in a rat model of obliterative airway disease. Transplantation 2000; 69: 661.
33. Ikonen TS, Brazelton TR, Berry GJ, et al. Epithelial re-growth is associated with the inhibition of obliterative airway disease in orthotopic tracheal allografts in non-immunosuppressed rats. Transplantation 2000; 70: 857.
34. Reichenspurner H, Girgis RE, Robbins RC, et al. Stanford experience with obliterative bronchiolitis after lung and heart-lung transplantation. Ann Thorac Surg 1996; 62: 1467.
35. El Gamel A, Awad M, Yonan N, et al. Does cyclosporine promote the secretion of transforming growth factor-beta 1 following pulmonary transplantation? Transplant Proc 1998; 30: 1525.
36. Hutchinson IV. The role of transforming growth factor-beta in transplant rejection. Transplant Proc 1999; 31: 9S.

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INDUCTION OF XENOGRAFT ACCOMMODATION BY MODULATION OF ELICITED ANTIBODY RESPONSES[1,2]

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Background. **We have established that the timing of splenectomy influences the magnitude of the xenoreactive antibody (XAb) response and thus hamster heart survival in cyclosporine (CyA)-treated rats. This model has been used to test our hypothesis that modulation of XAb responses without perturbation of complement may influence the development of graft accommodation.**

Methods. **Pretransplantation splenectomy (day −1/day 0) fully abrogated anti-graft IgM response, whereas a delayed procedure (day 1/day 2) caused significantly delayed (3–4 days) and decreased levels (two- to threefold) of XAb. Both interventions resulted in long-term graft survival. After surviving for 7 or more days, xenografts in CyA-treated rats with post-, but not pre-, transplantation splenectomy were also resistant to exogenous anti-graft XAb. Such grafts meet the criteria for accommodation. Accommodating hearts displayed progressive and increasing expression of protective genes, such as heme oxygense (HO)-1 and A20, in endothelial cells and smooth muscle cells.**

Results. **Our results suggest that XAb responses may influence the kinetics of accommodation development possibly by promoting protective gene expression. This hypothesis was directly tested in vitro. Pretreatment of porcine aortic endothelial cells with sublytic amounts of baboon anti-pig serum for 24 hr induced HO-1 expression; this was associated with cell resistance to lytic amounts of such serum. Overexpression of HO-1 by adenoviral-mediated gene transfer in porcine aortic endothelial cells resulted in similar protective effects.**

Conclusions. **Delayed and relatively low levels of XAb IgM promote expression of protective genes in the graft and thereby aid in the progress of accommodation. Expression of HO-1 protects xenoserum-mediated endothelial cell destruction.**

Hyperacute rejection of discordant xenografts may be prevented by pretransplantation deletion of xenoreactive antibodies (XAb); however, grafts are still rejected within a few days by a process referred to as *delayed xenograft rejection* (DXR) (*1*), alternatively termed *acute vascular rejection* (AVR) (*2*). DXR–AVR is mediated by a complex mechanism in which elicited XAb are thought to play a central role (*1–3*). Binding of XAb and activated complement components to graft endothelial cells (ECs) results in cellular activation (*4*, *5*), loss of barrier integrity (*6*, *7*), and promotion of procoagulatory responses (*8*). DXR–AVR also occurs in concordant xenografts, such as hamster or mouse organs transplanted into rats (*9*, *10*). In these concordant models, the recipients have very low levels of preformed XAb, but then rapidly produce anti-graft antibodies after the transplantation procedure, leading to graft rejection in untreated animals within 3 to 4 days (*9–12*).

Under some circumstances, a xenograft may survive continuously in the presence of anti-graft antibodies and complement that might otherwise cause rejection, a phenomenon referred to as accommodation (*13*). We and others suggest that accommodation may reflect a physiologic alteration in both graft ECs and host immune responses (*14*, *15*). One mechanism by which accommodated grafts protect themselves from antibodies and complement is to express in ECs a number of protective genes, such as A20, BcL-2 and Bcl-x_L, and heme oxygenase (HO)-1 (*13*). Overexpression of A20, Bcl-2, and Bcl-x_L in ECs in vitro blocks activation of transcription factor nuclear factor (NF)-κB and thereby sup-

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presses induction of proinflammatory genes associated with EC activation (*16*, *17*). HO-1, a stress-responsive gene, exhibits potent antiinflammatory and antiapoptotic activity (*18*, *19*). Critical to our hypothesis that the expression of protective genes is functionally associated with xenograft accommodation, we have recently shown that HO-1 knockout mouse hearts cannot accommodate in rats under conditions in which matched wild-type mouse hearts survive (*20*).

We hypothesized that if the antibody-mediated rejection response can be appropriately modulated for an adequate time period, xenografts may have a window of opportunity to up-regulate protective gene expression and therefore undergo accommodation (*14*). Thus, hamster heart accommodation in rats can be achieved by transient complement inactivation with cobra venom factor (CVF) plus the T cell immunosuppressive drug cyclosporine (CyA) (*13*, *21*). This intervention does not substantially interfere with anti-graft IgM antibody production that normally causes xenograft rejection in the presence of complement (*22*). Xenografts survive in the presence of IgM XAb and reconstituted complement activity that have the potential to precipitate rapid rejection of a second naïve hamster heart (*22*). Similarly, a state of porcine EC accommodation can be induced in vitro by incubation of the cells with given levels of human anti-pig IgM or IgG (*23*, *24*), or α-gal-binding lectin (*25*). Those results lead to the interpretation that XAb may play a role in promoting xenograft accommodation. However, there is little understanding of how XAb can facilitate accommodation of vascularized organ xenografts.

Previous studies have revealed that the spleen plays a critical role in the generation of T-cell–independent, B-cell–mediated immune responses in xenotransplantation (*26*–*28*). We have now extended these studies to demonstrate that timing of splenectomy with regard to hamster heart transplantation may determine the effects of this intervention in suppression of anti-graft XAb formation in CyA-treated rats. This model was used to evaluate whether accommodation can be induced by modulation of elicited antibody responses in the presence of normal serum complement levels. We show that the delaying and relatively low levels of XAb promote xenograft accommodation. This process is associated with graft expression of protective genes, such as HO-1 and A20. At least expression of one of these genes, HO-1, protects xenografts from the effect of XAb and complement and thereby aids in developing accommodation.

MATERIALS AND METHODS

Animals

Golden Syrian hamsters, weighing 68–80 g, were used for organ donors. Inbred male Lewis rats (RT1[l]; 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 protocol was approved by the International Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

Surgical Procedures

Heterotopic hamster-to-rat heart transplantation was performed using published techniques (*29*). The function of the graft was monitored by daily inspection and palpation. Rejection was diagnosed by cessation of visible and palpable ventricular contraction and confirmed by histology. Splenectomy was performed at different times from day −1 to day 3 of heart transplantation.

Experimental Reagents

CyA (Novartis, Basel, Switzerland) was diluted in normal saline and administered by daily i.m. injection (15 mg/kg), commencing from day −1 of transplantation. CVF (Quidel, San Diego, CA) was administrated i.p. in a single dose (60 U/kg) on day 3 after transplantation. HO-1 inducer hemin (ferriprotoporphyrin IX chloride) and HO-1 inhibitor tin-protoporphyrin IX (SnPPIX; Sigma Chemical Company, St. Louis, MO) were dissolved in 100 mM NaOH to a stock solution of 50 mM and kept at −70°C until use. Light exposure was avoided as much as possible. Both hemin and SnPPIX were administered intraperitoneally in phosphate-buffered saline (PBS) to the donor at day −1 (30 μM/kg) and to the recipient at the time of transplantation (day 0), and daily thereafter until day 14 (30 μM/kg). Administration of equivalent volumes of vehicles was used as a control.

Serum Transfer

Serum was prepared from pooled blood taken from untreated rats that had rejected a hamster heart 1 day earlier. Serum was heat inactivated at 56°C for 30 min and injected intravenously (400 μL) into a rat 30 min after transplantation of a hamster heart.

XAb Assay

The IgM and IgG isotypes of anti-hamster XAb were measured by flow cytometry using a technique described previously (*10*). Heparinized and washed hamster peripheral red blood cells were used as target cells. Aliquots of 1×10^7 red blood cells were incubated for 30 min at 4°C with 100 μl of 1/10 diluted rat serum in PBS. To determine the titer of anti-hamster IgG, the cells were further incubated with monoclonal antibody (mAb) against rat IgG 1 (MARG1–2), IgG2a (MARG2a-1), IgG2b (MARG2d-8), and IgG2c (MARG2c-5; mouse IgG1, kind gifts of Prof. Bazin, University of Louvain, Belgium) or a combination of those antibodies. After addition of goat-anti-rat IgM antiserum–fluorescein isothiocyanate (FITC; Cappel, Durham, NC) or rat-anti-mouse IgG1 antiserum-FITC (Zymed, South San Francisco, CA), the cells were examined by flow cytometry using CellQuest software (FACScan; BD Biosciences, Franklin Lakes, NJ). Results were expressed as the mean channel fluorescence of stained cells divided by the mean channel fluorescence of cells incubated with control serum and secondary antibodies conjugated with FITC.

Cell Cultures

Porcine aortic endothelial cells (PAEC) were isolated and cultured in Dulbecco's minimum essential medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS), penicillin 100 U/mL, streptomycin 100 U/mL, and L-glutamine 2 mM (*30*). Confluent cultures were split in the ratio 1:3 as necessary using trypsin-EDTA (Invitrogen). Cells from between passes 5 and 9 were used in the subsequent experiments.

