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ORIGINAL ARTICLE

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Donor-specific renal, but not cardiac, allograft tolerance promotes engraftment of the normally rejected rat skin graft

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Abstract This study examined whether a heart or kidney graft could provide protection for the more resistant skin graft. Buffalo rat recipients were given a single dose of RIB 5/2 (non-depleting anti-CD4 mAb) plus i.v. Lewis splenocytes 21 days before being given Lewis heart or kidney grafts. Lewis skin was grafted either simultaneously with, or after, long-term (> 50 days) Lewis heart or kidney allograft acceptance. Immune responsiveness was analyzed by in vitro mixed lymphocyte culture (MLC), cytotoxic T lymphocytes (CTLs), and limiting dilution analysis (LDA). While i.v. alloantigen plus RIB 5/2 resulted in long-term acceptance of heart and kidney, survival of skin grafts alone was not prolonged. However, simultaneous transplantation with kidney, but not heart, resulted in long-term skin graft acceptance, while skin grafts subsequently grafted to recipients tolerant to kidney or heart were not accepted. In vitro analysis revealed a down-regulation of proliferation, cytotoxicity, and precursor T-helper cells (pThs)/precursor cytotoxic T lymphocytes (pCTLs) in Buffalo recipients accepting Lewis kidney and skin allografts. While RIB 5/2 plus Lewis splenocytes do not prolong the survival of skin grafts, Lewis skin grafted simultaneously with a kidney, but not heart, is accepted indefinitely and provides donor-specific protection for a subsequent skin graft.

Keywords RIB 5/2 · Donor-specific transfusion · Tolerance

Introduction

CD4+ T lymphocytes participate in MHC class II antigen recognition [24] and contribute to class II restriction [34]. The CD4 molecule plays an important role in thymocyte differentiation and T-cell repertoire selection [37, 40]. The interaction of alloantigen through the CD4+ T-cell receptor results in T-cell activation followed by cellular proliferation and production of cytokines. Prior studies have revealed the immunosuppressive potency of in vivo depleting anti-CD4 monoclonal antibodies (mAbs) for allografts [7, 13, 26, 27]. The efficacy of these depleting CD4 mAbs is closely related to the degree of CD4+ depletion, but predisposes to the problems of non-specific immunosuppression [8, 15, 35, 38]. The ultimate goal in organ transplantation is the achievement of donor-specific unresponsiveness without this non-specific effect. The anti-rat non-depleting CD4 mAb, RIB 5/2, has been shown to modulate the CD4 glycoprotein without eliminating the CD4-positive T cells [12].

Skin allografts have been the most difficult of transplanted tissues to achieve prolonged survival [19]. In addition, kidney grafts generally evoke a more vigorous response than do rat hearts [16, 20] and, in several species, liver graft acceptance is relatively easily

achieved [4, 6, 11]. Donor antigen plus the non-depleting anti-CD4 mAb, RIB 5/2, has been shown to induce donor-specific acceptance of heart or kidney allografts, but not skin allografts [1, 16, 17]. Variations in the survival of different tissue grafts may be related to the presence of organ-specific antigens or to differences in the level of expression of major or minor histocompatibility antigens in each organ [22]. For example, Poindexter et al. [22] have eluted biologically active immunogenic peptides from a human HLA-A3+ kidney, but not from the matched spleen. The presence of skin-specific antigens, such as the murine Epa-1 and Sk antigens, which are not expressed on splenocytes, could result in the greater immunogenicity of skin grafts, and thus their resistance to tolerance [30, 31]. This study examined whether acceptance of a heart or kidney allograft would promote the acceptance of the more resistant skin grafts.

Materials and methods

Animals

Male Buffalo (BUF) ($RT1^b$), Lewis (LEW) ($RT1^l$), Dark Agouti (DA) ($RT1^a$), and Brown Norway (BN) ($RT1^n$) rats were purchased from Harlan Sprague Dawley (Indianapolis, Ind., USA) and used at 6–8 weeks of age. All animals were fed a balanced rodent diet and water ad libitum and were cared for according to specific National Institutes of Health guidelines (*Principles of Laboratory Animal Care*, NIH publication no. 86-23, revised 1985).

