapy that attenuated the rate of Ab return. Under such conditions, the surviving hearts showed expression in endothelial cells and smooth muscle cells of protective genes and an intragraft Th2 immune response. Th2 responses and protective genes are associated with resistance to IgM- and IgG-mediated, complement-dependent and -independent forms of rejection. The Journal of Immunology, 2000, 164: 4883–4892.

If hyperacute rejection of a discordant xenograft, such as from pig to primate, is overcome by inhibition of xenoreactive natural Abs (XNA) and/or complement, the graft is still rejected by a process referred to as delayed xenograft rejection (DXR). Among the factors that lead to DXR are elicited xenoreactive Abs (EXA) of both the IgM and IgG isotypes evoked in the recipient by the presence of the xenograft (1–4). In addition to the ability of these Abs to provoke activation of complement (1–4) or directly interact with graft endothelial cells leading to their activation (2, 5), IgG can mediate Ab-dependent cellular cytotoxicity (ADCC) in vitro and contribute to Ab-dependent cell-mediated rejection (ADCR) in vivo (6, 7). No procedure has been developed that allows one to overcome the consequences of the IgG EXA in the experimental systems tested to date (8–10).

Although there are also IgM and IgG EXA that lead to DXR in discordant xenotransplantation such as hamster to rat, treatment of the recipient with a number of immunosuppressive protocols that suppress IgG EXA production induces long-term survival of the graft (11–14). We have shown that treatment with just two doses of cobra venom factor (CVF) plus daily cyclosporin A (CyA) blocks complement activation for a few days and IgG synthesis permanently, respectively, and results in long-term survival of the grafts (15, 16). The grafts continue to survive in the presence of anti-donor IgM Abs and complement, a phenomenon we refer to as accommodation (17). However, in the presensitized situation, the CyA does not suppress the production of anti-hamster IgG, and thus, the same protocol that results in long-term survival of concordant grafts does not do so for discordant grafts transplanted to presensitized recipients or in the discordant xenograft models (8–10).

The preformed XNA that mediate hyperacute rejection of discordant xenografts are similar to preformed Abs normally found in serum directed at a variety of Ags expressed on normal tissue or infectious agents (18). Thus, XNA are thought to be associated with B cell sensitization due to infection with agents sharing immunologically cross-reactive Ags such as Gal-α (1, 3) Galβ (1, 4), GlcNac, or α-gal epitopes expressed on pig organs and recognized by human XNA (18, 19). Newborn baboons have no significant levels of anti-α-gal Abs and do not hyperacutely reject pig organ grafts (20). If the recipient of a discordant organ transplant is sensitized to donor Ags, anti-donor Abs are present pretransplantation in the recipient serum and participate in promoting hyperacute rejection of donor species organ grafts (21, 22), a situation mimicking discordant xenotransplantation. EXA and preformed XNA are also similar in that EXA in discordant combinations recognize the same epitopes as the preformed XNA (23), and that the same genes encode the EXA and XNA (24). Thus, transplantation of hamster grafts to presensitized rats may serve as a good model for studying discordant xenotransplantation.

Because of the refractoriness to immunosuppression of the return of anti-hamster Abs after their depletion in presensitized rats,
as is also true in discordant models (8–10), we have focused on attempts to develop a protocol that will allow us to achieve accommodation, i.e., survival of the organ in the presence of anti-graft Abs and complement. Accommodation may lessen the need for prolonged, heavy immunosuppression. A key feature of accommodation as we have studied it in the hamster-to-rat or mouse-to-rat models is expression in the endothelial cells (EC) and smooth muscle cells (SMC) of the accommodated grafts of protective genes (15, 25). Based on past studies by others and ourselves (26–29), the expression of these genes in EC and perhaps SMC may well protect the graft from an insult such as that delivered by anti-graft (anti-EC) Abs plus complement. For one protective gene, heme oxygenase-1 (HO-1), we have direct evidence that expression of the gene in the graft can be essential for graft survival (30). In the present study, we have used as one hypothesis our earlier stated idea that a slow return of anti-graft Abs after their depletion may help allow the graft to accommodate (15, 31).