Baboon Anti-Pig Serum

Baboon anti-pig serum was collected from pooled blood of sensitized baboons that were immunized with porcine bone marrow cells for 7 days (*31*, *32*). After a solid clot had formed at room temperature, the serum was removed and frozen at −70°C as a source of baboon XAb against PAEC. Heat-inactivated baboon anti-pig serum was prepared by incubation of the serum at 56°C water bath for 30 min to eliminate complement activity.

Cell Treatment and Reagents

PAEC (70% confluence) in 25-mm culture flasks were pretreated with sublytic amounts of baboon anti-pig serum for 24 hr to evaluate

whether this procedure could up-regulate protective gene expression. The sublytic amounts of baboon anti-pig serum were prepared by heat-inactivation of the serum followed by serial titrations for their capacity to induce PAEC membrane damage. A concentration of 5% (v/v) of the serum was chosen to achieve optimal stimulation of HO-1 expression with minimal preexisting cytotoxicity. Pretreated PAEC were then exposed for 6 hr to lytic baboon anti-pig serum (20%, non–heat-inactivated). Equal concentrations of heat-inactivated FCS were used as controls for baboon serum. SnPPIX was used to modulate HO enzymatic activity in PAEC. SnPPIX was dissolved (10 mM) in 0.01 M NaOH and added to the culture medium (50 μM) at the time when pretreatment started.

Adenovirus

The recombinant HO-1 adenovirus has been described previously (*19*). The recombinant β-galactosidase (β-gal) adenovirus was a kind gift of Dr. Robert Gerard (University of Texas Southwestern Medical Center, Dallas, TX). Adenoviruses were produced, extracted, purified, and titrated as described previously (*33*). PAEC in 70% confluence were infected with a multiplicity of infection (MOI) of 100 plaque-forming units per cell (PFU/cell) as described previously (*33*). This MOI was chosen to achieve maximal HO-1 expression without detectable cytotoxicity. Adenoviral infections proceeded in DMEM (1 hr at 37°C, 5% CO_2, and 95% humidity) under agitation. Cells were then washed with DMEM and incubated for an additional 24 hr in DMEM supplemented with 10% FCS.

Western Blot Analysis

Cell extracts were prepared and electrophoresed under denaturing conditions using 10% polyacrylamide gels (*34*). Proteins were transferred into a polyvinyl difluoridine (PVDF) membrane (Immobilon P, Millipore, Bedford, MA) by electroblotting (0.8 mA/cm^2; 1 hr) and detected after blocking in 5% non-fat dry milk in PBS 0.1% Tween-20 (v/v) at 4°C overnight. Rabbit anti-HO-1 polyclonal antibody (StreeGene Biotechnologies Corp., Victoria, BC, Canada) was used to detect HO-1. Anti-β-tubulin mAb (Boehringer Mannheim, Indianapolis, IN) was used to detect β-tubulin. Primary antibodies were detected using horseradish peroxidase–conjugated donkey anti-rabbit or rabbit anti-mouse IgG secondary antibodies (Pierce Chemical Co., Rockford, IL). Bands were visualized using the enhanced chemiluminescence assay (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions, and stored in the form of photoradiographs (Biomax MS; Eastman Kodak Co., Rochester, NY). Digital images were obtained using an image scanner (Arcus II, Agfa, Ridgefield Park, NJ) equipped with FotoLook and Photoshop software (Adobe Systems, San Jose, CA).

Flow Cytometric Analysis of Cell Membrane Damage

PAEC were harvested using trypsin-EDTA, and aliquots of 5×10^5 cells were suspended in 0.5 mL of ice-cold PBS. Immediately before flow cytometric analysis, 25 μL of propidium iodide (1 μg/mL in PBS; Sigma) was added to each sample to allow uptake by cells with injured membrane and thus increased permeability. Cellular debris and doublets were excluded from analysis by their forward-light scatter and right-angle-light scatter properties (*10*, *35*).

Histopathology and Immunohistochemistry

Tissue samples for histology were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for light microscopy. Tissue samples for immunohistochemistry were snap-frozen in prechilled isopentane and stored at −70°C. Frozen samples were cut into 4-μm sections in a cryostat at −25°C and air dried. Rat anti-hamster Ig in the xenografts were detected by mAb directed against rat IgM (MARM-4), IgG1 (MARG1–2), IgG2a (MARG2a-1), IgG2b (MARG-2b-8), and IgG2c (MARG2c-5; gifts from Prof. Bazin). Intragraft complement deposition was detected by staining with an anti-rat C3 mAb (CD11, Serotec, Oxford, U.K.). Expression of cytoprotective genes was analyzed using rabbit polyclonal antibodies to HO-1 and A20 (StreeGene Biotechnologies). Cryostat sections were fixed in paraformaldehyde-lysine-periodate for demonstration of humoral reactions, or fixed in acetone for localization of cytoprotective genes, as described (*13*). Isotype-matched mAbs or normal serum and a control for residual endogenous peroxidase activity were included in each experiment.

Statistics

The results were analyzed by Student's *t* test or by Fisher's exact test.

RESULTS

Graft Survival in Splenectomized Rats

In keeping with previous studies (*22*), unmodified rats rejected hamster heart grafts after a mean survival time (MST) of 3.1±0.4 days (Table 1, group 1). Administration of CyA alone did not significantly influence graft survival (MST, 3.3±0.5; group 2), in keeping with the hypothesis that this type of rejection can proceed in a T cell–independent manner. Splenectomy alone caused marginal delays in rejection to 4.5±1.1 days (group 3). However, a combination of CyA and splenectomy resulted in long-term survival (>90 days) of xenografts in 100% of cases evaluated (group 4).

Given the timing of anti-graft IgM antibody elicitation and associated xenograft rejection, we tested how the timing of splenectomy influenced xenograft survival in CyA-treated rats. As shown in Table 2, splenectomy performed immediately before hamster heart transplantation (day 0; group 1) or on day 1 (group 2) or day 2 (group 3) after transplantation induced long-term survival of hamster hearts in 100% cases ($P<0.001$ vs. CyA alone-treated rats). However, further delays in splenectomy to day 3 after transplantation (group 4) were associated with rejection of all grafts (six of six cases) within 7 days. This outcome was attenuated by administration of CVF at the time of splenectomy (day 3), resulting in grafts surviving continuously (>30 days in five of five cases;

TABLE 1. Survival of hamster hearts in CyA-treated rats with pretransplantation splenectomy on day −1

Group	Treatment	n	Graft survival (days)	Mean±SD (days)	*P* value[b]
1	None	7	3, 3, 3, 3, 3, 3, 4	3.1±0.4	
2	CyA	6	3, 3, 3, 3, 4, 4	3.3±0.5	NS
3	Splenectomy	6	3, 4, 4, 5, 5, 6	4.5±1.1	NS
4	CyA	10	>30 (n=3)[a]	>90	<0.001
	Splenectomy		>90 (n=7)[a]		

[a] Grafts were removed for histology.
[b] Versus group 1.
NS, not significant.

TABLE 2. Xenograft survival in CyA-treated rats with pre- versus posttransplantation splenectomy

Group	Timing of splenectomy[a]	Graft survival (days)	*P* value[c]
1	Day 0	>90 (n=7)[b]	<0.001
2	Day 1	>90 (n=7)[b]	<0.001
3	Day 2	>90 (n=9)[b]	<0.001
4	Day 3	3, 3, 4, 5, 5, 7	NS

[a] With respect to the time of heart transplantation.
[b] Grafts were removed for histology.
[c] Versus CyA alone-treated rats (group 2 in Table 1).
NS, not significant.

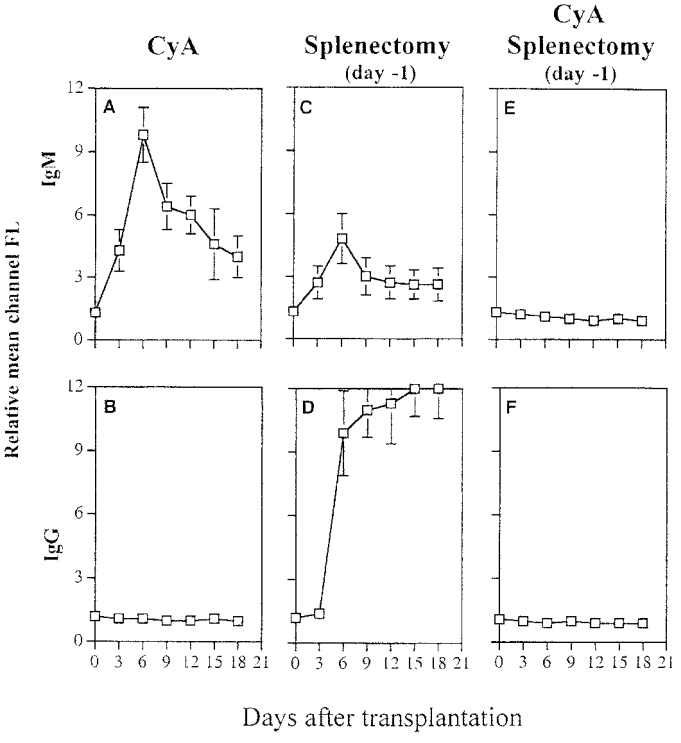

FIGURE 1. Flow cytometric analysis of anti-hamster antibody formation in rats treated with CyA in combination with pretransplantation splenectomy. (A) and (B) Anti-hamster IgM and IgG in rats treated with CyA alone. (C) and (D) Splenectomized rats. (E) and (F) Rats receiving a combination of both treatments . The data are the mean value ±SD of five animals in each group.