Monoclonal antibodies

RIB 5/2 mAb (IgG2a) was produced by the fusion of the mouse myeloma cells X63-Ag8.653 and splenocytes from mice immunized with concanavalin A-activated BDIX rat lymphocytes [12]. RIB 5/2 precipitates a 53-kDa polypeptide expressed on rat thymocytes and splenocytes. Simultaneous staining of lymphocytes with the non-depleting anti-CD4 mAb, RIB 5/2, and either of the depleting anti-CD4 mAbs, W3/25 or OX-35, results in the same fluorescence-activated cell sorting (FACS) binding pattern as that observed with either single depleting mAb alone, thus indicating that all three antibodies detect the same molecule [29]. However, RIB 5/2 defines a different epitope of the CD4 molecule, since there is no competition with the binding of RIB 5/2 by W3/25 or OX-35 [12]. The IgG2a content of the ascites (12 mg/ml) was determined by ELISA [12].

Flow-cytometry analysis

Peripheral blood lymphocytes were prepared by centrifugation over a Ficoll/sodium diatrizoate (Histopaque 1083; Sigma Chemicals, St. Louis, Mo., USA) gradient. The relative peripheral blood T-lymphocyte subset frequencies and their CD4 mean channel fluorescence (MCF) in Buffalo rats treated with a single i.p. injection of RIB 5/2 were analyzed 24 h and 21 days after mAb treatment in order for us to determine the length of time that CD4 expression was modulated.

Donor cells

Donor spleen was passed through a 60-mm screen, and erythrocytes were lysed with Tris-NH_4Cl (0.83%) at 37°C. The remaining splenocytes were washed three times with Hank's balanced salt solution (HBSS) and re-suspended in HBSS at 25×10^6cells/ml for i.v. injection via the penile vein. Under ketamine (86.98 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) plus xylazine (13.04 mg/kg; Burns Veterinary Supply, Rockville Center, N.Y., USA) anesthesia, recipients also simultaneously received a single i.p. injection of 20 mg/kg RIB 5/2 anti-CD4 mAb.

Cardiac transplantation

Twenty-one days after they had received antigen plus RIB 5/2 mAb, the recipients were shown to have recovered from the non-specific suppressive effects of the mAb [29]. Microsurgical intra-abdominal heterotopic cardiac transplantation was then performed in accordance with a modification of the method of Ono and Lindsey [21]. The aorta and pulmonary artery were anastomosed to the infrarenal aorta and inferior vena cava, respectively. Graft survival was monitored by daily palpation, and rejection was considered to be complete at the time of cessation of a palpable heartbeat and was confirmed by histology.

Renal transplantation

Orthotopic renal transplantation was performed in accordance with the modified technique of Fabre [5]. A segment of the left renal donor aorta was anastomosed end-to-side to the recipient abdominal aorta, and a segment of the donor vena cava end-to-side to the recipient vena cava. The ureters were anastomosed end-to-end over a PE-10 stent in accordance with Savas' technique [25]. Contralateral nephrectomy was performed 7 days after transplantation. Renal graft rejection was defined as the recipient animal's death from uremia and was confirmed histologically.

Skin transplantation

Skin grafting was performed in accordance with the method of Billingham and Medawar [2]. Donor tail skin was grafted onto the dorsal thorax of the recipient. An occlusive dressing was removed on the 7th post-operative day, and daily inspection defined rejection as more than 50% graft necrosis.

Mixed lymphocyte culture

Splenocytes (1×10^6) from na (control) or experimental rats were co-cultured with 1×10^6 allogeneic irradiated splenocytes (total volume of 200 µl) in a 96-well flat-bottomed microtiter plate. After incubation for 5 days at 37°C in a 95% air-/5% CO_2-humidified atmosphere, [^{3}H] thymidine (2.0 Ci/mmol [^{3}H] TdR; ICN Pharmaceuticals, Costa Mesa, Calif., USA) (2.0 µCi) was added to each well 16 h before harvesting, and [^{3}H] TdR incorporation determined in a liquid scintillation counter (1214 Rackbeta; LKB, Turku, Finland).