We used a therapeutic protocol involving pretransplantation blood exchange and splenectomy in combination with immunosuppression consisting of brief treatment with CVF and cyclophosphamide (CyP) plus daily CyA. This protocol effectively lowered the level of anti-donor Abs pretransplantation, delayed their return posttransplantation (as compared with rats treated with CVF + CyA + blood exchange), and resulted in long-term survival of hamster heart transplants in sensitized recipients. The grafts survived in the presence of anti-donor both IgM and IgG Abs that had returned and complement, i.e., they accommodated. Previous studies have only achieved accommodation in the presence of anti-graft IgM Abs, without the presence of IgG Abs (15, 16). Our present data demonstrate that xenografts can be induced to accommodate in the presence of preexisting anti-donor Abs of both types in a model that we believe is most likely relevant to discordant xenotransplantation. The accommodated grafts become resistant to Ab and complement-mediated rejection, as well as IgG Ab-mediated ADR.

Materials and Methods

Animals

Golden Syrian hamsters, weighing 60–80 g, were used as organ donors, and inbred Lewis rats (RTI1) (Harlan Sprague-Dawley, Indianapolis, IN), weighing 150–250 g, were used as recipients. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. The research protocols were approved by the International Animal Care and Use Committees of the Beth Israel Deaconess Medical Center.

Heterotopic heart transplantation

Cervical and abdominal heterotopic heart transplantation was performed using a technique described previously (14). A second or third donor heart was placed to the contralateral side of the recipient neck or to the abdomen using a technique described previously (14). A second or third donor heart was implanted to the contralateral side or to the abdominopelvic region (9). Donors were killed by cervical dislocation, incised ventrally, and both hearts were removed in toto. Hearts were washed briefly in saline and suspended on a pedicle in crystalline medium. Recipient thorax was incised ventrally and placed on a warming plate. Pericardium was incised and the heart was placed into the thoracic region. Aortas were sutured to the abdominal aorta. Recipient chest was closed using 3-0 Prolene. Recipients were maintained on a ventilator (14). A volume of 100 ml/kg of the supernatant is transferred to another 96-well plate and estimated for hemoglobin in a spectrophotometer (A = 415 nm). One hundred percent hemolysis and 0% hemolysis were included by incubation of the cells with 0.15 M NaCl, 1 mM KHCO3, 0.1 mM Na2 EDTA) and 200 

Complement-dependent cytotoxicity

Complement-dependent cytotoxicity (CDC) was measured by a hemolytic assay using modified techniques described previously (9). Briefly, in the hamster was bled into Alsevers solution (Sigma) (1:1, v/v). After washing in GVB, the RBC were suspended in GVB at a concentration of 1% (v/v). Sera collected from rat-clotted blood samples were heat-inactivated at 56°C for 30 min. A volume of 50 ml of RBC suspension was mixed with equal volumes of serially diluted rat anti-hamster serum from 1/10 to 1/100 in 96-well round-bottom microtiter plates. After incubation for 30 min at 37°C, the plates were centrifuged for 5 min at 350 × g. A volume of 100 ml of the supernatant was transferred to another 96-well plate and estimated for hemoglobin in a spectrophotometer (A = 415 nm). One hundred percent hemolysis and 0% hemolysis were included by incubation of the cells with 200 ml of ACK lysing buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2 EDTA) and 200 ml of GVB without serum, respectively. The 50% hemolytic end point is usually in the region of 1/200 to 1/400 in a volume of 200 ml (i.e., about 1000–2000 CH50/ml) (34).
The cytotoxic Ab titers were expressed by the maximal dilution of the serum that resulted in 50% specific hemolysis.

Ab-dependent cell-mediated cytotoxicity

The Ab-dependent cell-mediated cytotoxicity (ADCC) was measured by flow cytometry analysis using modified techniques described previously (35, 36). In brief, lymphokine-activated killer cells (LAK) were generated by incubation of rat splenocytes (5 × 10^6/ml) in a bulk lymphocyte culture in the presence of rIL-2 (100 U/ml) (Serotec, Oxford, U.K.) at 37°C in 5% CO₂ for 3 days. The cells were harvested and washed in ice-cold PBS. Immediately before flow cytometry analysis, 25 μl of 1 μg/ml propidium iodide (Sigma) in PBS was added to each sample to label nonviable cells. The target HAK cells were gated based on their larger size and therefore higher light scatter.