$P<0.01$), whereas CVF alone given to CyA-treated rats at day 3 was ineffective (six of six grafts rejected between 3 and 6 days).

Anti-Graft Antibodies in Splenectomized Rats

Unmodified rats produce high titers of anti-hamster IgM antibodies 3 days after hamster heart transplantation (*22*, *36*). Anti-hamster IgG antibodies also occur at readily detectable levels more than 3 days after transplantation (*22*, *36*). In keeping with the hypothesis that the IgM response in this model is T cell–independent (*22*, *36*), CyA-treated rats showed a progressive increase of IgM XAb by approximately three- to fourfold around day 3, with peak levels around day 6 after transplantation (Fig. 1A). CyA, however, completely blocked anti-graft IgG (Fig. 1B), indicating that this IgG XAb response is T cell–dependent (*22*, *36*). Pretransplantation splenectomy (day −1) alone decreased anti-graft IgM levels to approximately half of the level seen in CyA alone-treated rats (Fig. 1C), whereas high levels of anti-graft IgG XAb were detected after day 4, peaking after day 6 after transplantation (Fig. 1D). A combination of CyA and splenectomy resulted in a profound suppression of both IgM and IgG XAb to pretransplantation levels (Fig. 1, E and F). These procedures did not influence the circulating complement activity as measured by the CH50 assay (data not shown).

We next examined how the timing of splenectomy influences suppression of XAb formation after xenotransplantation to CyA-treated rats. Splenectomy performed immediately before transplantation (day 0) completely blocked XAb formation (Fig. 2A). Splenectomy on day 1 (Fig. 2B) or day 2 (Fig. 2C) after transplantation resulted in a marked delay (by 3 to 4 days for peak levels) and decrease (by two- to threefold) of IgM production when compared with CyA alone-treated rats. A further delay in performing splenectomy to day 3 substantially abrogated the ability of this intervention to suppress IgM XAb production (Fig. 2D).

Survival of Grafts After Hyperimmune Serum Transfer

Hamster hearts survived continuously in the presence of considerable levels of anti-graft XAb in CyA-treated rats with posttransplantation splenectomy (day 1 or day 2), suggesting accommodation. To test the resistance of the graft to antibody plus complement at different times after transplantation, we have performed experiments in which hyperimmune serum taken from unmodified rats that rejected a hamster heart 1 day earlier was given to the recipients (Fig. 3). A dose of 400 μl of such serum was selected on the basis of our previous results showing this amount of hyperimmune serum could result in hyperacute rejection of naïve hamster hearts, but allow accommodated hearts to survive continuously (*22*). Adoptive serum transfer resulted in rapid rejection of hamster hearts in CyA-treated rats with pretransplantation splenectomy on day −1. Rejection occurred when the hyperacute serum was injected immediately after transplantation (20–40 min; n=5; Fig. 3A), on day 3 after transplantation (60–240 min; n=5; Fig. 3B), or on day 7 or later after transplantation (12–24 hr; n=5; Fig. 3C). Similar results were seen with CyA-treated rats with pretransplantation splenectomy at day 0. Rejection occurred in CyA-treated rats with posttransplantation splenectomy on day 2 when hyperimmune serum was injected immediately (20–60 min; n=5; Fig. 3D) or on day 3 after transplantation (24–72 hr; n=5; Fig. 3E). However, when serum injection was given on day 7 or later after transplantation, all grafts survived continuously (>100 days; n=5; Fig. 3F). Similar results were obtained in CyA-treated rats with posttransplantation splenectomy on day 1.

Survival of Hamster Hearts After Retransplantation

To further evaluate the extent to which accommodating hearts were resistant to effects of XAb and complement under pathophysiologic conditions, we retransplanted accommodating hearts into CyA alone-treated rats that had rejected a hamster heart 1 day earlier (Table 3). Naïve hamster hearts were hyperacutely rejected by the sensitized animals (30–240 min; n=5; group 1). Rejection was delayed up to 1–2

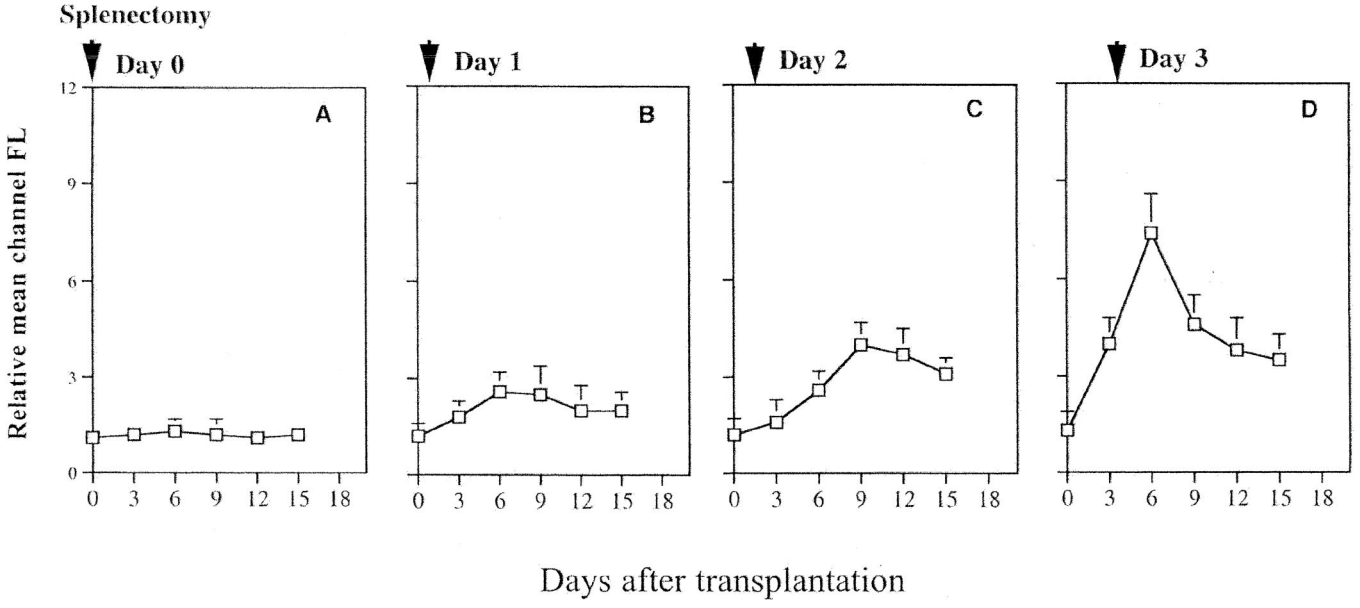

FIGURE 2. Flow cytometric analysis of anti-hamster IgM antibody formation in rats treated with CyA in combination with pretransplant versus posttransplantation splenectomy. Splenectomy was performed immediately before transplantation (day 0) (A), or on day 1 (B), day 2 (C), and day 3 (D) after transplantation. The data are the mean value ±SD of five animals in each group.

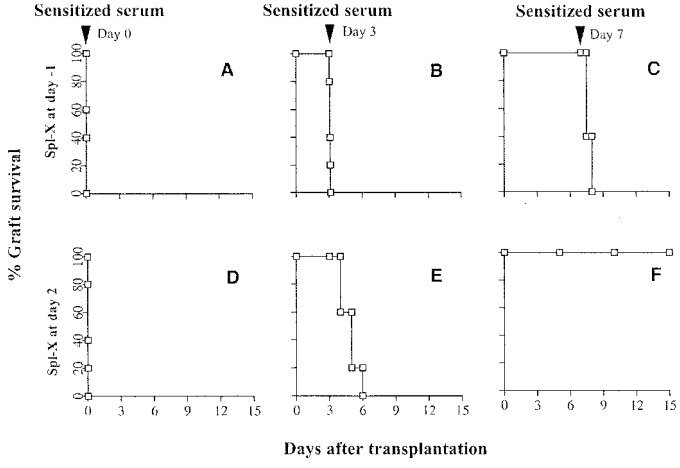

FIGURE 3. Survival of hamster hearts in rats after hyperimmune serum transfer. Hyperimmune serum taken from untreated rats that rejected a hamster heart 1 day earlier was injected intravenously into CyA-treated, splenectomized rats immediately before (on day 0) or on days 3 to 7 after transplantation. Graft survival in CyA-treated rats with pretransplantation splenectomy (Spl-X) on day −1 (A–C) or with posttransplantation splenectomy on day 2 (D–F).

days when the accommodating hearts had survived in a first recipient for 3 days (group 2). Accommodating hearts that had survived for 7 or more days in the first recipient survived continuously in majority of cases (five of six) after retransplantation (group 3).

Histopathology and Immunohistochemistry

H&E staining of hearts rejected by untreated rats or CyA-treated alone showed that rejection is associated with hemorrhage, edema, mononuclear cell infiltrate, and myocardial fiber disruption with focal necrosis (Fig. 4A). Immunoperoxidase staining demonstrated vessel wall deposition of IgM (Fig. 4B) and associated complement component C3 (Fig. 4C).

