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TACROLIMUS PRETREATMENT ATTENUATES PREEXISTING XENOSPECIFIC IMMUNITY AND ABROGATES HYPERACUTE REJECTION IN A PRESENSITIZED HAMSTER TO RAT LIVER TRANSPLANT MODEL[1]

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In the hamster to rat liver transplant model, we determined the efficacy of tacrolimus in attenuating natural xenospecific humoral immunity and in abrogating the hyperacute liver rejection that is produced by presensitizing the Lewis rat recipient. Hamster livers, transplanted orthotopically into naive rats (controls), were rejected with animal death after 6.4±0.5 (SD) days. The infusion on (day −6) of 1.5×10^7 hamster hepatocytes, or of 1.5×10^8 nonparenchymal cells (NPC), resulted in hyperacute rejection and death in ≤1.9 days. However, when the rats were pretreated with 1 mg/kg/day tacrolimus from days −6 to −1, survival of non-presensitized animals was prolonged to 25±20 days and that of recipients presensitized with hamster hepatocytes to 36±16 days or with NPC to 32±1.7 days. The tacrolimus pretreatment significantly reduced the hamster-specific complement-dependent cytotoxic antibodies response directed to liver NPC but not to lymph node cell targets. These observations suggest that the prolongation of survival by appropriately timed treatment with this T cell directed drug model is caused by the inhibition of humoral as well as cellular xenograft rejection.

The consequences of humoral immunity in xenotransplant models are dictated by the species combination (*1*, *2*), the kind of organ that is engrafted (*3*), and the direction of organ transfer between the species (*4*, *5*). With the appropriate selection of these variables, xenograft survival in an unaltered recipient may range from a few minutes (hyperacute rejection [HAR*]) to several days. In the longer survival models, species-specific xenosensitization of the recipient produces HAR (*6–10*). For example, the humoral rejection in 3 days of abdominally placed hamster hearts is converted in presensitized rats to HAR that is complete in <10 min (*6*, *8–10*). In contrast, when the antibody resistant hamster liver is transplanted under the same circumstances of presensitization, the reduction in survival is from 7 to <2.0 days (*11*).

Untreated nude (T-cell deficient) rats reject hamster heart grafts at the same 3 days as in normal rats (*12–14*), ostensibly in accord with a T-cell independent mechanism of humoral xenograft rejection. However, the presensitization procedure that caused HAR within 10 min in normal rats resulted in prolonged xenograft survival in all nude rat recipients, and, in 4 of 10 experiments, survival exceeded 100 days (*15*). This observation, coupled with the demonstration of a remarkably obtunded antibody response in the presensitized nude recipients, prompted the experiments in normal Lewis rats reported here in which the nude rat environment was mimicked with a 6-day pretransplant course of the T-cell directed immunosuppressant tacrolimus. Tacrolimus pretreatment alone did not reduce the titer of preformed xenospecific antibodies. However, it abrogated HAR of hamster hepatic xenografts by presensitized rats, and it prolonged the survival of the transplanted livers several times beyond that in untreated recipients, whether or not the presensitization protocol had been applied. The results suggest that, contrary to current consensus, an antibody subclass(es) important for humoral xenograft rejection is T-cell dependent, thereby exposing a potential seam in the xenotransplant barrier that may be susceptible to classic immunologic manipulation including tolerance induction.

MATERIALS AND METHODS

Animals and Procedures

Animals. Outbred male Syrian Golden hamsters (110–120 g) and male Lewis rats (LEW, RT1[1], 180–220 g) obtained from Charles Rivers Laboratories (Wilmington, MA) were used as donors and recipients, respectively. The animals were maintained in the pathogen-free facilities of the University of Pittsburgh Medical Center and provided with Purina rodent chow and tap water ad libitum.

