Brief Definitive Report

Thromboxane A_2 Receptor Blocking Abrogates Donor-specific Unresponsiveness to Renal Allografts Induced by Thymic Recognition of Major Histocompatibility Allopeptides

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Summary

Recent in vitro studies have documented that thromboxane (Tx)A_2 induces thymocyte apoptosis by acting on specific receptors abundantly expressed on the surface of immature T lymphocytes. No information is available on the in vivo relevance of this observation in development of self- or acquired tolerance. We and others have previously documented that injection of donor cells into adult thymus of experimental animals induced specific systemic unresponsiveness to allografts in the rat and mouse models. More recently, we have shown that intrathymic injection of synthetic class II major histocompatibility complex (MHC) allopeptides resulted in donor-specific unresponsiveness to renal allografts. The induction of unresponsiveness was abrogated by recipient thymectomy within the first week. We now report the effect of TxA_2 blockade on acquired thymic tolerance to renal allografts induced by intrathymic injection of synthetic class II MHC allopeptides in the Wistar-Furth (WF) to Lewis rat strain combination. Administration of the TxA_2 receptor blocker prior to transplantation or 2 wk postengraftment completely abrogated the unresponsive state. In addition, inhibiting the TxA_2-forming enzyme by aspirin or dexamethasone also abolished the induction of acquired thymic tolerance. Evidence is also provided for a critical "dose" of peptides to be injected into the thymus to induce systemic unresponsiveness to renal allografts. These data, coupled with observations that activated peripheral T cells can circulate through the thymus, provide evidence that TxA_2/TxA_2 receptor interaction in the thymic microenvironment, leading to anergy/programmed cell death of activated T cells, may play an important role in the development of acquired unresponsiveness in vivo.

In a previous study, we have shown that intrathymic injection of allogenic cells (glomeruli or peripheral mononuclear cells) into MHC incompatible recipients allowed long-term survival of a subsequent kidney graft from the same donor strain but not from a third party donor (1, 2). More recently we have shown that thymic recognition of immunogenic class II MHC allopeptides is sufficient to achieve systemic unresponsiveness and prolong allograft survival in high responder Wistar-Furth (WF) ($RT1^u$) to Lewis rat strain combinations (3, 4). Early (within 1 wk) but not late (after 2 wk) thymectomy abolishes this unresponsive state, indicating the central role of the thymus in the process of induction of acquired systemic tolerance to alloantigens in this model.

There are two recognized mechanisms by which T cells are rendered tolerant to self-antigens during their maturation in the thymus: one is apoptosis, which physically eliminates reactive T cell clones; the other one is clonal anergy, which functionally inactivates T cells (5–7). Both mechanisms can operate in the thymus, where given the cellular heterogeneity of thymic microenvironment variable degrees of apoptosis may be associated with anergy (7). However the complexity of cellular interactions underlying these events and the soluble mediators involved in the communication that ultimately generates tolerance have not been established.

That thymic microenvironment plays a major role in autoreactive T cell elimination is supported, among other demonstrations, by studies that clonal deletion does not occur in nude mice (which congenitally lack the thymus) (6). It also appears that soluble factors establish whether a given clone is suppressed or will mature to a functional T cell and leave the thymus as such. The observation that maturing thymocytes, in the earliest stage of T cell development, but not

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later on, have very high cell surface expression of receptors for thromboxane (Tx)A_2 (8), a potent aggregating agent and vasoconstrictor (9), suggested that TxA_2 elaborated by thymic stromal cells might act as the key signal for cellular events related to T cell development. TxA_2 is the ideal messenger for cell–cell interaction in the thymus for at least two of its properties: (*a*) it is synthesized and released upon cell activation; and (*b*) has an half-life as short as 30 s (9) so that its effect is confined to those very cells close enough to the ones initially activated. TxA_2 binding to its specific receptors on cell membranes activates protein kinase C and increases intracellular calcium as the result of phosphatidyl inositol breakdown (9–12). It is relevant here that both protein kinase C activation and enhanced intracellular calcium are the two single events that in developing thymocytes serve to promote DNA fragmentation and apoptosis (13–15). Recent data indicate that a TxA_2 agonist (but not an inactive metabolite), induced concentration-dependent DNA fragmentation in $CD4^+CD8^+$ cells (8), which was inhibited by a specific antagonist. We now present direct evidence based on in vivo experiments that TxA_2 mediates acquired unresponsiveness to renal allograft induced by thymic recognition of donor MHC allopeptides.

