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24 Use of Tacrolimus (FK506) and Antimetabolites as Immunosuppressants for Xenotransplantation Across Closely Related Rodent Species

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Introduction

In the present chapter, we will review our studies of the potential role of tacrolimus in xenotransplantation based largely on experiments in which rats were recipients of vascularized organs from either hamsters or mice. Because these xenografts after transplantation elicit a strong humoral response and are rejected within a few days, these models were considered until the last few years to be moderately difficult [1–10], more so than the sheep-to-goat combination of Perper and Najarian [11] or the wolf-to-dog model of Hammer et al. [12].

Hamster-to-Rat Model

This model was added to the transplant research armamentarium by Clyde Barker and Rupert Billingham at the University of Pennsylvania [1]. However, 16 years passed before significant progress was made in breaking down this xenogeneic barrier. Then, Knechtle et al. [13] reported extended survival of hamster hearts in rat recipients treated with total lymphoid irradiation followed by cyclosporine. However, a precise explanation for this limited success was not evident. The advent of tacrolimus, a more potent T cell-directed immunosuppressant [14–21] has permitted better insight into the mechanisms of xenograft rejection in this model, especially when it has been combined with other agents that disrupt the xenogeneic immune reaction at a different site. The use of such drug combinations has consistently led to indefinite survival in the hamster-to-rat and other closely related rodent organ xenotransplant models as will be described in the following pages. We will also discuss how these experiments have cast light on the mechanisms of xenograft rejection.

Humoral Responses

Using complement-dependent cytotoxicity assays, low titers (1:16–1:32) of preformed anti-hamster cytotoxic antibodies have been demonstrated in the sera of naive Lewis rats [22]. Isotyping studies using flow cytometric analysis invariably reveal a greater preponderance of IgM rather than IgG antibodies. Rejection of liver xenografts is accompanied by astronomical elevations of antihamster cytotoxic antibodies and massive enlargement of the spleen in untreated recipi-

ents [23]. Within the spleen, this is associated with marked stimulation of $IgM^{++\ bright}/IgD^{+\ dull}$ B cells, which in the rat are preferentially localized in the marginal zone and red pulp. Given its marked diminution following tacrolimus therapy, this response has appeared to be at least partially T cell dependent [22]. Nevertheless, the localization and phenotypic profile of the responding recipient splenocytes also have suggested the involvement of a subpopulation of B cells which are mediators of a T-independent immune response [22, 24]. Since the host's primitive defense against blood-borne polysaccharide antigens is mediated by B cells of similar phenotype [25], it is therefore tempting to speculate that splenic IgM^{++}/IgD^{+} B cells in the rat studied by Langer et al. [22] are analogues of human $CD5^{+}$and mouse Ly 1^{+} B cell subsets [26]. These cells have also been implicated to play a seminal role in the generation of destructive xenoreactive antibodies in other models [27], although recent experiments by Pitre et al. [28] contradict this assumption.

Fate of Different Organs

Heart

Hamster hearts are rejected by antibody-mediated mechanisms within 3–4 days [1–10]. There is evidence for progressive platelet and fibrin deposition in the microvasculature and margination of neutrophils in larger vessels, with accompanying endothelial cell hypertrophy and focal denudation. There is also evidence for increased deposition of IgM, IgG, and C_3 in both the endothelium of the larger vessels and in that of the microvasculature [22]. These events lead to widespread hemorrhagic necrosis precipitating xenograft loss. The use of tacrolimus (1–2 mg/kg per day until rejection) alone does not significantly prolong graft survival nor does it alter the histopathological or immunofluorescence observations detailed above [5, 7, 22]. It also fails to attenuate the gradually increasing xenospecific cytotoxic antibody titers in the recipients lending further credence to earlier contentions that the generation of xenoantibodies by a T cell-independent mechanism, along with complement, serve as main effectors in this model [29]. Further corroboration of this assertion is obtained when an increase in the titers of anti-hamster antibodies is noted in T cell-deficient nude rats receiving hamster heart xenografts [30–32]. In addition, Van den Bogaerde et al. [29] have shown that inhibition of recipient complement by cobra venom factor results in prolonged survival of hamster heart xenografts only when combined with a T-cell immunosuppressant like cyclosporine, pointing out the duality of the humoral and cellular mechanisms of xenograft rejection.