Orthotopic liver transplantation. Hamster livers were transplanted orthotopically into LEW recipients and revascularized with a combination of suture and cuff techniques (*3*, *16*). All operative procedures were performed under methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether, Pitman-Moore, Mundelein, IL) anesthesia. The diagnosis of HAR was made when after reperfusion; the liver surface appeared mottled with residual red patches culminating in the early death of the recipient. Confirmatory histopathological examination was done in all experiments.

Isolation of Cells

Hepatocytes. Hepatocytes (HC) from hamster livers were isolated using the in situ collagenase (Type A, 0.5 mg/ml, Boehringer Mannheim, IN) perfusion technique (*17*). Briefly, livers were initially perfused with a calcium-free solution containing EGTA (Sigma

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* Abbreviations: Ab, antibody; HAR, hyperacute rejection; HC, hepatocyte; LEW, Lewis; LN, lymph node; NPC, nonparenchymal cells; TDD, thoracic duct drainage; TI, thymus independent.

Chemical Co., St. Louis, MO) followed by collagenase for 30 min. The HC were then purified by differential centrifugation (50×*g*) for 2 min using a 30% Percoll (Sigma) gradient. Hepatocyte purity as assessed by microscopic analysis was consistently more than 98% and the viability as determined by trypan blue dye exclusion exceeded 95%.

Nonparenchymal cells. Nonparenchymal cells (NPC) from hamster liver were isolated with the ex vivo protease and deoxyribonuclease method (*18*). After in situ perfusion with Gey's balanced salt solution (Life Technologies Inc., Grand Island, NY), the liver was excised and minced. The fragments were treated by constant stirring for 10 min at 37°C in 100 ml of a solution containing protease (2 mg/ml) and DNAase (0.5 mg/ml), to which fresh DNAase solution was added every 20 min. NPC were purified from dead cells and hepatocytes by centrifugal elutriation (Beckman Elutriator Model #J-6M, Beckman Instruments Inc., Palo Alto, CA). This procedure yielded NPC with a purity and viability of >95%. The leukocyte composition was principally mononuclear cells of myeloid lineage with a minority population of lymphocytes. The NPCs also included endothelial cells.

Experimental Design

The experimental design is summarized in Table 1. In experiments involving tacrolimus (a gift from Fujisawa Pharmaceutical Co., Osaka, Japan), the drug was administered at 1 mg/kg/day from days −6 to −1 followed by orthotopic liver transplantation on day 0 (Table 1). No postoperative treatment was given.

Intravenous donor species cell infusion was with 1.5×10^7 NPC or HC on day −6 except in group 4 (Table 1) where a 10-fold dose of NPC was administered (1.5×10^8).

Antibody Studies

Complement-dependent cytotoxicity. These studies were performed only in the animals of group 1 (control) and groups 2, 5, and 6. The titers of serum anti-hamster antibodies (Ab) on the day of liver transplantation (day 0) were determined with a 2-step CDC assay in which hamster lymph node (LN) and liver NPC (which included endothelial cells) were the targets. Duplicate samples of 5×10^3 cells in 1 μl of RPMI were placed into each well of a 72-well tissue-typing tray (Robbin Scientific, Sunnyvale, CA). Decomplemented recipient serum (1 μl per well) was then added, and the plates were incubated for 30 min at room temperature. At the end of this incubation, 2 μl of baby rabbit complement (1:10, Cedarlane Labs, Hornby, Ontario, Canada) were added to each well and incubated for an additional 30 min at 37°C in 5% CO_2 in air. Cell viability was determined by adding 5 μl of 0.4% trypan blue solution to each well. The Ab titer was defined as the highest dilution of serum in which there was lysis of 30% of the cells.