Materials and Methods

Materials. BMS 180291 (1S-(1α, 2α, 3α, 4)-2-[[3-[4-[(pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo[2.2.1]hept-2-yl]methyl] benzenepropanoic acid) was a gift from Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Sequences of eight overlapping WF peptides of 25 amino acids each (1-25, 20-44, 39-64, and 68-92 for RT1.B, and 1-25, 20-44, 39-64, and 60-84 for RT1.D) were recently published (16).

Kidney Transplantation. Orthotopic rat renal transplants were performed as previously described (2). Briefly, ether anesthesia was used throughout. The WF donor left renal vein was anastomosed end to end to the recipient Lewis left renal vein. The donor renal artery was anastomosed to the aorta with the small patch of donor aorta, while the left ureter of donor rats was pulled through the posterior wall of the dome of the bladder. The right kidney of the recipient was then removed. Ischemic time ranged from 20 to 40 min (mean 30 min).

TxA_2 Receptor Blockade and Acquired Thymic Tolerance. Adult male Lewis ($RT1^l$) rat recipients (225–250 g body weight) were given the TxA_2 receptor antagonist BMS 180291 (10 mg/kg twice daily orally) or vehicle. BMS 180291 is a potent, highly selective antagonist of TxA_2/prostaglandin endoperoxide receptors which is long-acting when given orally to experimental animals including rats (17). Evidence for specific activity is based on inhibition of platelet aggregation, and contraction of trachea or aorta induced by a TxA_2 mimetic but not other agonists (17). 4 h later, the same animals were injected intrathymically (50 μg in each lobe) with a mixture of class II MHC allopeptides from a MHC-incompatible WF ($RT1^u$) rat. Four overlapping peptides of 25 amino acids for each locus were synthesized using published sequences of RT1.Bβ and RT1.Dβ distal domains of $RT1^u$ (WF) dissolved in PBS (16). The animals received no immunosuppressive drugs. After 48 h they underwent bilateral nephrectomy, and received a WF ($RT1^u$) renal allograft. Treatment with the TxA_2 receptor antagonist or vehicle was continued daily thereafter. Animals were housed in metabolic cages and monitored for daily urine output. Allograft rejection was defined as anuric renal failure. Renal allograft function was assessed by measuring serial serum creatinine levels at different intervals post-transplantation. Serum was obtained by tail vein puncture and serum creatinine was measured by the alkaline picrate method (18).

Additional experiments with the TxA_2 receptor antagonist were performed starting the treatment 15 d after transplantation ($n = 5$).

Preparation of Spleen Cells and Adoptive Transfer Experiments. The spleen was teased through a 500-μm gauge sieve into a 10-ml falcon tube, and the large particles were allowed to settle. The supernatant was centrifuged at 400 *g* for 10 min, and the cells washed twice in PBS. Contaminating red blood cells were lysed by resuspending the cells in 2 ml of Tris-HCl (17 mM), pH 7.2, containing 0.16 M ammonium chloride, incubated at room temperature for 5 min. The suspension was then diluted to 50 ml with 0.1% bovine serum albumin in PBS, and centrifuged at 400 *g* for 10 min. The cells were washed twice in PBS and resuspended in 0.5% (wt/vol) BSA/PBS. Immediately before intravenous injection into the Lewis recipient animals, the cells were washed and resuspended in saline. To investigate whether unresponsiveness to kidney allograft involved a suppressor effector mechanism, adoptive transfer experiments were performed. Two Lewis rats injected in the thymus with WF class II MHC allopeptides mixture and with kidney graft surviving >300 d were killed and their spleen removed. After isolation of spleen cells from the two rats they were pooled and injected intravenously (100×10^6 cells) in native Lewis rats ($n = 3$). 48 h later they were bilaterally nephrectomized and were transplanted orthotopically with MHC incompatible WF kidney. Graft survival was monitored as above. As control the same experiments were performed in Lewis rats ($n = 3$) injected with splenocytes from normal Lewis animals.