In an attempt to abate cardiac xenograft rejection, thereby prolonging survival, Murase et al. [7] have also tested tacrolimus in combination with antimetabolites such as cyclophosphamide, methotrexate, brequinar sodium (BQR) and RS-61443. Of the antimetabolites used, cyclophosphamide, a purine antimetabolite with pronounced B cell specificity [33], and BQR, which inhibits de novo pyrimidine [34], consistently allow for protracted heart xenograft survival (Table 1). More prolonged graft survival is witnessed when cyclophosphamide plus tacroli-

mus immunosuppression therapy is used. Similarly, when RS-61443, a purine synthesis inhibitor [35], is used in combination with tacrolimus a dramatic prolongation in xenograft survival is observed [7]. When used alone, mizorbine, an inhibitor of purine synthesis [36], and deoxyspergualin (DSG), an analogue of the antitumor antibiotic spergualin [37], have no discernible effect on cardiac xenograft survival, which is, however, prolonged with the addition of tacrolimus to the immunosuppressive regimen (Table 1). Methotrexate when used alone leads to modest prolongation of survival; however, when used in combination with tacrolimus results in indefinite graft survival. Interestingly, the use of tacrolimus alone in splenectomized recipients also results in indefinite survival of subsequently transplanted cardiac xenografts [38].

Table 1. Survival of hamster heart, liver, and kidney xenografts in Lewis rats immunosuppressed with tacrolimus and antiproliferative agents

Organ	Treatment	Dose (mg/kg per day)	Median survival (days)
Heart [7]	None	-	3.0
	FK506	2.0	4.0
	BQR	4.5	>42.0
	RS-61443	40.0	7.0
	Cyclophosphamide	15.0	56.0
	Methotrexate	0.5	13.0
	Mizorbine	7.5	4.0
	DSG	5.0	4.0
	FK506 + BQR[a]	1.0 + 4.0	>100.0
	FK506 + RS-61443[a]	2.0 + 20.0	>100.0
	FK506 + cyclophosphamide[a]	2.0 + 7.5	>100.0
	FK506+methotrexate[a]	2.0 + 1.0	>100.0
	FK506 + mizorbine[a]	2.0 + 7.5	>52.5
	FK506 + DSG[a]	2.0 + 5.0	>100.0
	FK506 + cyclophosphamide[b]	2.0 + 80.0	>100.0
	Splenectomy [38]	-	5.0
	FK506 + splenectomy [38]	2.0	>100.0
Liver [7]	None	-	7.0
	BQR	3.0	12.0
	RS-61443	20.0	7.0
	Cyclophosphamide[a]	7.5	9.0
	FK506 + BQR[a]	1.0 + 3.0	>100.0
	FK506 + RS-61443[a]	1.0 + 20.0	>100.0
	FK506 + cyclophosphamide[a]	1.0 + 7.5	>100.0
	FK506 + cyclophosphamide[b]	1.0 + 80.0	>100.0
Kidney [67]	None	-	6.0
	FK506	2.0	6.0
	Cyclophosphamide	7.5	8.0
	FK506 + cyclophosphamide[a]	2.0 + 10.0	11.5
	FK506 + cyclophosphamide[a]	1.0 + 15.0	79.0

BQR, brequinar sodium; DSG, deoxyspergualin.
[a]Antimetabolites were given in either 14- or 30-day course.
[b]Cyclophosphamide was given as a single injection 10 days before organ transplantation.

Consistent with the findings of Kumararatne et al. [39], we also observed that a single dose of cyclophosphamide (500 mg/m^2, i.p.) results after 10 days in the depletion primarily of the IgM^{++}/IgD$^+$ cells normally residing in the marginal zone of the spleen [22]. To elucidate the role played by these B cells in xenograft rejection, hamster hearts were transplanted into rats with or without tacrolimus therapy 10 days after a single bolus injection of cyclophosphamide. Although slight prolongation of xenograft survival is observed in animals pretreated with cyclophosphamide alone, the addition of tacrolimus nevertheless results in indefinite graft survival (Table 1). These observations suggest that splenic B cells of this distinct phenotype may mediate an important effector function culminating in xenograft rejection in this model. However, there is a T cell-mediated mechanism of graft injury which neither cyclophosphamide nor splenectomy, when used alone (23, 38, 40, 41), are able to attenuate. Accordingly, the use of strategies which allow for inhibition of both B and T cell function provides an optimal means to control xenograft rejection in this model [6, 7].