Characterization of serum immunoglobulins. Using a sandwich ELISA, the concentrations of total rat IgG and IgM were determined on day 0 in the sera of animals in each experimental group. Goat anti-rat IgM or IgG Ab (Accurate Chemical and Scientific Co., Westbury, NY), 100 μl (10 μg/ml in Na_2CO_3 buffer, pH 9.6), were added to each well of a 96-well microtiter plate, incubated overnight at 4°C and washed 3 times with PBS containing 0.05% Tween-20 (Sigma). Nonspecific binding was blocked by adding 200 ml of 5% goat serum (in PBS) for 30 min. After washing, 100 μl of the sample being assessed was placed in each well and incubated for 2 hr at room temperature. Each well was washed 4 times and incubated for an additional 3 hr at room temperature with 100 μl of affinity-isolated peroxidase-conjugated goat anti-rat IgM or IgG Ab ($1:10^4$ dilution, Accurate).

The reaction was developed using 200 μl/well of *O*-phenylenediamine dihydrochloride (0.4 mg/ml SIGMAFAST OPD Tablet, Sigma) solution as a substrate followed, by measurement of optical density at 490 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The results obtained were compared with those of known rat IgM or IgG standards that were run simultaneously with the test samples.

Assay for hamster NPC-specific antibodies. NPC (2×10^6) isolated from hamster liver were incubated for 60 min with 500 μl of serum obtained from animals in each group. At the end of this incubation, the cells were lysed by suspending them in 3M KCl solution for 3 min. The presence of hamster NPC-specific IgM and IgG Ab was determined by sandwich ELISA assay (see *Characterization of serum immunoglobulin* section).

Immunocytochemistry. A direct immunofluorescence technique was used to visualize the deposition of rat immunoglobulins (IgM or IgG) in hamster liver xenograft samples obtained 10, 30, 60, 180, and 360 min after their revascularization. Cryosections were incubated for 60 min with FITC-conjugated goat anti-rat IgM or IgG Ab. After washing, the slides were viewed on a Nikon epifluorescent microscope (Nikon Instrument Group, Melville, NJ) equipped with a DAPI (FITC) Texas-Red triple band pass filter (Fryer Co., Huntley, IL).

Anti-hamster Ab in rat recipients also was determined by incubating their sera (1:20) with cryosections obtained from normal hamster livers. Their binding was then visualized using FITC-conjugated goat anti-rat IgG or IgM Ab. The degree of deposition was evaluated as fluorescence intensity from weak (−) to strong (+++).

TABLE 1. Hamster to rat liver xenograft survival

Group	Treatment	n	Survival (days) (X±SD)	*P*
Without Immunosuppression:				
1	Untreated	10	6.4±0.5	
				<0.05
2	HC[a]	10	1.9±1.4	
3	NPC[a]	10	5.3±0.7	
				<0.05
4	NPC[b]	4	1.7±1.2	
With Immunosuppression[c]:				
5	Tacrolimus	11	25±20	
				<0.002
6	HC[1] + Tacrolimus	11	36±16	
7	NPC[1] + Tacrolimus	10	32±1.7	

[a] 1.5×10^7 of hepatocytes (HC) or nonparenchymal cells (NPC) were infused iv on day −6.
[b] 1.5×10^8 NPC infused iv on day −6.
[c] 1 mg/kg/day of tacrolimus im from −6 to −1.

RESULTS

Xenograft Survival

Without immunosuppression. Hamster livers, when transplanted into naive rat recipients, were rejected in 6.4±0.5 (SD) days (group 1, Table 1). Recipients pretreated on day −6 with 1.5×10^7 HC third party hamster HC had HAR of the subsequently transplanted hamster livers with death after 1.9±1.4 days (group 2, Table 1, Fig. 1A). The HAR was obvious immediately after revascularization. Although the same dose of NPC did not cause HAR (group 3), a 10-fold increase of NPC also caused HAR in 1.7±1.2 days (group 4, Table 1).

With immunosuppression. The preoperative course of tacrolimus resulted in significantly prolonged survival (25±20d) of the otherwise unaltered xenograft recipients (group 5, Table 1). Of greater interest, tacrolimus prevented HAR in animals presensitized with HC (Fig. 1B). The 36±16 day survival of the HC-augmented animals exceeded by 11 days that of recipients pretreated with tacrolimus only (P=0.1 NS).