Serum transfer

Serum was prepared from pooled blood taken at day 30 posttransplantation from presensitized rats without treatment or receiving full immunosuppressive regimen and carrying an accommodating heart. Serum was heat inactivated at 56°C for 30 min pretransfer, 25 μl of 1 μg/ml propidium iodide (Sigma) in PBS. The percentage of nonviable cells due to the ADCC was calculated as follows: % specific hemolysis = [% maximal nonviable background]/(% maximal nonviable cells ~ background) × 100.

Statistics

The results were statistically analyzed by the Student t test, or by the Fisher exact test.

Results

Survival of hamster hearts in presensitized rats

We and others have previously shown that rats do not have sufficient preformed anti-hamster Abs to provoke hyperacute rejection of hamster hearts (11–14, 37). After sensitization (Table I), rats hyperacutely (within minutes) reject hamster hearts (group 1). CyA does not affect the occurrence or tempo of hyperacute rejection (group 2). Blockade of complement activation prevented hyperacute rejection and prolonged graft survival to 2–4 days in CVF-treated (group 3) and CVF + CyA-treated rats (group 4).

Graft survival in rats presensitized in the presence of CyA

To test the hypothesis that ongoing T cell activation and T cell-dependent Ab production in presensitized rats contributed to the rejection of hamster hearts, we sensitized animals in the presence of CyA to block any T cell-dependent response during the sensitization procedure (Table II). CyA did not influence IgM Ab production, but it completely inhibited IgG Ab formation, as we and others have previously shown (11, 14, 38, 39). Whereas IgM Abs elicited during sensitization still led to rejection of hamster hearts within 24 h (group 1), addition of CVF to the CyA treatment in-
overcome. The graft shown in group 2 that stopped beating 8 days after transplantation showed widespread vacular thrombi without other evidence for the cause of rejection, based on the measurement of Ab titers and complement activity. We would suggest that the treatment we gave is sufficient to obtain long-term survival in the great majority of animals, but due to variation in the anti-graft response among animals is not sufficient for the occasional recipient.

**Graft survival in presensitized rats depleted of monocytes/macrophages and NK cells**

To evaluate a possible role played by NK cells and monocytes/macrophages in rejection of xenografts in presensitized rats given CVF + CyA, we investigated whether depletion of recipient NK cells and monocytes/macrophages could influence graft survival (Table III). Anti-asialo GM1 antiserum was used to deplete NK cells. We showed previously that this protocol eliminated rat NK cells to an undetectable level over the period of treatment (40). Liposome-encapsulated alendronate was used to deplete monocytes/macrophages (32). The efficacy of this depletion was evaluated by immunofluorescence staining and FACScan analysis of ED1+ mononuclear cells (monocytes/macrophages) in the recipient spleen. Treatment led to a decrease of ~90% (compared with pretreatment levels) of those cells over the period of treatment (data not shown). Elimination of both cell types significantly enhanced graft survival in CVF + CyA-treated rats, as compared with CVF + CyA treatment alone (see Table I) (7 ± 3 days vs 3 ± 0.6 days, p < 0.001).

**Graft survival in presensitized rats receiving blood exchange-based therapy**

To lower the titer of preexisting Abs and achieve long-term survival, we performed blood exchange in combination with other immunosuppressive therapies (Table IV). Blood exchange alone significantly decreased the titer of preexisting anti-donor Abs (see below), and prolonged graft survival somewhat to 4.2 ± 2.4 h. This treatment, however, did not prevent hyperacute rejection (group 1). The documentation of hyperacute rejection after blood exchange indicated that even the low level of remaining Abs from the sensitization procedure in the presence of complement was sufficient to provoke rapid destruction of the graft. Blood exchange in combination with CVF + CyA prolonged graft survival to 5 ± 1.2 days (group 2). Rejection under this treatment protocol was associated with a rapid return of anti-donor Abs (see below).

Splenectomy or CyP alone or in combination did not affect the occurrence of hyperacute rejection, because the high levels of preexisting xenoreactive Abs were not influenced by these therapies. Hyperacute rejection even occurred when splenectomy and CyP were combined with blood exchange. These data are consistent with a role in rejection for the low level of preexisting, complement-fixing Abs that were not removed by blood exchange.