Liver

Unlike heart xenografts, hamster livers are acutely rejected by untreated Lewis rat recipients in 7 days by a composite humoral and cell-mediated immunity [5, 7, 22, 23]. The early deposition of anti-hamster antibodies in the sinusoids and portal tracts is followed by cellular infiltration in these areas composed largely of recipient CD8$^+$ T and few natural killer cells. This histopathological finding differs from that witnessed during liver allograft rejection, where the initial cellular infiltrate is localized primarily in the portal or perivenular areas [42]. These findings provide support for a role of antibody-mediated cellular cytotoxicity in xenograft rejection [43–45]. When tacrolimus is used alone, it significantly prolongs xenograft survival with 10 %–30 % of the recipients surviving indefinitely [5, 7, 46]. Single drug therapy using either BQR, RS-61443 or cyclophosphamide has minimal impact on graft survival, whereas the addition of tacrolimus to BQR, RS-61443, or cyclophosphamide-treated recipients leads to their indefinite survival (Table 1). As with hearts, pretreatment of liver xenograft recipients with a single bolus injection of cyclophosphamide 10 days prior to organ transplantation also results in their indefinite survival (Table 1) [7, 22].

Despite its moderate efficacy in prolonging liver xenograft survival, tacrolimus when used alone does not mitigate the extreme elevation of xenospecific cytotoxic antibody titers in the recipient serum, which reach a peak around day 7 post-transplantation. However, by day 30 post-transplantation, these antibodies become undetectable in tacrolimus-treated recipients, the majority of whom nevertheless succumb to late graft failure subsequent to biliary obstruction [22]. We have hypothesized that this outcome represents a delayed manifestation of humoral injury which may have transpired early during the period following transplantation [47]. This assertion is further substantiated by the obvious lack of biliary complications and long-term survival of tacrolimus-treated recipients who had received a short perioperative course of BQR or RS-61443; both of which are known to partially abate the initial peak antibody response [7].

Although tacrolimus does not have an effect on xenoantibody production when therapy starts on the day of transplantation, Tsugita et al. [48] have shown that this immunosuppressant not only abrogates the hyperacute liver xenograft rejection induced by pretransplant infusion of hamster hepatocytes or liver nonparenchymal cells (NPC, which include liver sinusoidal cells), but also prolongs graft survival beyond that achieved by pretransplant treatment with tacrolimus alone. The interesting observation is that, although the cytotoxicity (IgM) of the recipient's serum against hamster lymphocytes remains high despite tacrolimus therapy, that against hamster-specific NPC is blunted. Similarly, we have shown recently that T cell-deficient nude rats that receive hamster whole blood 1 week prior to transplantation with a hamster heart never reject the graft in a hyperacute fashion. Indeed, the heart xenografts survive indefinitely in most of the cases [49]. These observations indicate the importance of T cell help in the induction of xenosensitization and hyperacute rejection in this xenograft model.

Our achievement of long-term survival in liver xenograft recipients has afforded us the opportunity to study several physiological and immunological features uniquely associated with this organ. We have specifically investigated the metabolic and coagulatory changes [50, 51] that occur in the liver xenograft recipients and the cellular events that lead to the induction of donor-specific tolerance [46]. Rats who accept hamster liver xenografts exhibit species-specific unresponsiveness to subsequently transplanted donor organs. It must be emphasized that while they retain donor-specific tolerance, the liver recipients are fully capable of rejecting organs from other animal species [46].

This species-specific immunological unresponsiveness, afforded best by the liver but also witnessed after heart xenotransplantation as reported by Tanaka et al. [52], may be ascribed to the establishment of chimerism, which has been shown to play a seminal role in the induction of donor-specific tolerance [53–56]. Additionally, it is also well known that given their species-specific restriction, certain membrane-associated complement (C) regulatory proteins, such as decay-accelerating factor (DAF), membrane cofactor protein (MCP), and CD59 antigen, among others, play an important role in protecting autologous or homologous tissues from collateral damage that may be prompted by C activation [57–62]. Therefore, the production by the liver of species-specific circulating C proteins may provide yet another mechanism by which this organ may exert its observed protective effects.