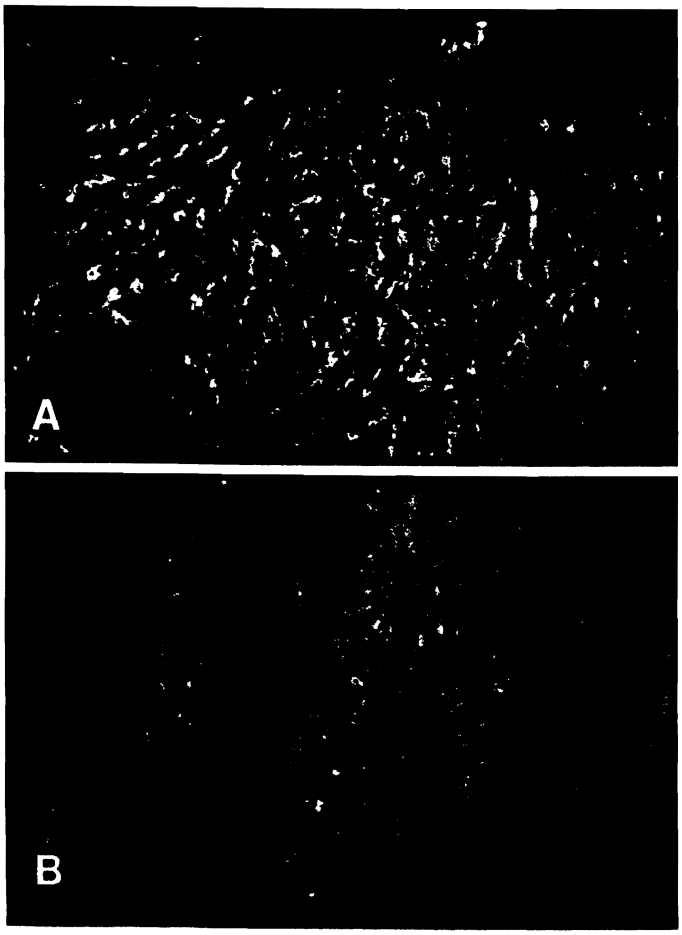

FIGURE 1. Rat IgM on cryosections of hamster liver xenograft 10 min after reperfusion in rat recipients. (A) Marked deposition of IgM on cells lining the sinusoids of recipients presensitization with hamster HC. (B) Minimal IgM deposition after HC pretreatment but with a 6-day preoperative course of tacrolimus. No staining was observed when sections of livers obtained from normal hamsters were stained with FITC-conjugated goat anti-rat IgM antibodies (negative controls) (×200).

Antibody Studies

Cytotoxic antibodies. Anti-hamster Ab titers (2-step complement-dependent cytotoxicity assay) are shown in Figure 2. The liver NPC targets were less susceptible to lysis than the LN cell targets when tested against preoperative sera obtained from either untreated (Fig. 2A) or tacrolimus-treated recipients (Fig. 2D). The NPC susceptibility was increased almost to that of the LN cells in animals presensitized with HC (Fig. 2B), but this was reduced to control levels in rats also pretreated with tacrolimus (Fig. 2C). The anti-NPC and anti-LN cell cytotoxic activity shown in all 4 panels was mediated by IgM Ab, because it was largely eliminated by dithiothreitol (DDT; Eastman Fine Co., Rochester, NY) (data not shown).

Total and NPC-specific IgM and IgG. Pretreatment with neither tacrolimus nor HC affected the concentration of total IgM or IgG concentration in the rat preoperative sera in the different groups (Fig. 3).

The specificity of IgM binding in the hamster livers 10 min after reperfusion is shown in Figure 4. This was significantly increased in the rats infused with HC. Paradoxically, binding was increased slightly when the recipient animals had been pretreated with tacrolimus alone or in conjunction with HC (Fig. 4A). NPC-specific IgG Ab followed the same pattern, but the changes compared with control were not significant (Fig. 4B).