Cytotoxic T lymphocytes

The rat target blasts were generated by the culturing of lymph-node cells with 10 µg/ml concanavalin A for 48 h. Target cells (5×10^6)were labeled with 200 µCi^{51}Cr ($Na^{51}CrO4$; Dupont NEN,

Wilmington, Del., USA) in 100 µl 10% FCS-DMEM for 1 h at 37°C in 5% CO_2. We used effector-to-target ratios of 100:1 to 3:1 to measure specific cytotoxicity of cells recovered from a 7-day MLC and incubated at 37°C for 4 h.

Limiting dilution analysis

Responding splenocytes were added in dilutions ranging from 7.8×10^3 to 500×10^3 cells per well to 96-well U-bottomed plates in 24-well replicates. Irradiated (2,000 rad) normal spleen cells (250×10^3 per well) were added in complete Iscove's medium containing 100 U/ml of human recombinant IL-2 (Genzyme, Boston, Mass., USA). After 7 days of culture at 37°C in a 95% air/5% CO_2 atmosphere, all cells were gently re-suspended, and 3×10^3 ^{51}Cr-labeled target cells in 50 µl were added to each well. The culture plates were centrifuged at 35 *g* for 2 min and the cells incubated at 37°C for 4 h. After centrifugation at 1,000 *g* for 10 min, cell-free supernatants (100 µl) were harvested from each well and counted in a gamma counter (1272 Clingamma; LKB).

Experimental groups

Simultaneous mAb/donor-specific transfusion + transplant

BUF rats received either no treatment (groups 1–3, Table 1) or i.p. injection of 20 mg/kg RIB 5/2 at the time of LEW skin, heart, or kidney transplantation (groups 4–6, Table 1). To demonstrate the suppressive effect of RIB 5/2 in another strain combination, we transplanted BN skin, heart, or kidney into BUF rats treated on the day of transplantation with RIB 5/2 (groups 7–9, Table 1).

Delayed allograft

To separate the specific effect of donor antigen from the non-specific effects of the RIB 5/2 mAb, we used FACS analysis to determine that CD4 MCF had recovered to normal by 21 days. Therefore, groups 16–19 (Table 2) received 25×10^6 LEW splenocytes i.v. with i.p. injection of 20 mg/kg RIB 5/2 antibody 21 days before LEW skin, heart, or kidney grafts or third-party BN kidney graft. Control BUF animals were treated 21 days before LEW skin, heart, or kidney grafts with either 25×10^6 LEW splenocytes i.v. alone (groups 10–12, Table 2) or i.p. RIB 5/2 alone (groups 13–15, Table 2).

Simultaneous transplants

To determine whether tolerance to a heart or kidney graft would protect a skin graft, we gave BUF recipients a simultaneous LEW skin graft with heart or kidney, treated at the same time with IP RIB 5/2 alone (groups 20–21, Table 3) or 21 days following 25×10^6 LEW splenocytes i.v. plus i.p. RIB 5/2 (groups 23–24, Table 3). Third-party BN skin was also transplanted simultaneously with LEW kidney after each of these treatments (groups 22 and 25, respectively, Table 3) in order for us to demonstrate the donor antigenic specificity.

To investigate whether a greater cardiac mass would enhance skin graft survival, we transplanted two LEW hearts simultaneously with LEW skin 21 days following LEW i.v. antigen plus i.p. RIB 5/2 pre-treatment with or without an additional LEW i.v. infusion of 25×10^6 cells (group 26a–b, Table 4).

Subsequent grafting to kidney, heart, or skin

BUF recipients tolerant to a single LEW heart were given a subsequent LEW skin, heart, or kidney graft (group 27a–c, Table 5). BUF recipients tolerant to a LEW kidney were given subsequent

Table 1 Simultaneous RIB 5/2 mAb alone non-specifically promotes heart and kidney, but not skin, allograft acceptance