Sections of surviving xenografts harvested 7 days or later after transplantation from CyA-treated rats with pre- or posttransplantation splenectomy showed normal cardiac histology (Fig. 4, D and G). Xenografts in CyA-treated rats with pretransplantation splenectomy showed absence of IgM (Fig. 4E) and C3 (Fig. 4F) deposition. ECs and smooth muscle cells (SMCs) of those grafts expressed undetectable HO-1 and A20 by day 3, and very low levels of HO-1 after day 7 (Fig. 5, A–F). Xenografts in CyA-treated rats with posttransplantation splenectomy exhibited a moderate deposition of IgM (Fig. 4H) and C3 (Fig. 4I) along the surface of graft ECs. ECs and SMCs of those grafts expressed increasing levels of HO-1 (Fig. 5, G–I) and A20 (Fig. 5, J–L) after transplantation. Importantly, both HO-1 and A20 were easily detectable 3 days after transplantation (Fig. 5, H and K), and HO-1 was expressed at high levels after day 7 (Fig. 5, I and L).

TABLE 3. Survival of accommodating hearts after retransplantation

Group	Graft survival time before retransplantation[a]	Graft survival time after retransplantation[b]
1	0 day	30, 30, 60, 120, 240 min
2	3 days	1, 1, 1, 2 days
3	7 days	2, >30 (n=5) days

[a] In CyA-treated rats with posttransplantation splenectomy on day 2.
[b] In CyA alone-treated rats that rejected a xenograft 1 day earlier.

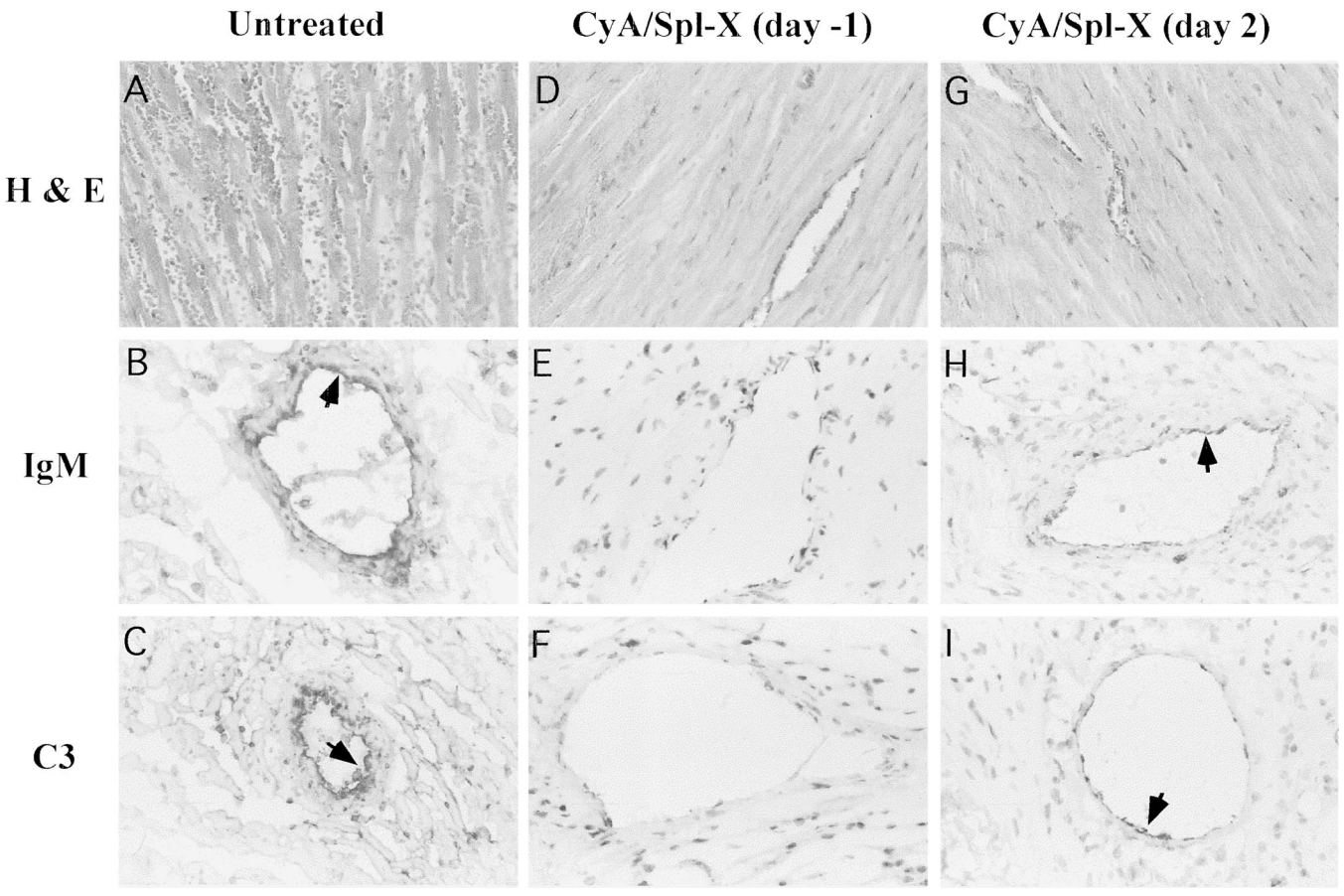

FIGURE 4. Histology and immunohistochemistry of grafts in CyA-treated rats with pre- versus posttransplantation splenectomy. Grafts were harvested after rejection or 7 days after transplantation. IgM and C3 deposit along the surface of graft ECs in untreated rats (A–C), in CyA-treated rats with pretransplantation splenectomy on day −1 (D–F) and in CyA-treated rats with posttransplantation splenectomy on day 2 (G–I) are presented. Positive staining when appropriate (*arrows*). (Hematoxylin-eosin stain)

Effects of HO-1 Expression on Graft Survival

We have provided evidence to suggest that expression of protective genes facilitates xenograft accommodation. To evaluate this hypothesis, we conducted a model in which induction of HO-1 expression was produced by treatment of the donor and recipient with hemin, a specific inducer of HO-1 (Table 4). We showed above that delayed splenectomy at day 3 after transplantation failed to prevent rejection of hamster hearts transplanted in CyA-treated rats (Table 2). Hemin treatment did not significantly alter graft survival in CyA-treated rats (MST, 4±0.7 days; n=5; $P>0.05$), as compared with rats treated with CyA alone (MST, 3.3±0.5 days). Hemin treatment, however, reversed graft rejection in CyA-treated rats with posttransplantation splenectomy at day 3 (MST>7 days with four of six grafts surviving continuously; group 3; $P<0.001$), as compared with vehicle controls (MST, 3.6±0.9 days; group 1). This effect was abrogated by blockade of HO-1 enzymatic activity with SnPPIX (group 4).

HO-1 Expression in PAEC After Pretreatment With Sublytic Baboon Anti-Pig Serum

To directly test our hypothesis that exposure to given levels of anti-graft XAb promotes protective gene expression in xenograft ECs, we developed and studied an experimental model in which PAEC were pretreated with baboon anti-pig serum in vitro (Fig. 6). Although this model may not exactly reflect the situation of hamster-to-rat combination, it may provide a means to understand the mechanism underlying the development of accommodation. Non-pretreated PAEC expressed very low levels of HO-1 (lane 1). After pretreatment with sublytic amounts of baboon anti-pig serum without complement (5%; with heat-inactivation), PAEC showed a time-dependent up-regulation of HO-1 expression. Easily detectable levels of HO-1 occurred 12 hr after pretreatment (lane 2), with peak levels after 24 hr (lanes 3 and 4).

HO-1 Expression Protects PAEC From Lytic Baboon Anti-Pig Serum

We examined the effects of HO-1 in the protection of PAEC from effects of lytic baboon anti-pig serum (20%; without heat-inactivation). A 6-hr incubation resulted in a significant percentage of cells with membrane damage as manifested by increased membrane permeability (approximately 55%; Fig. 7A*ii*) as compared with PAEC incubated with control FCS (approximately 6%; Fig. 7A*i*). Pretreatment of PAEC with sublytic amounts of baboon anti-pig serum (5%, heart-inactivated) for 24 hr essentially did not cause membrane damage in PAEC (approximately 7%; Fig. 7A*iii*). However, such