We also evaluated whether reducing the amount of peptide mixture injected into the thymus allowed permanent engraftment of the subsequent kidney allograft. Lewis rats received intrathymically 1 ($n = 4$) or 10 ($n = 4$) μg of the WF MHC peptide mixture 48 h before transplantation of the WF kidney into the left orthotopic site. Graft survival was evaluated thereafter.

Mixed Lymphocyte Response (MLR) Assay. Lymphocytes (PBLs) isolated from peripheral blood of responder Lewis and stimulator WF rats were suspended in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS%. The MLRs were set up by culturing an equal number of Lewis responder cells and irradiated (3,000 rads) WF stimulator cells in 96-well U-bottom plates at 37°C with 5% CO_2 for 4 d. Proliferation was assayed by [^{3}H]thymidine uptake and relative response was calculated as previously described (16).

Statistical Analysis. Results are expressed as mean ± SD. Data were analyzed by *t* test or by one way analysis of variance (repeated measures) with Fisher's protected least significance difference, and Dunnett's test for multiple comparisons, as appropriate. Statistical significance level was defined as $p < 0.05$.

Results and Discussion

Effect of TxA_2 Receptor Blockade on Acquired Thymic Tolerance. Lewis rats given vehicle and injected intrathymically with the MHC allopeptide mixture did not reject their grafts and are surviving (>110 d; Table 1). By contrast, rats treated with the TxA_2 receptor blocker and injected with the pep-

Table 1. *Effect of BMS 180291, Aspirin, Dexamethasone, or Vehicle on Kidney Allograft Survival in Lewis Rat Recipients Injected Intrathymically with MHC-WF Allopeptide Mixture*

	Graft survival	Median
	d	*d*
BMS 180291	6, 7, 6, 6, 6	6
Aspirin	7, 7, 7, 8, 9, 10	7.5
Dexamethasone	6, 7, 7, 7, 9, 9	7
Vehicle	>110, >110 >110, >110	>110

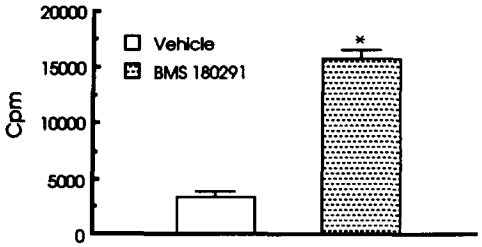

Figure 2. Proliferation of peripheral blood lymphocytes from Lewis recipients of WF renal allografts injected intrathymically with class II MHC peptide mixture and given vehicle or BMS 180291, in the standard one-way mixed lymphocyte response. Values are mean ± SD. $*p < 0.01$ vs. vehicle.

tides rejected their allografts within 6–7 d (Table 1). 30 d after transplant, renal grafts in untreated rats injected with the peptide mixture were functioning well, as documented by serum creatinine (0.62 ± 0.05 mg/dl) compared with age- and sex-matched control rats (Fig. 1). In rats given BMS 180291, however, serum creatinine was elevated when anuria developed (2.94 ± 0.15 mg/dl). To establish the functional status of alloreactive T cells of transplanted animals injected intrathymically with the peptides, peripheral blood lymphocytes from rats treated with BMS 180291 (on day 8–10) or vehicle-treated rats were tested in vitro for their ability to proliferate to WF stimulator cells in the standard one-way MLR (3). MLR proliferation was significantly ($p < 0.01$) reduced in tolerant animals, but a high MLR proliferative response was found in rats receiving the TxA_2 receptor blocker, which rejected the kidney (Fig. 2).

In an additional group of rats injected intrathymically with peptide mixture, BMS 180291 treatment was started 15 d after transplantation. At that time renal grafts were functioning well, as documented by serum creatinine concentration within the normal range (0.58 ± 0.07 mg/dl). 3–6 d