CyP or splenectomy, when added to the blood exchange + CVF + CyA protocol, prolonged survival of grafts to 14 ± 11 days (group 3) and 15 ± 11 days (group 4), respectively. Occasionally, those therapies induced long-term survival of xenografts (2/7 and 3/9 cases in each group). Again, we suggest that the variation in survival relates to the differential response of different animals (normal biological variation) to the graft in the presence of a treatment protocol that is not sufficient to ensure survival of all grafts. The full immunosuppressive regimen that we used involved the combination of splenectomy + CyP + blood exchange + CVF + CyA. This resulted in long-term survival of grafts in essentially all cases (9/10) (group 5). One graft that was lost from this group showed severe vascular thrombosis. The Ab titer and complement activity in this rat were comparable with other animals from the same group.

**Abs and complement activity**

Anti-donor Abs were quantified by FACScan analysis using hamster RBC as target cells. We (34) and others (41) have shown that hamster RBC express xenointgens recognized by rat anti-hamster IgM and IgG Abs at a level quantitatively and qualitatively comparable with that expressed on hamster PBMC. The advantage of using RBC as target cells is the decrease in nonspecific binding by Abs via cell surface Ig and Fc receptors (34, 42). Fig. 1A shows the

---

**Table III. Survival of hamster hearts in presensitized rats depleted of NK cells and monocytes/macrophages**

<table>
<thead>
<tr>
<th>CVF + CyA + Depletion</th>
<th>n</th>
<th>Graft Survival Time</th>
<th>MST ± SD</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>10, 15, 20, 25 min</td>
<td>24 ± 17 min</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>5, 6, 6, 7, 7, 9, 15 days</td>
<td>7 ± 3 days</td>
<td>&lt;0.001b</td>
</tr>
</tbody>
</table>

* See Materials and Methods.

---

**Table IV. Survival of hamster hearts in presensitized rats after whole blood exchange**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Graft Survival Time</th>
<th>MST ± SD</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood exchange</td>
<td>5</td>
<td>2, 3, 5, 8 h</td>
<td>4.2 ± 2.4 h</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Blood exchange, CVF + CyA</td>
<td>5</td>
<td>4, 4, 5, 5, 7 days</td>
<td>5 ± 1.2 days</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Blood exchange, CVF + CyA + CyP</td>
<td>7</td>
<td>4, 6, 8, 9, 9, &gt;30 (n = 2)b</td>
<td>14 ± 11 days</td>
<td>&gt;0.05c</td>
</tr>
<tr>
<td>4</td>
<td>Blood exchange, CVF + CyA, splenectomy</td>
<td>9</td>
<td>5, 6, 8, 9, 9, &gt;30 (n = 3)b</td>
<td>15 ± 11 days</td>
<td>&lt;0.05c</td>
</tr>
<tr>
<td>5</td>
<td>Blood exchange, CVF + CyA + CyP, splenectomy</td>
<td>10</td>
<td>8 &gt;30 (n = 2)c, &gt;60 (n = 3)c, &gt;90 (n = 4)c</td>
<td>&gt;60 days</td>
<td>&lt;0.001c</td>
</tr>
</tbody>
</table>

* See Materials and Methods.

b Grafts were removed for histology.

c Versus group 2.
time course of the anti-donor Ab response in presensitized rats treated with the CVF + CyA protocol. Five days following sensitization, rats developed significant levels of anti-donor IgM and IgG Abs (Fig. 1 Ai). IgM peaked at about day 8, thereafter declining to baseline levels by day 30 following sensitization. IgG Abs reached a maximal level at about day 10 and remained at this level for more than 40 days following sensitization (Fig. 1 Ai). CVF alone or in combination with CyA did not markedly influence the kinetics or quantity of Ab production (Fig. 1 Ai, ii and iii). In contrast, the presence of CyA totally suppressed anti-hamster IgG production in naive rats (13–15), suggesting that the IgG Abs are all T cell dependent. A short course (day 0, 2, 4, 6) of CVF eliminated complement activity to an undetectable level until day 12, followed by a progressive recovery to predepletion levels after day 20 following the first dose of CVF (Fig. 1B).