Immunocytochemistry. Livers transplanted into untreated animals had moderate deposition of IgM Ab (Table 2) and IgG Ab (data not shown) in serially biopsied samples, 10–360 min postreperfusion. The single color immunofluorescence staining was primarily observed along the sinusoids with much more IgM than IgG. Tacrolimus pretreatment did not reduce the intensity of staining (Table 2), suggesting that the drug had no effect on the production of natural Ab.

In contrast, the findings were dramatically changed by pretreatment with HC in the absence of tacrolimus. These xenografts had markedly increased deposition of IgM (but

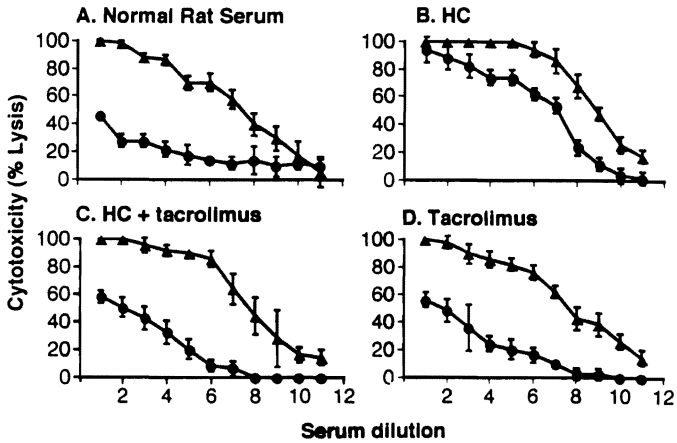

FIGURE 2. CDC assay of rat recipient serum on day 0 (pre-liver transplant) using hamster LN cells (▲) or liver NPC (●) as targets. (A) Naive rat sera. (B) Sera obtained from recipients presensitized with donor species HC. (C) Sera from rats presensitized with HC but given 6 day pre-operative course of tacrolimus. (D) Six day pretreatment with tacrolimus only. Note that selectively increased cytotoxicity to NPC caused by HC infusion (B) was prevented by course of tacrolimus (C).

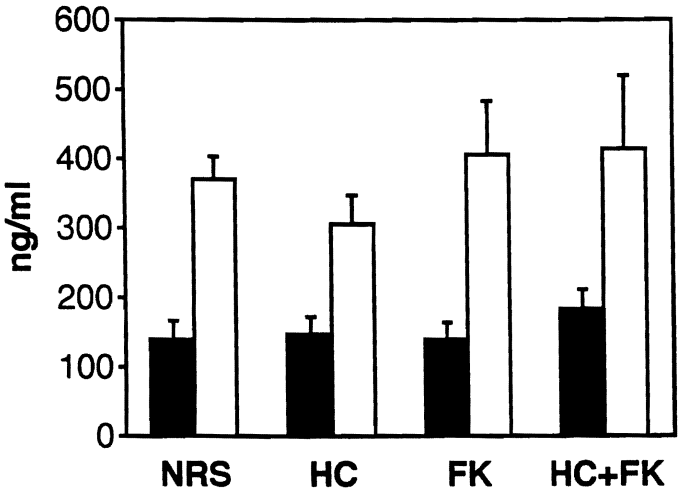

FIGURE 3. Total rat IgM (closed bars) and IgG antibodies (open bars) on day 0 (pre-liver transplant) in the sera of naive rats (NRS) and in animals pretreated with HC (group 2). The quantity of these antibodies remained unaltered in the sera of all these animals. Hepatocytes plus tacrolimus (HC + FK, group 6), or tacrolimus (FK) only (group 5).