Group	Strain combination	Pre-treatment	Allograft	Graft survival (days)	MST ± SD
1	LEW to BUF	None	LEW skin	7, 7, 7, 7, 8, 8, 8, 9	7.6±0.7
2	LEW to BUF	None	LEW heart	6, 7, 7, 7, 8, 8	7.2±0.8
3	LEW to BUF	None	LEW kidney	10, 10, 10	10±0
4	LEW to BUF	RIB 5/2 (day 0)	LEW skin	10, 12, 13, 13, 15	12.6±1.8
5	LEW to BUF	RIB 5/2 (day 0)	LEW heart	10, 11, 12, 35, >50×5	5 of 9>50±0
6	LEW to BUF	RIB 5/2 (day 0)	LEW kidney	>50×5	>50±0
7	BN to BUF	RIB 5/2 (day 0)	BN skin	9, 10	9.5±0.5
8	BN to BUF	RIB 5/2 (day 0)	BN heart	>50×4	>50±0
9	BN to BUF	RIB 5/2 (day 0)	BN kidney	33, >50×3	3 of 4>50±0

Table 2 The administration of i.v. LEW alloantigen plus RIB 5/2 mAb 21 days, before specifically promotes LEW heart and kidney, but not skin, allograft acceptance. The administration of i.v. LEW alloantigen alone or RIB 5/2 mAb alone 21 days before, does not promote LEW heart, kidney, or skin allograft acceptance

Group	Strain combination	Pre-treatment	Allograft	Graft survival (days)	MST ± SD
10	LEW to BUF	LEW i.v. (day-21)	LEW skin	7, 8, 8	7.7±0.6
11	LEW to BUF	LEW i.v. (day-21)	LEW heart	9, 10, 11, 12, 14	11.2±2.5
12	LEW to BUF	LEW i.v. (day-21)	LEW kidney	11, 12	11.5±0.5
13	LEW to BUF	RIB 5/2 (day-21)	LEW skin	8, 8	8±0
14	LEW to BUF	RIB 5/2 (day-21)	LEW heart	6, 6, 7, 7, 7	6.6±0.4
15	LEW to BUF	RIB 5/2 (day-21)	LEW kidney	10, 10, 10	10±0
16	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin	9, 9, 9, 9, 9	9±0
17	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW heart	7, 10, 17, >50×15	15 of 18>50±0
18	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW kidney	9, 12, 13, >50×11	11 of 14>50±0
19	BN to BUF	LEW i.v. + RIB 5/2 (day-21)	BN kidney	11, 12, 12	11.7±0.6

Table 3 LEW kidney, but not LEW heart, can protect simultaneous LEW skin transplant after either RIB 5/2 treatment alone on day 0 or 21 days after LEW i.v. plus RIB 5/2

Group	Strain combination	Pre-treatment	Allograft	Graft survival (days)	MST ± SD
20	LEW to BUF	RIB 5/2 (day 0)	LEW skin + LEW heart	13, 14, 17, 19 > 50×4	15.7 ± 2.8 > 50 ± 0
21	LEW to BUF	RIB 5/2 (day 0)	LEW skin + LEW kidney	34, > 50×4 > 50×5	4 of 5 > 50 ± 0 > 50 ± 0
22	BN/LEW to BUF	RIB 5/2 (day 0)	BN skin + LEW kidney	17, 18 > 50×2	17.5 ± 0.7 > 50 ± 0
23	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + LEW heart	9, 10, 12, 14, 15 > 50×5	12.0 ● 2.6 > 50 ± 0
24	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + LEW kidney	15, > 50×5 > 50×6	5 of 6 > 50 ± 0 > 50 ± 0
25	BN/LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	BN skin + LEW kidney	9, 10 > 50×2	9.5 ± 0.7 > 50 ± 0

Table 4 Lack of dose effect of LEW heart donor grafts on simultaneous LEW skin allografts

Group	Strain combination	Pre-treatment	Allograft	Graft survival (days)	MST ± SD
24	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + one LEW kidney	15, > 50×5 > 50×6	5 of 6 > 50 ± 0 > 50 ± 0
23	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + one LEW heart	9, 10, 12, 14, 15 > 50×5	12.0 ± 2.6 > 50 ± 0
26a	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + two LEW hearts	11, 14, 14, 15, 17, 24 9, 11, > 50×4	15.8 ± 6.6 4 of 6 > 50 ± 0
26b			LEW skin + two LEW hearts[a]	14, 14, 14 > 50×3	14.0 ± 0.0 > 50 ± 0