Blood exchange significantly reduced the preexisting Ab titers by ~70% of pretreatment levels. However, both IgM and IgG Ab titers quickly returned to pretreatment levels within 2–4 days (Fig. 2A). Addition of CVF + CyA did not significantly affect the speed with which Abs returned nor the magnitude of the return as compared with blood exchange alone (Fig. 2B). Whereas splenectomy or CyP alone only slightly delayed the Ab return (data not shown), the combination of these two agents resulted in a significant delay of Ab return for up to 6 days following blood exchange. Furthermore, the Abs returned relatively slowly (Fig. 2C) as compared with the rate of return when recipients were treated only with blood exchange (Fig. 2A) or blood exchange + CVF + CyA (Fig. 2B). When splenectomy + CyP in combination with CVF + CyA were used, both IgM and IgG were sustained at postdepletion levels until day 12 following blood exchange. In addition, the return of IgM Abs did not peak until about day 16, followed by a decline to baseline levels 30 days following the start of treatment. Returning IgG reached its maximal levels at about day 25 following the treatment (Fig. 2D).

**IgG Ab subclass**

Fig. 3 depicts FACS profiles illustrating the IgG subclasses present in the serum taken 30 days posttransplantation from presensitized rats that had rejected their grafts or from presensitized rats given the full immunosuppressive regimen and carrying a surviving hamster heart for 30 days. The untreated rats showed high levels of anti-donor total IgG with IgG subclasses IgG1, IgG2a, and IgG2b (Fig. 3, A–D). Rats carrying an accommodated heart showed high levels of total IgG and IgG subclasses IgG2a and IgG2b (Fig. 3, F, H, and I), although the levels of these Abs were substantially lower as compared with untreated controls. IgG1 was not seen in rats treated with the full immunosuppressive regimen (Fig. 3G). Neither set of sera contained detectable IgG2c (Fig. 3, E and J).
Complement-dependent cytotoxicity

We have previously shown that sera from naive rats exhibit undetectable or very low levels (<1/1) of anti-hamster cytotoxic Abs (14). Five days after sensitization, high titers of these Abs were detected (up to 1/2560), with peak levels after day 10 of sensitization (Fig. 4A). The blood exchange-based full immunosuppressive regimen remarkably decreased the cytotoxic Ab titers to less than 1/80 for ~12 days following the treatment. Thereafter, there was a progressive return of those cytotoxic Ab titers, reaching a maximal level 20 days following the treatment (Fig. 4A).

FIGURE 3. Flow cytometry analysis of the isotype of IgG subclasses in presensitized rats treated with full immunosuppressive regimen vs untreated controls. Sera were taken 30 days following transplantation from untreated rats that had rejected their grafts or from rats receiving the full immunosuppressive regimen and carrying accommodated grafts. Anti-hamster IgG and IgG subclasses IgG1, IgG2a, IgG2b, and IgG2c in untreated rats (A–E) or rats carrying an accommodated graft (F–J). Sera from naive rats were used as controls (open histogram). The data are representative of one of three separate tests in each group.

FIGURE 4. A, Complement-dependent cytotoxic Ab titer in presensitized rats treated with full immunosuppressive regimen vs untreated controls. B, Serum ADCC activity in presensitized rats treated with full immunosuppressive regimen vs untreated controls. The arrow shows the time point at which blood exchange was performed. The data are presented as mean value ± SD of four individual rats in each group.
Ab-dependent cell-mediated cytotoxicity

To evaluate the potential of anti-hamster IgG Abs to provoke ADCC, we tested rat LAK cell-mediated lysis against hamster HAK cells in the presence of those IgG Abs. Rat LAK cells caused ~23% lysis of hamster HAK cells at an E:T cell ratio of 40:1 (Fig. 4B). Addition to the culture of the serum (1/40 dilution) taken from naive rats did not markedly influence the percentage of the lysis (data not shown), whereas sera from presensitized rats enhanced lysis significantly in a time-dependent manner, with peak lysis occurring after 15 days following sensitization (Fig. 4B). The full immunosuppressive regimen decreased the serum ADCC activity close to the basal level (mediated by LAK cell alone) for up to 15 days following the treatment. Thereafter, a slow return of the ADCC activity of the serum from those animals occurred, peaking 25 days following the treatment.