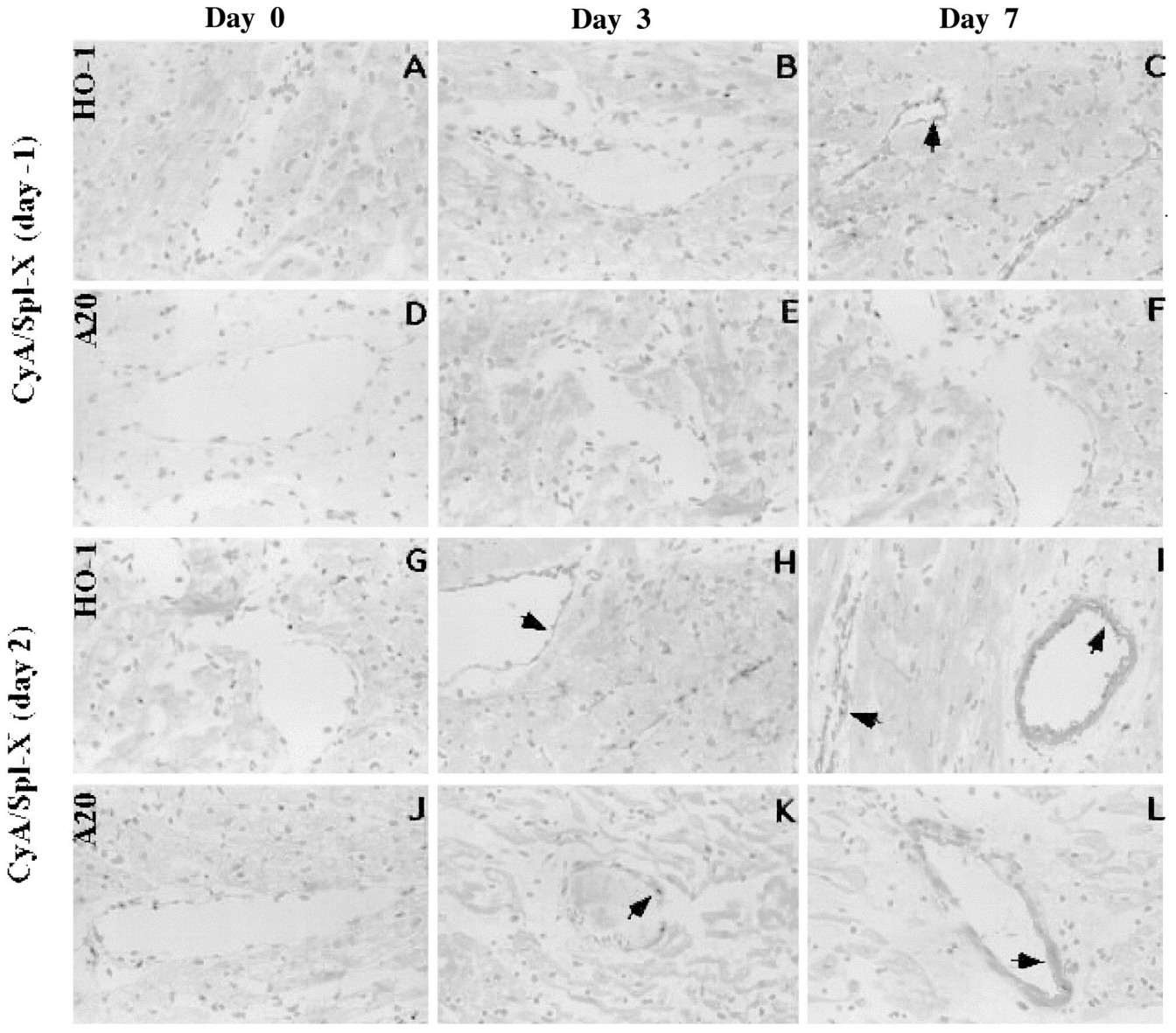

FIGURE 5. Immunoperoxidase staining for protective gene expression in grafts transplanted in CyA-treated rats with pretransplantation versus posttransplantation splenectomy (Spl-X). Surviving grafts were harvested on day 0, 3, or 7 after transplantation. HO-1 and A20 expressions in CyA-treated rats with pretransplantation splenectomy on day −1 (A–F) and with posttransplantation splenectomy on day 2 (G–L).

pretreated PAEC showed a marked decrease in the percentage of cells with membrane damage on exposure to lytic baboon anti-pig serum (approximately 21%; Fig. 7A*iv*). When the pretreatment of PAEC was performed in the presence of HO-1 activity inhibitor, SnPPIX, the protective effect was largely abrogated (approximately 40%; Fig. 7A*vi*). These results are summarized in Fig. 7B. Percentages of cells with membrane damage in pretreated cells (20±3.7%; n=4) were significantly lower than those seen in nonpretreated cells (49±7%; n=4; $P<0.001$). This pretreatment-induced protection was largely reversed by blockade of HO-1 activity with SnPPIX (41±4.8%; n=4; $P>0.05$ vs. nonpretreated cells).

The specific contribution of HO-1 in protecting cells from lytic baboon anti-pig serum-mediated cell membrane damage was further investigated in a model in which overexpression of HO-1 was induced by means of adenoviral-mediated gene infection (Fig. 8). HO-1–adenovirus-infected PAEC had high levels of expression of HO-1 (lane 3) when compared with noninfected (lane 1) or β-gal-infected cells (lane 2). HO-1 overexpression essentially did not influence binding of XAb and complement to those cells, as examined by FACS (data not shown). HO-1–infected PAEC exhibited resistance to lytic baboon anti-pig serum (approximately 13%; Fig. 9A*vi*) as compared with noninfected (approximately 38%; Fig. 9A*ii*) or β-gal-infected (approximately 39%; Fig. 9A*iv*) cells. These data are summarized in Figure 9B. Percentages of cells with membrane damage in HO-1–adenovirus-infected cells (14±2.8%; n=4) were significantly lower than those seen in noninfected (42±7%; n=4) or β-gal-adenovirus-infected (44±6.7%; n=4) cells ($P<0.001$ for both comparisons).

TABLE 4. Induction of HO-1 in grafts by hemin treatment facilitated graft survival in CyA-treated rats with delayed administration of splenectomy (day 3)

Group	Treatment[a]	n	Graft survival (days)	Mean±SD (days)	*P* value[b]
1	Vehicle	5	3, 3, 3, 4, 5	3.6±0.9	
2	SnPPIX	5	2, 3, 3, 3, 4	3±0.7	NS
3	Hemin	6	7, 8, >30 (n=4)	>30	<0.001
4	Hemin SnPPIX	6	3, 3, 3, 4, 4, 6	3.8±1.9	NS

[a] Administered to the donor at day −1 and to the recipient at the time of transplantation (day 0) and daily thereafter until day 14.
[b] Versus group 1.
NS, not significant.

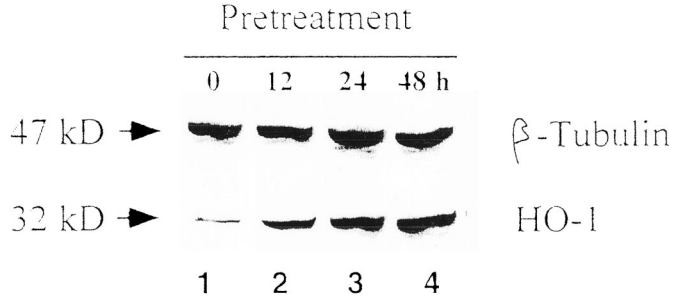

FIGURE 6. HO-1 expression in cultured PAEC after pretreatment with sublytic baboon anti-pig serum. PAEC (70% confluence) were incubated with sublytic amounts of baboon anti-pig serum (5%, heat-inactivated) for 24 hr. HO-1 expression was measured by Western blot analysis. Nonpretreated PAEC expressed very low levels of HO-1 (lane 1). Pretreated PAEC showed time-dependent up-regulation of HO-1 expression. Easily detectable levels of HO-1 occurred 12 hr after pretreatment (lane 2), with peak levels at or after 24 hr (lanes 3 and 4). Data depicted are representative of three separate analyses.

DISCUSSION

Long-term survival and accommodation of hamster-to-rat cardiac xenografts can be achieved by transient complement inactivation with CVF plus CyA maintenance therapy (*13*, *21*). This therapy essentially does not interfere with elicited anti-graft IgM XAb responses that normally cause xenograft rejection in a complement-dependent manner (*29*, *37*). In the present study, we have established a model in which timing of splenectomy controls the levels and kinetics of elicited XAb responses in CyA-treated rats after hamster heart transplantation. We have used this model to test our hypothesis that appropriate modulation of elicited XAb responses may induce xenograft accommodation in the presence of normal levels of complement activity.

The spleen has been implicated as a key lymphoid organ responsible for generation of B cell–mediated humoral immune responses in xenotransplantation (*26*–*28*). Antigen encounter recruits antigen-specific immune cells and immune regulatory cells in the spleen (*38*, *39*). The highly organized microstructure of the spleen is thought to facilitate antigen recognition and generation of antigen-specific effector cells (*38*, *39*). After hamster heart transplantation, rat splenic B cells in the marginal zone proliferate and express a maturation phenotype characterized by IgM^{high}, IgD^{low} associated with anti-graft XAb production and graft rejection (*27*, *28*). The generation of marginal zone B-cell activation occurs owing to T cell–independent antigenic stimulation (*40*) and is observed in nude and thymectomized rats (*41*). These data support the idea that the humoral response to transplanted xenografts can be T cell–independent.

Pretransplantation splenectomy (day −1 and day 0) in the presence of complement (no CVF) in the CyA-treated rats completely suppressed IgM antibody formation and resulted in long-term survival of xenografts. In keeping with our findings, previous studies have shown that splenectomy in combination with other T cell–directed immunosuppressive drugs, such as FK506 and anti-T cell antibody reagents, can promote hamster or mouse heart survival in rats (*42*–*44*). Moreover, splenectomy alone was able to induce profound inhibition of XAb formation in nude rats after hamster heart transplantation, associated with prolonged graft survival (*44*). Thus, splenectomy effectively suppresses T cell–independent antibody responses in rats after hamster heart transplantation.