[a] 25×10^6 LEW splenocytes i.v. infused at the time of transplantation

Table 5 Recipients tolerant to a LEW heart or kidney alone will accept a subsequent LEW kidney or heart, respectively, but will not accept a subsequent LEW skin graft. Recipients tolerant to a simultaneously transplanted LEW kidney and skin graft will accept a subsequent LEW, but not third-party BN, skin graft. Recipients tolerant to a LEW heart but not simultaneous LEW skin graft will also not accept a subsequent LEW skin graft

Group	Strain combination	Pre-treatment	First graft	Second graft[b]	Second graft survival (days)	Second graft MST ± SD
27a	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW heart	LEW skin	11, 15, 17, 21	16 ± 4.2
27b				LEW heart	> 50×3	> 50 ± 0
27c				LEW kidney	> 50×2	> 50 ± 0
28a	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW kidney	LEW skin	20, 21, 24	21.7 ± 2.1
28b				LEW heart	> 50×4	> 50 ± 0
29	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + LEW kidney	LEW skin	> 50×4	> 50 ± 0
30	BN/LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin + LEW kidney	BN skin	9, 9, 10	9.3 ± 0.6
31	LEW to BUF	LEW i.v. + RIB 5/2 (day-21)	LEW skin[a] + LEW heart	LEW skin	16, 17, 19	17.3 ± 1.5

[a] First LEW skin graft rejected with a MST of 12.0 ± 2.6 days, while the LEW heart was accepted indefinitely

[b] Second graft transplanted after acceptance of the original LEW heart or kidney for > 50 days

LEW skin and heart transplants (group 28a–b, Table 5), and those tolerant to simultaneous LEW kidney and skin were given either a subsequent second LEW skin transplant or a third-party BN skin transplant (groups 29 and 30, respectively, Table 5). BUF recipients who had rejected a LEW skin graft but tolerated a simultaneous LEW heart were given a subsequent second LEW skin graft (group 31, Table 5).

Statistics

Difference in days of graft survival between the groups was analyzed by ANOVA with the Scheffe's multiple comparison post-hoc test. A P value of less than 0.05 was considered significant.

Results

Concomitant treatment with non-depleting anti-CD4 mAb RIB 5/2 results in non-specific protection of heart and kidney, but not skin, allografts

The mean survival time (MST) of LEW ($RT1^l$) skin, heart, or kidney allografts in untreated control BUF ($RT1^b$) rats was 7.6 ± 0.7, 7.2 ± 0.8, and 10 ± 0 days, respectively (groups 1–3, Table 1). The i.p. injection of

RIB 5/2 at the time of transplantation prolonged both heart ($P < 0.05$, group 5 vs group 2, Table 1) and kidney ($P < 0.05$, group 6 vs group 3, Table 1), but not skin, grafts (NS, group 4 vs group 1, Table 1). The acceptance of third-party BN heart and kidney (groups 8–9, Table 1), but not BN skin (group 7, Table 1), demonstrated the effect of non-specific immunosuppression by RIB 5/2 administered at the time of heart or kidney transplantation, but not that of skin. Although indefinite survival is reported as greater than 50 days, a subset of animals was also allowed to survive with functioning grafts for more than 120 days in order for us to establish "indefinite survival." Animals killed at over 50 days for histological examination demonstrated no rejection.

CD4-positive T cells recover 21 days after i.p. injection of RIB 5/2

By flow cytometry, 46.8% (range 41.2–49.7%) of T cells were CD4-positive in the naive BUF rat, with an MCF of 1,800 (range 1,720–1,880). Twenty-four hours after in vivo administration of 20 mg/kg of the non-depleting anti-CD4, RIB 5/2, the MCF of CD4 cells was markedly reduced to 228 (range 217–240), while the CD4 cells remained at 44.6% (range 38.5–47.2%). By day 21 following administration of 20 mg/kg RIB 5/2, the MCF of CD4 cells had recovered to 1,572 (range 1,455–1,670), and CD4+ cells represented 34.4% (range 31.9–43.4%) of the total BUF peripheral blood lymphocytes. Thus, FACS analysis demonstrated that the mAb RIB 5/2 modulates the specific CD4 glycoprotein without eliminating the CD4 T cells. Motoyama et al. [18] showed by FACS analysis with FITC-conjugated IgG2a secondary antibody that RIB 5/2 mAb present on the lymphocyte cell surface at 1 day post-injection of RIB 5/2 decreased by approximately 60% at 10 days post-injection and was completely absent at 21 days post-injection.