IgG Abs in Ab-dependent cell-mediated rejection

We performed two experiments to determine the ability of returned anti-donor IgG Abs to influence the rejection process. First, we transplanted a second hamster heart into presensitized rats carrying an accommodated heart for 30 days, a time when the anti-donor IgG level was maximal and IgM were essentially undetectable (Fig. 2D). Second, 1 ml of serum from these rats carrying an accommodated heart for 30 days was adoptively transferred into naive recipients after hamster heart transplantation. In both experiments, naive hearts were rejected hyperacutely within 24 h (n = 6 in each group). The ability of those sera to provoke rejection upon adoptive transfer persisted even when the sera were treated with 2-ME (data not shown) to exclude any potential role for IgM Abs in mediating rejection.

Histopathology and immunohistochemistry

Grafts from the various treatment protocols were studied by immunohistopathology. Grafts that underwent hyperacute rejection in different experimental groups showed a picture of Ab and complement-mediated rejection, as we have described previously (43).

Histopathology of grafts that were rejected within a few days in presensitized rats despite CVF-based treatments generally showed the features of DXR, including severe endothelial destruction, hemorrhage, edema, monoclonal cell infiltration, intravascular thrombi, and myocardial necrosis. Immunoperoxidase staining demonstrated vessel wall deposition of IgM, IgG1, IgG2a, and IgG2b without complement factor C3. No IgG2c was detected (data not shown). There was dense leukocyte infiltration consisting of mainly Th1-like cells that were associated in the sections with IFN-γ, TNF-α, and faint IL-2, with essentially no IL-4 or IL-10 (Fig. 5, A–E). Up-regulated expression of P-selectin on endothelial cells, platelet aggregation, and extensive vascular deposition of fibrin were noticed (Fig. 6, E and F). Graft EC and SMC expressed no, or low levels of A20, Bcl-2, Bcl-xL, and HO-1 proteins that are seen in accommodated hearts (see immediately below) (Fig. 6, A–D).

Accommodated grafts that were harvested 30 days following transplantation from presensitized rats treated with the full immunosuppressive regimen revealed healthy appearing cardiac fibers and vascular endothelium, with monoclonal cell infiltration. There was vessel wall deposition of IgG2a, IgG2b, and complement component C3, essentially without IgM, IgG1, or IgG2c (data not shown). Infiltrating cells consisted of primarily Th2-like cells that were associated on sections with IL-4 and IL-10 with essentially no IFN-γ, TNF-α, or IL-2 (Fig. 5, F–J). These hearts expressed a high level of A20, Bcl-2, Bcl-xL, and HO-1 in their EC and SMC (Fig. 6, G–J), with no up-regulation of P-selectin on EC, nor deposition of fibrin on vessel walls (Fig. 6, K and L).

Discussion

In past studies, we and others have obtained long-term survival and accommodation of hamster hearts in rats following treatment with CVF + CyA (15, 44). In those models, grafts encounter a gradual increase of anti-donor IgM Abs and subsequently survive in the presence of these IgM Abs and complement. In an effort to mimic the situation existing in the discordant pig-to-primate combination, we have studied here a small animal model in which recipient rats are presensitized with donor hamster Ags. In this model, both IgM and IgG anti-donor Abs are present pretransplantation and rapidly return after depletion, a situation that is found with discordant models in which long-term survival has not been achieved (8–10).

In the present study, we have tested our hypothesis that a delayed and slow return of anti-donor Abs may allow xenografts to accommodate even in a model that has major similarities to a discordant xenograft (15, 45).

Preexisting anti-hamster Abs in the sensitized rats readily provoked hyperacute rejection of hamster hearts (Table I). The same CVF + CyA therapy we used to induce long-term survival of hamster hearts in naive rats prevented hyperacute rejection in the
presensitized rats, but the grafts were still rejected within 4 days, even without detectable complement activity in the host (Fig. 1B). Histopathologically, the rejected hearts showed features of DXR typically seen in discordant combinations when hyperacute rejection is averted by depletion of complement (46, 47), or by genetic modification of donor pig organs to express human complement-regulating proteins (9, 48, 49).