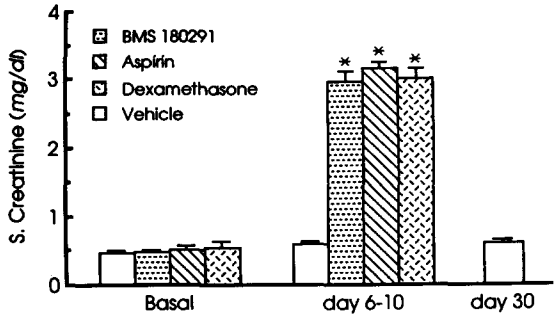

Figure 1. Time course of renal allograft function after intrathymic injection of class II MHC allopeptides in Lewis rat recipients given BMS 180291, aspirin, dexamethasone, or vehicle. Values are mean ± SD. $*p < 0.01$ vs. vehicle at the same time point, and at day 30 after kidney transplantation.

after beginning the TxA_2 receptor antagonist, the animals had a sudden decline in renal function and invariably rejected their kidneys within 10 d. Individual graft survival was 5, 6, 7, 9, and 10 d. When the animals developed oliguria serum creatinine level was 2.87 ± 0.21 mg/dl. Findings that BMS 180291 treatment eliminated thymic unresponsiveness in vivo and enhanced alloreactivity to donor lymphocytes in vitro would indicate that blocking TxA_2 activity into the thymus inhibits the intracellular events that result in functional anergy or apoptosis of alloreactive T cells.

Altogether these data suggest that TxA_2 is at least one of the soluble factors regulating events in the thymus that follow recognition of antigenic material (either self-antigens or allopeptides) leading to systemic unresponsiveness. TxA_2 forming enzymes are expressed in thymic stromal cells, including epithelial and dendritic cells. Recent data by Agus et al. (19) indicate that activated but not resting peripheral T cells circulate through the thymus. These observations provide a potential mechanism to explain the events that lead to acquired thymic tolerance in our model. We suggest that MHC-peptide recognition by alloactivated T cells circulating through the thymus presented by thymic antigen-presenting cells leads to a series of events that involve the TxA_2/TxA_2 receptor, thus triggering intracellular events that increase cytoplasmic calcium resulting in T cell anergy and ultimately deletion by apoptosis. This is consistent with our recent observation that although in vivo administration of recombinant (r)IL-2 abrogates the induction of the unresponsive state, it had no effect when administered in the maintenance phase of tolerance (4).

Effect of Cyclooxygenase Inhibitors on Acquired Thymic Tolerance. TxA_2 is synthesized via cyclooxygenase, the key regulatory enzyme of eicosanoid cascade (9, 10). Cyclooxygenase is present in all cell types and transforms arachidonic acid into unstable endoperoxide prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) (20). Formation of biologically active TxA_2 from PGH_2 occurs through the action of an additional enzyme called TxA_2 synthase (9, 20). Two isoforms of cyclooxygenase enzyme exist, a constitutive one and an inducible one (21). Cytokines and growth factors upregu-

late the inducible form which is highly expressed in the thymus of neonatal mice (22, 23) and participates in the early phase of thymocyte maturation by promoting the synthesis of prostaglandins. To identify the specific enzyme involved in TxA_2-mediated unresponsiveness upon thymic recognition of alloantigens, we have introduced a further pharmacological manipulation by giving to Lewis rats aspirin (100 mg/kg/day orally) or glucocorticoids (dexamethasone: 2.5 mg/kg/day subcutaneously) from the day of intrathymic injection of the MHC allopeptide mixture, and transplanting an incompatible WF kidney into them 48 h later. Aspirin by acetylating serine-530 of the prostaglandin H synthase molecule aspecifically inhibits both the constitutive and inducible cyclooxygenase enzymes (21), whereas glucocorticoids selectively inhibit the inducible but not the constitutive form (24, 25). This has been documented in vitro in human dermal fibroblasts (26) and cultured monocytes (27), and in vivo in mice concurrently treated with intravenous lipopolysaccharide (24). By contrast the activity of TxA_2 synthase enzyme is not inhibited by aspirin nor by glucocorticoids (28).