Pre-treatment with both RIB 5/2 and LEW donor antigen is required for specific LEW heart and kidney allograft acceptance

LEW antigen or anti-CD4 mAb RIB 5/2 administered alone to BUF rats 21 days prior to LEW skin, heart, or kidney transplantation did not promote LEW skin, heart, or kidney allograft survival (groups 10–15, Table 2). However, pre-treatment of BUF recipients with both i.v. LEW antigen and i.p. RIB 5/2 21 days prior could specifically promote indefinite acceptance of both LEW heart and kidney (groups 17–18, Table 2), but not LEW skin grafts (group 16, Table 2). The escalation of doses of i.v. LEW antigen (to 100×10^6) also did not result in skin acceptance (data not shown). The rejection of third-party BN kidney following pre-treatment of BUF recipients with i.v. LEW antigen and i.p. RIB 5/2 21 days prior (group 19, Table 2) demonstrated the donor antigen specificity of this tolerance induction. In all cases, histological examination of the long-term surviving grafts showed no rejection, whereas all rejected grafts demonstrated intense lymphocytic infiltration.

Acceptance of a LEW kidney, but not heart allograft, can specifically promote acceptance of a simultaneously transplanted LEW skin allograft

Simultaneous transplantation of a LEW skin allograft with a LEW kidney, but not with a LEW heart, resulted in indefinite acceptance when RIB 5/2 mAb alone was given at the time of transplantation ($P < 0.05$, group 21, Table 3, vs group 4, Table 1; NS, group 20, Table 3, vs group 4, Table 1, respectively) or if LEW splenocytes were given i.v. with the RIB 5/2 mAb 21 days prior to transplantation ($P < 0.05$, group 24, Table 3, vs group 4, Table 1; NS, group 23, Table 3, vs group 4, Table 1, respectively). On the other hand, third-party BN skin grafts transplanted simultaneously with LEW kidney, following either pre-treatment schedule, were rejected within 18 and 10 days, respectively, without rejection of the LEW kidney (groups 22 and 25, Table 3), thus, again demonstrating the donor antigen specificity.

We examined kidney, heart, and skin grafts histologically at serial time points to assess the pattern of cellular infiltration (slides not shown). Skin grafts destined to be rejected (i.e., those transplanted alone or with heart grafts) demonstrated severe cellular infiltration at 3–5 days post-transplantation. This was followed by progressive fibrotic changes, leading to complete rejection. Skin grafts that were indefinitely accepted (i.e., those transplanted simultaneously with kidney grafts) never demonstrated this cellular infiltration or fibrosis.

Double LEW heart transplantation does not protect LEW skin allografts

We have previously demonstrated that there are approximately 50% fewer passenger leukocytes in one LEW heart than in one LEW kidney (approximately 3–6 million/heart vs 8–14 million/kidney) [17]. We therefore transplanted two LEW hearts in order to control the number of passenger leukocytes. The transplantation of two LEW hearts simultaneously with a LEW skin graft to a BUF recipient 21 days after pre-treatment minimally increased LEW skin graft survival (15.8 ± 6.6, group 26a, Table 4, vs 12.0 ± 2.6, group 23, Table 4). In addition, infusion of an equivalent number of donor LEW splenocytes (25×10^6) at the time of transplantation also did not facilitate skin graft acceptance (14.0 ± 0.0, group 26b, Table 4, vs 12.0 ± 2.6,

Table 6 In vitro immune suppression following LEW-to-BUF kidney, heart, and skin transplantation. LDA was performed 20 days after transplantation. *SC* spleen cells, *CPM* counts per minute