Several possibilities exist to explain the occurrence of DXR in those rats depleted of complement activity. High levels of anti-donor IgM and IgG Abs present at the time of transplantation may directly perturb graft vascular EC (2, 5). Monocytes/macrophages and NK cells may react with graft EC (2, 40, 50–52), a process that would be enhanced by anti-graft IgG Abs via ADCR (6, 7, 40). We showed that depletion of either anti-graft Abs (Table IV) or the ADCR-associated effector cells (Table III) significantly aided survival of grafts. T cells that have been activated during sensitization most likely contribute to rejection, both by direct T cell-mediated rejection and by T cell promotion of Ab responses (53, 54). In fact, when the recipients were initially sensitized in the presence of CyA to block T cell activation, the anti-graft IgM response remained essentially intact, while IgG Ab formation that is T cell dependent (14) was inhibited. Although those elicited IgM Abs readily caused rejection of hamster hearts in the presence of complement, addition of CVF to the CyA treatment could induce long-term survival of grafts in those rats (Table II).

Pretransplantation blood exchange effectively decreased the level of the preexisting anti-graft Abs. However, both anti-graft IgM and IgG, which were associated with graft rejection within a few days despite CVF + CyA treatment, returned rapidly and reached their pretreatment levels within 2–4 days (Fig. 2). Ab synthesis by B cells with Th cells that had been sensitized and had not been depleted by blood exchange was most likely responsible for the rapidity of the Ab return, as seen in discordant recipients depleted of XNA pretransplantation (8–10). We searched for a therapy that would maintain a low level of EXA for a period of time after their depletion and lead to an eventual slow return of the Abs. We found a synergistic effect between splenectomy and CyP, an antiproliferative drug with potent immunosuppressive properties against B cells (55). When splenectomy and CyP were added to the blood exchange + CVF + CyA protocol, the return of Abs was substantially delayed for up to 12 days, the Abs returned relatively slowly as compared with the other treatments, and grafts survived long-term in the presensitized rats. The effect of splenectomy and CyP on impeding Ab return most likely relates to the capacity of these therapies to decrease the number and activity of xenoreactive T and B cells (56, 57), thereby minimizing ongoing activation of, and Ab production by those cells. Similar effects have been seen in other protocols using immunoabsorption, splenectomy, and CyP to deplete Abs and to promote survival of xenografts in discordant combinations (8–10, 46). In addition, the full immunosuppressive regimen resulted not only in the longest delay until Abs began to return to the circulation, but also to the lowest rate of return of the Abs (Fig. 2).

Long-term survival of xenografts was occasionally induced in rats receiving either splenectomy or CyP in combination with blood exchange + CVF + CyA. However, the return of Abs in those rats with the long-term surviving hearts was delayed for a relatively longer time as compared with other animals that rejected their grafts in the same group (data not shown). This finding is consistent with our hypothesis that to achieve long-term survival it will be necessary to have a relatively longer period of time during which Abs are not assaulting the endothelium of the graft, thereby giving the EC the opportunity to up-regulate the protective genes that will make them resistant to damage by Abs and complement, even in the presence of cells able to mediate ADCR. The different survival times of the organs in various animals in the group most likely reflect variation in underlying biological responses of the individual animals.

The hamster hearts survived in the presence of both high levels of anti-donor IgM and IgG Abs that gradually returned to the circulation following depletion, and returned complement activity, i.e., accommodation was induced. Isotype analysis demonstrated that these IgG Abs consisted mainly of IgG2a and IgG2b, as compared with the presence of IgG1, IgG2a, and IgG2b in the serum of untreated sensitized rats (Fig. 3). Immunohistopathological study consistently showed that the deposition of IgG1 in accommodating first hearts was essentially undetectable, ruling out the
possibility that absorption of this IgG subset by the surviving grafts was responsible for its absence. After recovery, IgG2a and IgG2b appeared to be sufficient to participate in rejection of xenografts in the absence of IgG1. We found that the IgG2a and IgG2b mediated CDC (Fig. 4A) and ADCC (Fig. 4B) in vitro. This is in keeping with the activity of rat IgG2b in fixing complement (37) and the ability of IgG2a and IgG2b to bind to the Fc receptor (58, 59). Both IgG subclasses are also likely to participate in promoting graft rejection in vivo. When a fresh heart was transplanted into presensitized rats that had already carried an accommodated heart for 30 days, a time when the IgG Ab level was maximal and the IgM was essentially undetectable (see Fig. 2), this second graft was hyperacutely rejected. Also, adoptive transfer of these IgG subclasses from the above rats provoked hyperacute rejection of fresh hearts in naive rats.