Further credence to the latter hypothesis is provided by exhaustive experiments in rats in which species-specific tolerance is successfully achieved by prior hamster liver xenotransplantation [63, 64]. The intravenous administration of C-depleted (but not enriched) rat anti-hamster hyperimmune serum into these tolerant animals has no deleterious effect on simultaneously transplanted hamster hearts which enjoy prolonged survival. These observations underscore the pernicious nature of heterologous C which, following xenotransplantation of organs other than livers, may play a major role in mediating the antibody-dependent rejection [65]. In view of this it would be reasonable to predict that clinical transplantation of organs, other than livers, from transgenic pigs expressing human C regulatory proteins on their endothelium would prevent hyperacute rejection as has already been demonstrated by White et al. [66]. Of concern, however, is

the potential risk to the liver recipient of heterologous C secreted by the xenografted organ, whose uninhibited activity may precipitate an autoimmune-like syndrome. This is an area of research which warrants further investigation.

Kidney

Similar to our observations for hearts, Miyazawa et al. [67] have reported that hamster kidney xenografts are rejected by untreated rat recipients in 5–6 days predominantly by a humoral response. The use of tacrolimus alone does not prolong graft survival nor has any appreciable effect on the generation of rat anti-hamster IgM antibodies. Although the additional use of cyclophosphamide along with tacrolimus extends xenograft survival, the results are not as dramatic and consistent as those obtained with heart and liver xenografts (Table 1). Attempts to deplete heterospecific antibodies by pre-transplant perfusion of recipient's blood through donor-strain kidneys or livers, as well as the use of the anti-complementary agent K76 [68, 69], result in marginal prolongation of survival of subsequently transplanted kidney xenografts in tacrolimus/cyclophosphamide-treated rat recipients (Table 1). Interestingly, Ye et al. [70] have observed that the majority of kidney xenograft recipients develop severe polyuria which is not corrected by restriction of water intake. Since the minimal histopathological changes in the kidney xenografts cannot account for the de novo development of diabetes insipidus witnessed in this model, it is speculated that there might be a functional incompatibility between transporter proteins expressed on cells in hamster kidney with that of their ligands in the rats. Attempts are underway to provide substantial evidence for the latter postulate.

Mouse-to-Rat Model

Similar to our observations in the hamster-to-rat model, mouse hearts when transplanted into unmodified rat recipients are rejected by antibody-mediated mechanisms within 2–3 days [71–73]. However, Pan et al. [73] have shown that liver xenografts when transplanted into untreated Lewis rats survive for approximately 7 days, succumbing eventually to combined antibody and cell-mediated rejection (Table 2). Although no appreciable difference in mouse heart xenograft

Table 2. Survival of B10.BR mouse hearts and livers xenografted into Lewis rat recipients with or without tacrolimus immunosuppressive therapy

Organ	Tacrolimus	Graft survival (days)	Median survival (days)
Heart	No	2, 2, 2, 2, 2, 2, 3, 3	2.7±1.7
Heart[a]	Yes	2, 2, 3, 3, 3	2.8±0.8
Liver	No	7, 7, 7, 7, 7	7.0
Liver[b]	Yes	18, 18, 28, 37, 61, 82, 93, 107, 110, 111, 117, 133, 203, 207, 221	103.9±64.7

[a] 1 mg/kg per day, i.m.
[b] 1 mg/kg per day ×30 days followed by 0.5 mg/kg every other day for the next 70 days.

survival is observed following tacrolimus administration, it does however, improve the outcome in mouse liver graft recipients, 50% surviving beyond 100 days. Despite the presence of elevated xenospecific antibodies, the observation of long-term liver xenograft survival in this model further emphasizes the inherent resistance of this organ to antibody-mediated injury. The donor-specific protective effect afforded by the transplanted liver following hamster-to-rat xenotransplantation is also witnessed in this model. This effect appears to be species-specific but non-major histocompatibility complex (MHC) restricted, allowing for transplanted mouse hearts obtained from donors of varying MHC phenotype to be equally protected, whereas hearts obtained from hamster are promptly rejected [74].

Comment

Our studies have demonstrated that the interdiction of the initial B cell proliferative response is the critical first step towards successful xenotransplantation in closely related species. Once the antibody barrier is breached, the need for the antiproliferative drugs apparently diminishes with tacrolimus-based monotherapy being sufficient to maintain graft function. The manipulation of the donor and/or the recipient with novel agents such as phosphatidic acid inhibitors [75] and the creation of chimeric donors [76, 77] are some of the more contemporary approaches that are currently being employed in our laboratory to make xenotransplantation a clinical reality.

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