When splenectomy was performed after transplantation, e.g., at day 1 or day 2, in CyA-treated rats, this intervention could still result in marked delays (by 3 to 4 days for peak levels) and decreases (by two- to threefold) of IgM production when compared with CyA treatment alone. These changes in humoral responses could also prevent graft rejection. These effects were thought to result in part from removal of antigen-specific B cells that had been activated within the spleen (*45*). However, further delays in splenectomy to day 3 after engraftment were insufficient to prevent transplant rejection. Rejection under these circumstances could be explained by high levels of antibodies already released to the circulation (Fig. 2), or by the release of activated B cells from the spleen (*45*). Previous studies have shown that the timing of splenectomy may critically influence hamster heart survival in rats; however, the underlying mechanism was unclear (*45*). A mechanism consistent with the present data would suggest that the rate of antibody production must be slowed and not allowed to exceed a given level early after transplantation. Thus, timing of splenectomy may determine the efficacy of this procedure in suppression of XAb responses and graft survival in xenotransplantation.

In CyA-treated rats with posttransplantation splenectomy (day 1 or day 2), grafts survived continuously in the presence of considerable levels of anti-graft antibodies and normal circulating complement levels, as well as having evidence for deposition of these factors along the surface of vessel walls. Our findings suggest that accommodation occurred at some steps. To test this hypothesis, we performed serum transfer experiments in which hyperimmune serum taken from untreated rats 1 day after hamster heart rejection was injected

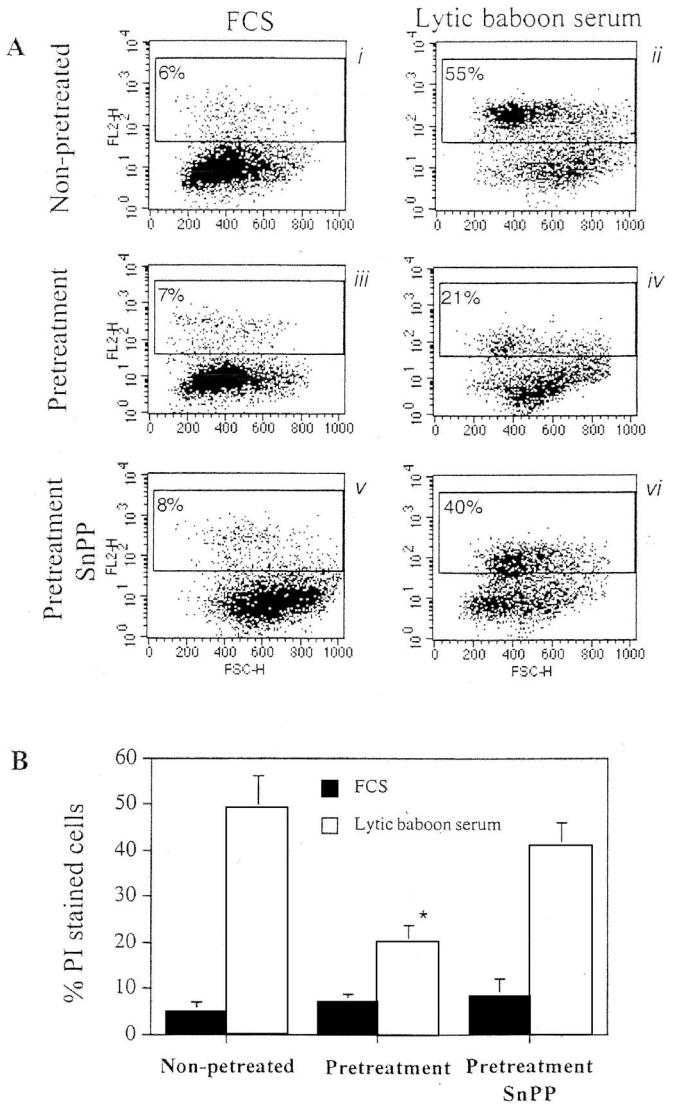

FIGURE 7. HO-1 expression in pretreated PAEC protected those cells from lytic baboon anti-pig serum. PAEC were pretreated with sublytic amounts of baboon anti-pig serum (5%, heat-inactivated) for 24 hr, followed by incubation with lytic amounts of baboon anti-pig serum (20%, non–heat-inactivated) for 6 hr. Cell membrane damage was determined by propidium iodide (PI) uptake by the cell and then by flow cytometric analysis. Equivalent concentrations of FCS were added to cultures as controls. (A) Nonpretreated cells (*i* and *ii*), pretreated cells (*iii* and *iv*), and cells pretreated in the presence of HO-1 inhibitor SnPPIX (*v* and *vi*) are presented. (B) Percentages of lytic baboon anti-pig serum-mediated membrane damage in different groups tested are presented. The data depicted are the mean value ±SD of four separate experiments. The asterisk indicates statistical significance when compared with nonpretreated cells ($P<0.01$).

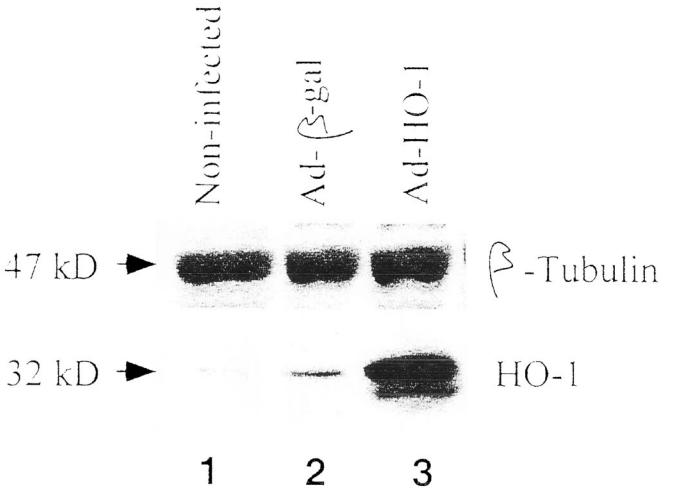

FIGURE 8. Overexpression of HO-1 by adenoviral-mediated gene infection. PAEC in 70% confluence were infected with a multiplicity of infection of 100 PFU/cell in DMEM (1 hr at 37°C, 5% CO_2, and 95% humidity) under agitation. Cells were then washed with DMEM and incubated for an additional 24 hr in DMEM supplemented with 10% FCS. HO-1 expressions in noninfected cells (lane 1), β-gal-infected cells (lane 2), and HO-1-infected cells (lane 3) are measured by Western blot. The data depicted are representative of three separate experiments.

into those rats carrying an accommodating xenograft on 0, 3, or 7 or more days after transplantation. Xenografts survived long-term only when they had been transplanted 7 or more days before serum injection. We would interpret these findings as suggesting that the xenografts were fully accommodated by day 7. In contrast, xenografts surviving in the absence of detectable anti-graft XAb in CyA-treated rats with pretransplantation splenectomy were invariably rejected at any time tested for hyperimmune serum injection up to day 7 or later. In this instance, the survival of the graft without serum injection may reflect a state of recipient adaptation as opposed to graft accommodation (*46*).

Our findings have two important implications. First, the presence of certain levels of anti-graft antibodies promotes the development of accommodation. In keeping with this view, previous studies have revealed that a state of PAEC accommodation could be induced in vitro by incubation with human anti-α-gal IgM or IgG (*23*, *24*) or α-gal-binding lectin (*25*). In addition, accommodation of hamster-to-rat hearts often occurs in the presence of anti-graft XAb (*47*). Alternative evidence for the contribution of anti-graft XAb to the development of accommodation arises from observations that a number of immunosuppressive drugs that result in profound inhibition of XAb formation indefinitely prolong xenograft survival of hamster hearts in rats, but do not induce true accommodation (*36*, *48*).

A further implication is that a given period is importantly required for the development of accommodation. We can show that accommodating xenografts developed resistance to exogenous XAb only after they had survived for 7 or more days. Additional evidence for this time-dependent process of accommodation may be derived from our previous observation that a 4- to 6-day duration of complement inactivation was needed for accommodation induction (*13*, *22*). To assess the extent to which accommodated xenografts resist the effects of XAb and complement in pathophysiologic conditions, we have retransplanted accommodating xenografts into CyA alone-treated rats 1 day after hamster heart rejection. Those sensitized rats rejected naïve hamster hearts, or accommodating hearts that had survived in a first recipient for 3 or fewer days. However, after surviving for 7 or more days in the