In the nonsensitized model we have used in the past, accommodated hamster hearts survive in CVF + CyA-treated naive rats that developed only anti-donor IgM Abs (15, 16). These IgM Abs precipitate rejection of a second, fresh hamster heart transplanted into rats already carrying an accommodating graft on day 10, when the recipient’s complement activity has partially recovered (34). Because CyA suppresses T cells, it has been impossible to test whether an accommodated heart can resist rejection mediated by T cell-dependent, anti-graft IgG-mediated reactions (14, 22, 37). Previous studies suggested that such resistance exists because serum from rats carrying an accommodated hamster heart contains Abs able to mediate donor-specific ADCC in vitro (60). In the present model, we were able to show that an accommodated xenograft is not rejected even when anti-graft IgG, as well as ADCR-associated effector cells are present. Together with the ability of those IgG Abs to mediate CDC and ADCC in vitro and rejection of naive xenografts in vivo, our data demonstrate that accommodated xenografts may develop resistance to IgG-mediated, complement-dependent, and complement-independent forms of rejection.

We have hypothesized that keeping anti-graft Abs at a low level for a sufficient period of time with a slow return of the Abs is critical for graft accommodation (15, 45). In this presensitized model, accommodation occurs in association with the longest delay and lowest rate of return of anti-graft Ab titers. Given the capacity of the rat IgG subclasses in mediating CDC (IgG2a and IgG2b) and ADCC (IgG1, IgG2a, and IgG2b) (37, 58, 59), the lack of IgG1 as well as the decrease in IgG2a and IgG2b may facilitate the development of accommodation. However, it is uncertain whether the lack of IgG1 is specifically required for accommodation. The returned IgG2a and IgG2b, although at a lower level as compared with untreated rats, could cause hyperacute rejection of naive grafts. These data may suggest that after surviving at a low level of anti-graft Abs, grafts undergo certain physiological alterations, as such becoming resistant to Ab-mediated rejection (15, 45). It is our model that the changes that underlie the resistance of the graft to rejection include expression in graft EC and SMC of protective genes (Fig. 6) and infiltration with Th2-like cells (Fig. 5). Expression of protective genes is anti-inflammatory and anti-apoptotic in vitro (26–29), and promotes survival of xenografts in vivo (30). Th2 cytokines may facilitate expression of protective genes (61) and counter Th1 cell-mediated rejection responses (62).

Conversely, in the presence of high levels of preexisting or rapidly returned anti-graft Abs, binding of those Abs to graft EC, especially in the absence of complement, may lead to EC activation and expression of a number of proinflammatory molecules (2, 5), i.e., P-selectin (Fig. 6), with recruitment of host proinflammatory cells (Fig. 5), platelet aggregation, and fibrin deposition (Fig. 6). These proinflammatory cells produced Th1-type cytokines such as IFN-γ and TNF-α (Fig. 5), promoting a proinflammatory microenvironment within the graft. Those responses may lead to DXR (63).

In summary, the present study shows that hamster hearts can undergo accommodation in presensitized rats. The accommodated hearts become resistant to rejection by complement-fixing IgM and IgG Abs, as well as IgG Abs that can mediate ADCC. Removal of the preexisting anti-donor Abs pretransplantation and the suppression of their rapid return would seem to be critical steps to allow the development of accommodation. The protocol we have developed in the present study may encourage a different approach to achieving survival of xenografts in discordant combinations, including induction of the Th2-polarized response and protective gene expression in the graft. There will likely be factors in discordant combinations different from those we have studied here, e.g., molecular incompatibilities that precipitate and/or accelerate rejection (64). However, given the similarities between this model and discordant ones, the fundamental findings in this study regarding the depletion-suppression of the anti-graft Abs may well provide guidance for further development of therapies for the discordant models. Studying survival under these conditions in discordant combinations may be informative as to which problems exist.

References