Responding SC from	Pre-treatment	Transplant	In vitro antigen	CPM by MLC	Percent CTL (percentlysis)	pTh freqency	pCTL freqency
BUF	None	None	LEW-SC	97,034 ± 16,041	43.0	1/37,409	1/52,793
BUF	LEW i.v. + RIB 5/2	None	LEW-SC	15,309 ± 4,259	39.5	1/43,134	1/48,320
BUF	LEW i.v. + RIB 5/2	LEW skin	LEW-SC	103,098 ± 3,986	63.5	1/27,270	1/37,566
BUF	LEW i.v. + RIB 5/2	LEW kidney	LEW-SC	1,817 ± 337	0	1/381,151	0
BUF	LEW i.v. + RIB 5/2	LEW kidney + LEW skin[a]	LEW-SC	7,548 ± 2,026	10.1	1/136,680	0
BUF	LEW i.v. + RIB 5/2	LEW kidney + LEW skin[b]	LEW-SC	3,647 ± 1,123	3.2	1/250,159	1/586,692
BUF	LEW i.v. + RIB 5/2	LEW heart	LEW-SC	2,406 ± 958	4.5	1/122,010	0
BUF	LEW i.v. + RIB 5/2	LEW heart + LEW skin[c]	LEW-SC	12,102 ± 873	8.5	1/100,735	0

[a]Both simultaneously transplanted LEW kidney and LEW skin are accepted long term (assayed 20 days after transplantation)
[b]Long-term (> 50 days) acceptance of LEW kidney followed by subsequent (after 50 days) LEW skin; LEW kidney persists despite rejection of the LEW skin (assayed 20 days after subsequent skin transplantation)
[c]Long-term (> 50 days) acceptance of LEW heart; rejection of the LEW skin (assayed 20 days after transplantation)

group 23, Table 4). This suggested that the increasing of the dose of heart tissue antigens or the number of heart passenger leukocytes was not beneficial to skin graft survival.

BUF recipients tolerant to a single LEW heart or kidney will not accept a subsequent LEW skin graft, while simultaneous transplantation of both a LEW kidney and skin is accepted and protects a subsequent second LEW skin allograft

BUF recipients tolerant to a LEW heart alone will accept a subsequent LEW heart or kidney (groups 27b–c, Table 5), and those tolerant to a LEW kidney alone will accept a subsequent LEW heart (group 28b, Table 5). However, recipients tolerized to either a LEW heart or kidney alone, by the i.v. injection of LEW antigen plus RIB 5/2 21 days before, will not accept a subsequent LEW skin graft (groups 27a and 28a, Table 5, respectively). In contrast, recipients tolerant to both simultaneously transplanted LEW skin and kidney grafts will subsequently accept a second LEW (group 29, Table 5), but not a third-party BN (group 30, Table 5), skin allograft. Of note, BUF rats given simultaneous LEW skin and heart allografts following pre-treatment will not only accept the heart and reject the first LEW skin graft (group 23, Table 3), but will also continue to accept the heart graft during rejection of a subsequent second LEW skin allograft (group 31, Table 5).

LEW-to-BUF kidney and heart transplantation down-regulates in vitro anti-donor reactivity independent of skin transplantation

Pre-treatment with intravenous LEW alloantigen and RIB 5/2, plus transplantation of a LEW skin graft alone, resulted in a sensitization response with increased MLC proliferation, CTL lysis, and pTh/pCTL frequencies. However, the same pre-treatment followed by kidney or heart graft alone resulted in down-regulation in vitro. Simultaneous LEW kidney and skin graft acceptance also resulted in down-regulation. In fact, despite the rejection of subsequently transplanted LEW skin following long-term acceptance of LEW kidney, immune suppression was not significantly altered (i.e., it continued to be down-regulated when compared with controls). Similarly, immune suppression was also not altered following LEW heart acceptance when simultaneously transplanted skin was rejected (Table 6). We believe activity that was measured in the MLC, CTL, and LDA assays preferentially measures responses to lymphocyte antigens and not to skin antigens.

Discussion

Tolerance to allografts can be induced by the selective manipulation of the CD4+ T-cell subset. CD4-depleting mAb abrogates rejection and prolongs the survival of allografts [7, 13, 26, 27], but the depletion of CD4+ T cells is long lasting. The use of non-depleting anti-CD4 mAbs, such as RIB 5/2, results in disruption of T-cell function without reducing the number of CD4+ T cells. Effective immunosuppression has been shown for pancreas, heart, and skin transplants after treatment with non-depleting CD4 mAbs or $F(ab')_2$ mAb fragments [28].