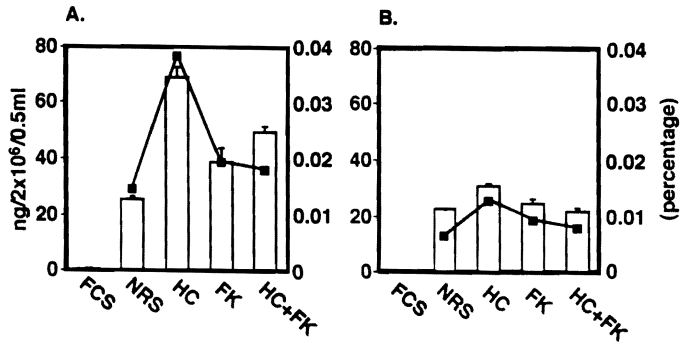

FIGURE 4. The concentration of NPC-specific rat anti-hamster (A) IgM and (B) IgG antibodies, determined in the same experimental groups shown in Figure 3. The bar graph depicts the quantity of NPC-specific immunoglobulins, and the line indicates their percentage of the same sera. Note the differing effect on NPC-specific IgM by different pretreatment regimens without comparable modulation of NPC-specific IgG.

TABLE 2. Rat anti-hamster IgM antibody deposition by direct immunofluorescence staining and hamster to rat xenotransplanted livers to rat xenotransplanted Livers[a]

Time Post-perfusion (min)	Untreated (group 1)	Tacrolimus (group 5)	HC (group 2)	HC + Tacrolimus (group 6)
10	±	±	+++	±
30	+	+	+++	±
60	+	+	++	+
180	+	+	++	±
360	+	+	++	±

[a] Scored as weak (−) to strong (+++) deposition. The treatment regimens were as in Table 1.

not IgG) at all times sampled (group 2 in Table 2 and Fig. 1). Remarkably, postperfusion IgM deposition in the livers of animals pretreated with the combination of HC and tacrolimus was less than in either untreated controls or in animals treated with tacrolimus only (group 6 in Table 2 and Fig. 1).

The titer and isotype of rat anti-hamster Ab obtained preoperatively from the recipients in different groups was also determined by staining cryosections of normal rat livers with these recipient sera (data not shown). All serum samples tested contained more IgM than IgG Ab. However, the titer of these pretransplant Ab was greatest in recipients pretreated with HC only; intermediate and similar in naive animals and those pretreated only with tacrolimus; and least when both HC and tacrolimus were used for treatment.

DISCUSSION

Although a different organ was used for the present liver transplant experiments, the observations support the conclusion from our earlier nude rat studies with heart transplantation (*15*) that humoral rejection is T-cell modulated in the hamster to rat models. This was easier to demonstrate with the liver model because the unusual resistance of hepatic allografts (*19*, *20*) and xenografts (*21*) to antibody injury expands the observational time frame.

The 6-day pretransplant course of tacrolimus in normal rats had no demonstrable effect on the preexisting rat anti-hamster IgM Ab. However, this course of T-cell directed treatment prevented the rise in xenospecific antibody titer otherwise caused by the sensitization protocol or by transplantation. Avoidance of the antibody storm correlated with avoidance of hyperacute rejection, and with a paucity of Ig deposition in the graft. The duration of survival in the absence of any posttransplant therapy far exceeded that in naive recipients and was at least as good (especially in animals with the presensitization protocol) as can be achieved by giving continuous postoperative tacrolimus in the same daily dose range (*22*).

It is a general rule in adaptive immunity that the activation by protein antigen of most naive B cells is thymus-dependent and requires accessory signals that come from an armed helper T cell (*23*). However, many bacterial lipopolysaccharides, polysaccharides, and polymeric proteins are classified as thymus-independent (TI) antigens because in the absence of T cells they can directly induce B cells to produce antigen specific antibody, or in higher concentration induce polyclonal activation (*24–28*). According to the consensus literature, there are 2 classes of TI antigens: TI_1, which can directly activate immature and mature B cells; and TI_2 (limited to polysaccharides), which can activate mature B cells only.