All animals pretreated with aspirin rejected the graft within 7–10 d (Table 1), with severe renal failure as documented by high serum creatinine (3.15 ± 0.06 mg/dl) the day before they became anuric (Fig. 1). Similarly, after daily dexamethasone the kidney allograft failed within 6–9 d (Table 1). At the time of graft rejection, serum creatinine was again elevated (3.04 ± 0.11 mg/dl) (Fig. 1). The fact that both aspirin and dexamethasone eliminate renal allograft unresponsiveness mediated by thymic recognition of class II MHC peptides might indicate that upregulation of inducible cyclooxygenase in stromal cells, the only cell population in the thymus expressing the enzyme, is instrumental in generating cellular events (T cell apoptosis or functional anergy) that promote tolerance in the long run. The present data have major implications for future studies that might be planned to investigate the feasibility of the thymic technique to induce tolerance in humans. One option that has been considered by most investigators so far is the use the thymus inoculation of donor cells or peptides in association with conventional immunosuppression to start with, in order to avoid that a failure of the thymus procedure exposes the patients to an acute irreversible rejection. From the present data, it is clear that glucocorticoids interfere with the mechanism(s) that allow to achieve thymic tolerance. This has therefore to be considered when planning human studies.

Effect of Adoptive Transfer Experiments on Graft Survival. In our model intrathymic injection of class II MHC allopeptides alone was sufficient to induce unresponsiveness to class I and class II MHC incompatible renal allografts. Induction of tolerance through indirect T cell recognition of alloantigens (29), also called "dominant tolerance" has been observed in the cardiac allograft model of tolerance with systemic injection of class I MHC transfected L cells (30) in the mouse and intrathymic injection of soluble class I MHC molecules (31) in the rat. Such a state of dominant tolerance may suggest the presence of a regulatory or suppressor effector mechanism. To investigate this possibility we performed adoptive transfer experiments. A single intravenous dose of spleen cells from long-term kidney graft–tolerized Lewis rats injected into naive Lewis rats that received a WF kidney allograft 48 h later failed to prolong renal graft survival. Thus in all kidney recipients the graft was invariably rejected within 7–9 d (median graft survival: 7 d). Similarly, Lewis rats injected intravenously with spleen cells from normal Lewis rats rejected a subsequent WF kidney allograft within 10 d (median graft survival: 8 d). In both group of rats serum creatinine was elevated at the time of the development of anuria (3.07 ± 0.18 and 3.15 ± 0.13 mg/dl). These findings suggest that the state of tolerance is not due to the presence of suppressor cells, but indicate that the effector mechanisms are most likely related to apoptosis of functionally anergic T cells that circulate through the thymus (4, 19, 32).

Evidence That Acquired Thymic Unresponsiveness to Renal Allograft Is Dependent on the Dose of Peptides Injected into the Thymus. Our initial studies showed that a single intrathymic injection of 100 μg of a mixture of eight synthetic RT1.B$^u\beta$ plus RT1.D$^u\beta$ allopeptides into adult male Lewis rats 48 h before receiving MHC-incompatible WF vascularized renal allograft induced antigen-specific unresponsiveness and prolonged renal allograft survival (3). Recent evidence has became available that the amount of self-antigens presented to maturing thymocytes by stromal cells within the thymic microenvironment is critical for determining the fate of $CD4^+8^+$ immature thymocytes (33). Thus high concentration of these peptides activates the series of events at the early stages of thymocyte development ultimately leads to negative selection of potentially autoreactive cells. By contrast low concentration of the same peptides complexed to self-MHC on stromal cells serves to deliver signals that promote positive selection and full maturation of thymocytes to lymphocytes. In our model we tested the tolerogenicity of lower doses of the same class II MHC allopeptide mixture. Animals intrathymically injected with 1 and 10 μg RT1.B$^u\beta$ plus RT1.D$^u\beta$ allopeptides and receiving WF kidney allografts 48 h later acutely rejected their grafts within 12 and 11 d, respectively (Table 2). All animals had elevated serum creatinine level at the time of development of anuria (1 μg: 2.99 ±

Table 2. *Survival of WF Renal Allografts in Lewis Recipients Intrathymically Injected with Lower Doses of Class II MHC Allopeptides*

	Graft survival	Median
	d	*d*
Peptides (1 μg)	7, 11, 11, 12	11
Peptides (10 μg)	9, 9, 9, 11	9

0.15 mg/dl; 10 μg: 2.89 ± 0.28 mg/dl). These data indicate that lowering the dose of allopeptides injected into the thymus did not allow permanent engraftment of donor kidney allograft and suggest that the amount of donor antigen presented to T cells is critical for triggering intracellular events that promotes functional anergy and or apoptosis of T cells.

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