In the present study, injection of only the anti-CD4 mAb, RIB 5/2, at the time of transplantation promoted graft acceptance, but was ineffective when given 21 days prior to transplantation, unless it was combined with LEW splenocytes given i.v. With both anti-CD4 mAb and donor antigen, indefinite survival of LEW kidney and heart allografts, but not skin, resulted. In addition, a LEW kidney, but not a LEW heart, allograft specifically protected simultaneously, but not subsequently,

transplanted LEW, but not BN, skin grafts. It is generally accepted that skin is more resistant to the induction of tolerance than kidney and heart allografts are. While kidney and heart transplants are primarily vascularized, skin grafts are secondarily re-vascularized. However, this obvious difference in vascularization appears insufficient to explain the significant differences in organ allograft survival [39].

While passenger leukocytes have been shown to be responsible for graft immunogenicity [10], they can also promote the development of tolerance. Sun et al. [33] reported the importance of dose effect of transplanted organ and donor leukocytes in liver tolerance induction. DA recipients spontaneously accepted PVG liver allografts indefinitely, while acutely rejecting PVG kidney or heart. However, if two PVG hearts or two PVG kidneys with associated donor leukocytes were transplanted into naive DA rats, the heart or kidney grafts were also spontaneously accepted. In contrast, we found that double LEW-heart-to-BUF transplantation did not significantly prolong a simultaneously transplanted LEW skin graft.

Tissue-specific antigens are recognized for their importance in immunogenicity [3]. T lymphocytes recognize epitopes formed by a specific peptide bound to the target cell MHC molecule. Because the associated peptide is typically derived from the endogenous pool of proteins, tissue-specific cells express proteins that are unique to the organ of origin [9]. Absence of that bound peptide can attenuate or even completely abrogate the specific T-cell recognition in another organ. On the other hand, the sharing of common peptides may promote similar immune recognition of different tissues. While this response may be characteristic of rejection, under permissive conditions, acceptance of two different organ grafts may result. While monoclonal antibody to CD4+ T cells can non-specifically promote graft acceptance, the addition of donor alloantigen provides delayed MHC specificity, as is demonstrated by our induction of donor-specific tolerance 21 days after RIB 5/2 mAb is combined with LEW donor antigen.

Johnson et al. [9] studied the H-Y antigen and minor H antigens in eight C57BL/6By-congenic mouse strains for their distribution among 13 different tissues. They found that no two strains had identical patterns of expression of their distinguishing antigens, providing evidence for differences in levels of minor H-antigen expression among different tissues. Steinmuller et al. [32] showed that the epidermal alloantigen-1 (Epa-1) is a strong determinant of skin graft rejection, but weakly influences heart allograft rejection. They postulated that Epa-1 was a peptide fragment of an intracellular protein that was recognized in the context of cell surface MHC molecules by self-restricted T cells. Takeuchi et al. [36] demonstrated that $RT1^l$ rats express Thy-1 within renal glomeruli, but not cardiac myocytes. In addition, Thy-1 antigen is up-regulated on dermal vascular endothelial cells during allogeneic skin graft rejection. Poindexter et al. [23] similarly isolated a sub-population of human T cells that recognized donor kidney-specific epithelial antigens from acutely rejecting renal allografts. These studies provide evidence for the existence of tissue-specific allograft-infiltrating CTLs. Specificity has also been demonstrated by the finding of CTLs that lyse both donor kidney epithelial cells and skin fibroblasts, but do not affect blood leukocytes [14]. Thus, LEW kidney, but not heart, may share an antigen (e.g., epithelial peptide) with skin that results in protection of both a LEW kidney and a LEW skin graft.

Down-regulation of donor-reactive T cells by exposure to kidney-specific antigen appears to be necessary at the time of transplantation to allow the acceptance of both kidney and skin grafts. This is supported by the observation that the skin graft is protected only when simultaneously transplanted with, and not after, the kidney graft. This suggests that a subset of skin-directed CTLs may not be sufficiently suppressed by a kidney graft alone to subsequently allow acceptance of a skin graft. In contrast, skin simultaneously transplanted with kidney resulted in adequate suppression of these CTLs and acceptance of a subsequent second skin graft. Thus, a more complete understanding of differences in peptide processing and antigen presentation between different tissue allografts should provide insight into the mechanisms underlying the development of tissue-specific graft rejection or tolerance.

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