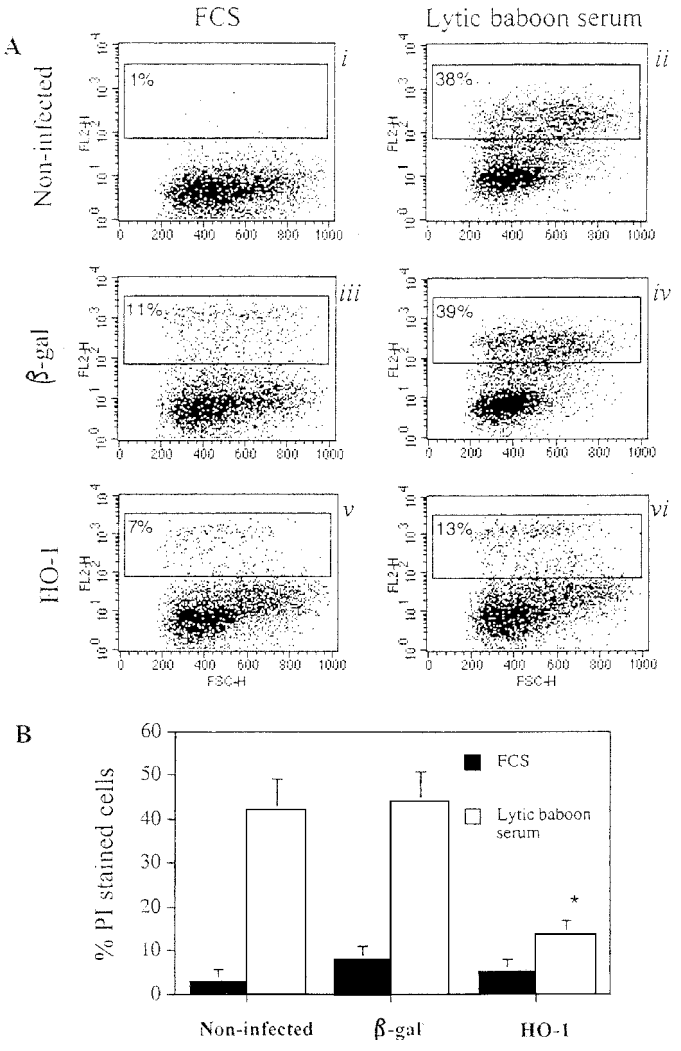

FIGURE 9. Adenoviral-mediated HO-1 expression protects PAEC from lytic baboon anti-pig serum. After infection with adenovirus preparations, PAEC were incubated with lytic amounts of baboon anti-pig serum (20%, non–heat-inactivated) for 6 hr. Cell membrane damage was measured by propidium iodide (PI) uptake by cells and flow cytometric analysis. Equivalent concentrations of FCS were added to cultures as controls. (A) Noninfected (*i* and *ii*), β-gal-infected (*iii* and *iv*), and HO-1-infected (*v* and *vi*) cells are presented. (B) Percentages of lytic baboon anti-pig serum-mediated membrane damage in different groups tested are presented. The data depicted are the mean value ±SD of four separate experiments. The asterisk indicates statistical significance when compared with noninfected cells ($P<0.01$).

first recipient, the majority of the accommodating grafts (five of six cases) could survive continuously in second recipients that had been previously sensitized to xenoantigens.

We have previously observed that xenografts undergoing accommodation express a number of genes, termed protective genes on the basis of their antiinflammatory and antiapoptotic properties (*13*). Here, we showed that there was a progressive up-regulation of protective gene expression, including A20 and HO-1, in the accommodating graft EC and SMC. The stronger expression of those genes at 7 days or later after transplantation when compared with that on day 3 or earlier may be correlated with increasing resistance of the grafts to XAb and complement. Moreover, we provided evidence that specific induction of HO-1 aided in graft survival and accommodation in rats receiving suboptimal immunosuppressive regimen, such as CyA plus delayed administration of splenectomy. These findings warrant further evaluation of the therapeutic potential of protective genes in xenotransplantation.

To test the hypothesis that anti-graft XAb promote protective gene expression, we studied induction and function of HO-1 in an experimental model in which PAEC were incubated with baboon anti-pig serum. Pretreatment of PAEC with sublytic mounts of baboon anti-pig serum (5%, heat-inactivated) for 24 hr up-regulated HO-1 expression in the cells, which was associated with PAEC resistance to lytic amounts of baboon anti-pig serum (20%, without heat-inactivation). The specificity of HO-1–mediated protection against effects of lytic baboon anti-pig serum was further supported by two observations: (1) Blockade of HO-1 activity with SnPPIX, a specific inhibitor of HO-1, restored sensitivity of pretreated PAEC to lytic baboon anti-pig serum; and (2) expression of HO-1 by means of adenoviral-mediated gene infection rendered PAEC resistant to lytic baboon anti-pig serum in a manner comparable to that achieved by the pretreatment.

HO-1–mediated protection may result from multiple mechanisms associated with unique enzymatic activities. HO-1 is the rate-limiting enzyme in the catabolism of heme into three products: free iron, biliverdin, and carbon monoxide, with biliverdin being subsequently catabolized into bilirubin (*18*, *49*). The antiinflammatory activity of HO-1 is suggested by the observation that expression of HO-1 in vitro prevents EC injury mediated by polymorphonuclear cells (*50*), hydrogen peroxide (*50*), or heme (*51*). We have recently reported that HO-1 can suppress EC apoptosis via the activation of p38 mitogen-activated protein kinase directly by the generation of carbon monoxide (*19*). In vivo, expression of HO-1 suppresses a variety of inflammatory and apoptotic responses, including endotoxic shock (*52*), hyperoxia (*53*), ischemia-reperfusion injury (*54*), and graft rejection (*20*, *55*).

In addition to the above-mentioned protective gene responses, previous studies have shown that pretreatment of porcine ECs with human anti-pig IgM or IgG antibodies or α-gal-binding lectin can render the cells resistant to lytic complement-mediated killing (*23–25*). This resistance is related to decreased EC expression of vascular cell adhesion molecule (*24*), a cell surface adhesion molecule associated with EC activation and EC–leukocyte interaction (*56*). The induced protective reaction is protein synthesis dependent, and associated with up-regulation of several molecules including inducible nitric oxide synthase (*57*) and CD59 (*25*). The cytoprotective inducible nitric oxide synthase uses L-arginine as a substrate to generate nitric oxide, a major component in signal transduction pathways controlling smooth muscle tone, platelet aggregation, and EC proinflammatory and apoptotic responses (*58–60*) associated with xenograft transplantation. As expected, expression of CD59, a known complement regulatory protein, has been shown to prevent lytic complement-mediated cell membrane injury (*61*). In addition, CD39, or vascular adenosine triphosphate diphosphohydrolase-1, has been recently suggested to play a pivotal role in regulation of intravascular platelet sequestra-

tion and fibrin deposition associated with xenotransplantation (*62*, *63*). The relative contribution of these genes to EC accommodation remains to be further identified. In this regard, our observation that HO-1, for example, can be induced and protect ECs in response to XAb plus complement-mediated effects is important. This finding provides strong evidence that HO-1, or other genes, can be crucial for the induction of EC accommodation.

CONCLUSION

We have shown that splenectomy of rats, which suppresses IgM production in response to a transplanted hamster heart, can play a key role in allowing the heart to survive. However, the timing of splenectomy with respect to the time of transplantation is critical. Delaying anti-graft antibody production and not allowing high levels of antibodies to develop as compared with nonsplenectomized animals seems key. Antibodies elicited under conditions that lead to long-term survival appear to elicit protective gene expression in the ECs and SMCs of the transplanted organs. Specific induction of at least one of those protective genes, HO-1, in the graft aids in graft survival and accommodation. Expression of HO-1 protects the ECs from antibody plus complement–mediated membrane injury in vitro, further suggesting that expression of this gene in vivo may explain long-term survival of the grafts. That accommodation is different from other conditions in which grafts survive is shown by our experiments in which no detectable XAb production occurred. Under these conditions there was also long-term graft survival, however, without the expression of protective genes or resistance to the exogenous anti-graft antibodies and complement. Further understanding the mechanism leading to xenograft accommodation may allow genetic engineering of the xenograft ECs to achieve long-term xenograft acceptance without deleterious interventions that may otherwise be needed.