However, there is a possibility that the thymus independence of these TI B cells is not absolute. Although nude mice respond to TI_2 antigens, depletion of all T cells in culture eliminates these responses, which can be restored by adding back very small numbers of T cells (*30*). Responses to several TI_2 polysaccharide antigens have been shown to be dominated by a subpopulation of B cells that have CD5 and $IgM^{++bright}/IgD^{dim}$ surface expression. These cells arise from an immature stem cell early in ontogeny, secrete IgM >>> IgG, and may, under some conditions, respond to protein as well as polysaccharide antigen (*29*). Their responses do not induce significant Ig class switching or somatic hypermutation of immunoglobulin variable (V) regions. As a consequence, their predominant antibody isotype is IgM, which is augmented by IL-5 and to an unknown extent by T cells.

Much of the immunoglobulin found in normal serum is produced by these cells whose function is not yet fully known (*31*).

It has been suspected for several years that the CD5 cells may be important for, or critical to, the xenogenic response. We have reported a striking and specific activation of $IgM^{++bright}/IgD^{dim}$ cells in the spleen after hamster to rat heart and liver transplantation (*32*). Congruent with these findings, Wu et al. (*33*) and Borie et al. (*34*) have identified 6 monoclonal IgM secretory products after hamster to rat heart transplantation, of which 4 with essentially identical sequence caused hyperacute rejection with passive transfer. These antibodies were strongly reactive with the sialyl-Lew^a epitope of a glycoprotein and less so with several other oligosaccharides. These observations and the similarity of the hamster epitope to the α-galactosyl carbohydrate target of pig to human xenotransplantation (*35–37*) suggest that there is a family of polysaccharide epitopes (with variations) that constitutes the prohibitive barrier to interspecies transplantation.

It was shown in the investigations of Wu et al. (*33*) and Borie et al. (*34*) that the V gene segments that encode CD5 B-cell receptors in the rat are evolutionarily selected to recognize common bacterial antigens and contribute to early phases of (innate) immunity. It goes without saying that the drastic modification of this "innate" response with the T cell deficient state (*15*) or by a T-cell directed immunosuppressant in the present study is surprising and potentially relevant clinically. It is noteworthy that Sablinski et al. (*10*) observed the same effect of abrogated HAR in the sensitized hamster to rat heart model using an anti-CD4 mAb. The ability of an immunosuppressant to prevent donor species presensitization and coincidentally induce short-term species specific tolerance was first described with deoxyspergualin (*9*) and ascribed at the time to a direct B cell inhibition. However, it is now recognized that the manifold actions of DSG include potent T-cell suppression (*38, 39*).

The evidence that the xenospecific humoral response can be T-cell modulated may be the soft spot in the xenograft barrier for which experimentalists have been probing. It is important, therefore, to determine if these findings are relevant to other species combinations including those in which humans are recipients. Most preclinical immunosuppressive regimens or other strategies developed for xenotransplantation have been based on the assumption that control of T- and B-cell activation requires drugs or interventions (such as splenectomy) that are specific to each lymphocyte population (*22*). The use of B-cell selective drugs will undoubtedly continue to play a role in xenotransplant cocktails designed for potential clinical use. However, treatment protocols exploiting pretransplant and continued postoperative T-cell modulation of humoral immunity may have an unexpectedly important role.

Although established only in the hamster to rat combination, this strategy may be applicable to other species combinations and to specific allotransplant syndromes of HAR (i.e., ABO incompatibility and presensitization), which we believe to have the same pathogenesis in principle as the HAR of xenografts (*5*). Of interest, T-cell deletion with thoracic duct drainage (TDD), in patients with preformed antibody states was attempted 15–20 years ago with moderate success in avoidance of HAR (*40, 41*), based on the demonstration by MacGregor and Gowans (*42*) that TDD in rats sharply reduced humoral as well as cellular immune responsiveness within 5 days. In a classic human study of nontransplant patients, Machleder and Paulus (*43*) established that the required duration of TDD to virtually eliminate ABO isoagglutinins (which resemble anti-α-galactosyl antibodies [*37*]) was 30 days. Although TDD is an obsolete method of lymphoid depletion, the lessons from these trials should not be forgotten.

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