REFERENCES

1. Bach FH, Winkler H, Ferran C, Hancock WW, Robson SC. Delayed xenograft rejection. Immunol Today 1996; 17: 379.
2. Platt JL. The immunological barriers to xenotransplantation. Crit Rev Immunol 1996; 16: 331.
3. Lin SS, Weidner BC, Byrne GW, et al. The role of antibodies in acute vascular rejection of pig-to-baboon cardiac transplants. J Clin Invest 1998; 101: 1745.
4. Blakely ML, Van der Werf WJ, Berndt MC, Dalmasso AP, Bach FH, Hancock WW. Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection. Transplantation 1994; 58: 1059.
5. Platt JL, Lindman BJ, Geller RL, et al. The role of natural antibodies in the activation of xenogenic endothelial cells. Transplantation 1991; 52: 1037.
6. Robson SC, Candinas D, Hancock WW, Wrighton C, Winkler H, Bach FH. Role of endothelial cells in transplantation. Int Arch Allergy Immunol 1995; 106: 305.
7. Shimizu A, Meehan SM, Kozlowski T, et al. Acute humoral xenograft rejection: destruction of the microvascular capillary endothelium in pig-to-nonhuman primate renal grafts. Lab Invest 2000; 80: 815.
8. Bach FH, Robson SC, Ferran C, et al. Endothelial cell activation and thromboregulation during xenograft rejection. Immunol Rev 1994; 141: 5.
9. Chong AS, Shen J, Xiao F, et al. Delayed xenograft rejection in the concordant hamster heart into Lewis rat model. Transplantation 1996; 62: 90.
10. Lin Y, Soares MP, Sato K, et al. Long-term survival of hamster hearts in presensitized rats. J Immunol 2000; 164: 4883.
11. Sato K, Takigami K, Miyatake T, et al. Suppression of delayed xenograft rejection by specific depletion of elicited antibodies of the IgM isotype. Transplantation 1999; 68: 844.
12. van den Bogaerde J, Aspinall R, Wang MW, et al. Induction of long-term survival of hamster heart xenografts in rats. Transplantation 1991; 52: 15.
13. Bach FH, Ferran C, Hechenleitner P, et al. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; 3: 196.
14. Bach FH, Hancock WW, Ferran C. Protective genes expressed in endothelial cells: a regulatory response to injury. Immunol Today 1997; 18: 483.
15. Platt JL. A perspective on xenograft rejection and accommodation. Immunol Rev 1994; 141: 127.
16. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB J Clin Invest 1999; 103: 543.
17. Ferran C, Stroka DM, Badrichani AZ, et al. A20 inhibits NF-kappaB activation in endothelial cells without sensitizing to tumor necrosis factor-mediated apoptosis. Blood 1998; 91: 2249.
18. Choi AM, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996; 15: 9.
19. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 2000; 7: 1015.
20. Soares MP, Lin Y, Anrather J, et al. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998; 4: 1073.
21. Hasan RI, Sriwatanawangosa V, Wallwork J, White DJ. Graft adaptation in hamster-to-rat cardiac xenografts. Transplant Proc 1994; 26: 1282.
22. Lin Y, Soares MP, Sato K, et al. Accommodated xenografts survive in the presence of anti-donor antibodies and complement that precipitate rejection of naive xenografts. J Immunol 1999; 163: 2850.
23. Dalmasso AP, He T, Benson BA. Human IgM xenoreactive natural antibodies can induce resistance of porcine endothelial cells to complement-mediated injury. Xenotransplantation 1996; 3: 54.
24. Dorling A, Stocker C, Tsao T, Haskard DO, Lechler RI. In vitro accommodation of immortalized porcine endothelial cells: resistance to complement mediated lysis and down-regulation of VCAM expression induced by low concentrations of polyclonal human IgG anti-pig antibodies. Transplantation 1996; 62: 1127.
25. Dalmasso AP, Benson BA, Johnson JS, Lancto C, Abrahamsen MS. Resistance against the membrane attack complex of complement induced in porcine endothelial cells with a Gal alpha(1–3)Gal binding lectin: upregulation of CD59 expression. J Immunol 2000; 164: 3764.
26. Carobbi A, Araneda D, Thomas F, Quarantillo P, Thomas J. Splenectomy is a potent immunosuppressive modality for cardiac xenografting. Transplant Proc 1993; 25: 419.
27. Langer A, Valdivia LA, Murase N, et al. Humoral and cellular immunopathology of hepatic and cardiac hamster-into-rat xenograft rejection: marked stimulation of $IgM^{++bright}/IgD^{+dull}$ splenic B cells. Am J Pathol 1993; 143: 85.
28. Shen J, Short J, Blinder Let al. Quantitation of the changes in splenic architecture during the rejection of cardiac allografts or xenografts. Transplantation 1997; 64: 448.
29. Lin Y, Vandeputte M, Waer M. Suppression of T-independent IgM xenoantibody formation by leflunomide during xenografting of hamster hearts in rats. Transplantation 1998; 65: 332.
30. Kopp CW, Grey ST, Siegel JB, et al. Expression of human thrombomodulin cofactor activity in porcine endothelial cells. Transplantation 1998; 66: 244.
31. Buhler L, Awwad M, Treter S, et al. Induction of mixed hematopoietic chimerism in the pig-to-baboon model. Transplant Proc 2000; 32: 1101.
32. Kozlowski T, Monroy R, Xu Y, et al. Anti-Gal(alpha)1–3Gal antibody response to porcine bone marrow in unmodified baboons and baboons conditioned for tolerance induction. Transplantation 1998; 66: 176.
33. Soares MP, Muniappan A, Kaczmarek E, et al. Adenovirus-mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. J Immunol 1998; 161: 4572.
34. Stuhlmeier KM, Lin Y. Camouflaging endothelial cells: dose it prolong graft survival? Biochim Biophys Acta 1999; 1428: 177.
35. Zarcone D, Tilden AB, Cloud G, Friedman HM, Landay A, Grossi CE. Flow cytometry evaluation of cell-mediated cytotoxicity. J Immunol Methods 1986; 94: 247.
36. Chong AS, Ma LL, Shen J, Blinder L, Yin DP, Williams JW. Modification of humoral responses by the combination of leflunomide and cyclosporine in Lewis rats transplanted with hamster hearts. Transplantation 1997; 64: 1650.
37. Miyatake T, Sato K, Takigami K, et al. Complement-fixing elicited antibodies are a major component in the pathogenesis of xenograft rejection. J Immunol 1998; 160: 4114.

38. Witmer MD, Steinman RM. The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light-microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patch. Am J Anat 1984; 170: 465.
39. Fu YX, Chaplin DD. Development and maturation of secondary lymphoid tissues. Annu Rev Immunol 1999; 17: 399.
40. Oliver AM, Martin F, Kearney JF. IgMhighCD21 high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J Immunol 1999; 162: 7198.
41. Kumararatne DS, MacLennan IC. The origin of marginal-zone cells. Adv Exp Med Biol 1982; 149: 83.
42. Carobbi A, Araneda D, Patselas T, Thomas J, Mosca F, Thomas F. Effect of splenectomy in combination with FK 506 and 15-deoxyspergualin on cardiac xenograft survival. Transplant Proc 1991; 23: 549.
43. Haga M, Tsuchida M, Hirahara H, et al. Synergistic effect of anti-T cell receptor monoclonal antibody and 15-deoxyspergualin on cardiac xenograft survival in a mouse-to-rat model. Transplantation 2000; 69: 2613.
44. Araneda D, Carobbi A, Thomas J, Thomas F. Paradoxical effects of xenografting in the nude rat: effect of splenectomy on aberrant graft rejection. Transplant Proc 1992; 24: 495.
45. Araneda D, Carobbi A, Patselas T, Thomas F, Thomas J. Timing of splenectomy is critical in prolongation of cardiac xenografts. Transplant Proc 1992; 24: 523.
46. Yin D, Ma LL, Blinder L, et al. Induction of species-specific host accommodation in the hamster-to-rat xenotransplantation model. J Immunol 1998; 161: 2044.
47. Hasan R, Van den Bogaerde J, Forty J, Wright L, Wallwork J, White DJ. Xenograft adaptation is dependent on the presence of antispecies antibody, not prolonged residence in the recipient. Transplant Proc 1992; 24: 531.
48. Ribas Y, Mollevi DG, Mestre M, et al. Lack of accommodation after long-term survival of hamster xenografts in rats. Transplant Proc 1999; 31: 2633.
49. Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997; 37: 517.
50. Yang L, Quan S, Abraham NG. Retrovirus-mediated HO gene transfer into endothelial cells protects against oxidant-induced injury. Am J Physiol 1999; 277: L127.
51. Abraham NG, Lavrovsky Y, Schwartzman ML, et al. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. Proc Natl Acad Sci USA 1995; 92: 6798.
52. Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 1997; 94: 10925.
53. Otterbein LE, Kolls JK, Mantell LL, Cook JL, Alam J, Choi AM. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. J Clin Invest 1999; 103: 1047.
54. Amersi F, Buelow R, Kato H, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury [see comments]. J Clin Invest 1999; 104: 1631.
55. Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nat Med 1998; 4: 1392.
56. Lin Y, Kirby JA, Browell DA, et al. Renal allograft rejection: expression and function of VCAM-1 on tubular epithelial cells. Clin Exp Immunol 1993; 92: 145.
57. Dorling A, Delikouras A, Nohadani M, Polak J, Lechler RI. In vitro accommodation of porcine endothelial cells by low dose human anti-pig antibody: reduced binding of human lymphocytes by accommodated cells associated with increased nitric oxide production. Xenotransplantation 1998; 5: 84.
58. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109.
59. Radomski MW, Palmer RM, Moncada S. Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. Trends Pharmacol Sci 1991; 12: 87.
60. Amezcua JL, Palmer RM, de Souza BM, Moncada S. Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br J Pharmacol 1989; 97: 1119.
61. McCurry KR, Kooyman DL, Diamond LE, et al. Human complement regulatory proteins in transgenic animals regulate complement activation in xenoperfused organs. Transplant Proc 1995; 27: 317.
62. Enjyoji K, Sevigny J, Lin Y, et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation [see comments]. Nat Med 1999; 5: 1010.
63. Imai M, Takigami K, Guckelberger O, et al. Modulation of nucleotide triphosphate diphosphohydrolase-1 (NTPDase-1)cd39 in xenograft rejection. Mol Med 1999; 5: 743.

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ALLOGRAFT TOLERANCE INDUCED BY INTACT ACTIVE BONE CO-TRANSPLANTATION AND ANTI-CD40L MONOCLONAL ANTIBODY THERAPY[1]

DENGPING YIN, LIANLI MA, HUASONG ZENG, JIKUN SHEN, AND ANITA S. CHONG[2]

Background. **One of the most promising approaches to achieving allograft tolerance involves the transient inhibition of co-stimulatory signals in T cells. There is, however, increasing evidence that this approach alone cannot universally elicit allograft tolerance and that adjunct therapies capable of synergizing with co-stimulation blockade may be necessary.**

Methods. **We developed a novel tolerance strategy involving co-transplantation of intact allogeneic bone fragments containing active bone marrow (intact active bone [IAB]) with heart allograft and transient anti-CD40L monoclonal antibody therapy.**

Results. **Mice treated with IAB and anti-CD40L were tolerant to major histocompatibility complex and minor antigen-mismatched cardiac and skin allografts. Heart allografts had normal histology up to 270 days posttransplantation, and the production of graft-reactive antibodies was inhibited. Microchimerism, but no macrochimerism, of donor cells was detected in the peripheral blood or lymphoid organs of tolerant mice receiving IAB and anti-CD40L. Lymphocytes from tolerant mice retained normal proliferative responsiveness to donor cells in vitro but demonstrated a donor-**

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