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Evidence for clonal anergy as a mechanism responsible for the maintenance of transplantation tolerance*

The main stimulus triggering early acute allograft rejection is known to be delivered by the allogeneic "passenger" leukocytes present within the grafts. Once these cells have been replaced by cells of recipient origin, subsequent rejection episodes are generally less frequent and less acutely destructive. How this replacement affects the cell populations responsible for allograft rejection is not known. Here we report that rat alloreactive non-cytotoxic AS ($RT1^l$) anti-August ($RT1^c$) $CD4^+$ T cells, that were shown to be specific for $RT1.B^{c+}$ August spleen stimulators, were able to cause acute rejection of normal August kidney allografts transplanted into sublethally irradiated AS recipients. These cells, however, failed to reject passenger cell-depleted (PCD) August kidneys, despite the substantial expression of $RT1.B^{c+}$ products on the graft tubular epithelium. In experiments *in vitro*, August kidney tubular epithelial cells expressing $RT1.B^{c+}$ antigens were found to be unable to stimulate the alloreactive T cells to proliferate. Moreover, preincubation with class II-positive August kidney epithelial cells specifically abrogated the alloreactivity of the T cells. Adding recombinant interleukin-2, however, restored the response to alloantigens. These results are consistent with the hypothesis that T cell populations capable of mediating early acute allograft rejection are different from those mediating late rejection, when donor passenger leukocytes are no longer present. They also suggest clonal anergy as one of the mechanisms responsible for maintaining long-term transplantation tolerance.

1 Introduction

It has been proposed that the recognition of donor alloantigens during the process of allograft rejection by the recipient's $CD4^+$ T cells is achieved through two different pathways [1, 2]. The first pathway, or direct pathway, involves the direct recognition by T cells of the polymorphism of MHC class II alloantigens displayed at the surface of donor allogeneic stimulators. In the second pathway, or indirect pathway, donor alloantigens are recognized by the recipient's T cells in the same way as any nominal protein antigens, *i.e.* as processed peptides associated with self-MHC class II molecules. Although both types of cells have been shown to be present in alloreactive T cell populations *in vitro* as well as *in vivo* [3–7], the extent of their involvement in allograft rejection is unknown. Recently, in an experimental model of kidney transplantation in the rat, it was observed that the ability of direct pathway-sensitized $CD4^+$ T cells to cause the rejection of renal allografts was strictly dependent on the presence within the grafts of donor passenger leukocytes [8]. Once these cells were no longer present, the direct-pathway-sensitized $CD4^+$ T cells failed to cause rejection. These observations are consistent with the idea that direct-pathway $CD4^+$ T cells play a dominant role in early acute rejection of kidney allografts, but do not intervene in late rejections when incompatible donor passenger cells are presumably no longer present within the allograft.

A lack of expression of MHC class II molecules by the target cannot explain the failure of direct-pathway $CD4^+$ T cells to cause rejection of passenger cell-depleted (PCD) kidney allografts. It has been shown previously that PCD kidneys contain at least an equal amount of donor MHC class II alloantigen as found in normal allografts [9, 10], and these molecules are expressed by the epithelial cells (EC) of the renal tubules [8–10]. The reason why PCD allografts, although expressing the correct ligand for the antigen receptor of direct pathway $CD4^+$ T cells, fail to activate these cells is not known. However, if T cells require two signals for activation, as has been suggested [11, 12], it is possible that PCD allografts, although expressing MHC class II products, do not deliver the "second" signal.

To verify this hypothesis, we tested isolated rat kidney tubular EC for their capacity to stimulate direct pathway-sensitized alloreactive $CD4^+$ T cells. Here, we report that the epithelial cells did not stimulate a proliferative response among the alloreactive T cells. Instead they were able to induce a state of specific unresponsiveness, or anergy, that made the T cells refractory to subsequent challenge with alloantigen. These data provide evidence for clonal anergy being one of the mechanisms involved in the long-term maintenance of unresponsiveness observed in several models of vascularized organ allografts.

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Abbreviations: **EC:** Kidney tubular epithelial cells **PCD:** Passenger cell-depleted kidney

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2 Materials and methods

2.1 Animals

Male AS (RT1.A^{l}.B^{l}) rats were supplied by the National Institute for Medical Research (Mill Hill, GB). Male August (RT1.A^{c}.B^{c}) rats were purchased from Olac Harlan Ltd (Oxon, GB). (AS × August)F_1 were raised in the breeding unit of the Royal Postgraduate Medical School.

2.2 Reagents

Human rIL-2 (200 U/ml in PBS with 1 mg/ml BSA) was obtained from Boehringer Mannheim (Lewes, GB). Collagenase A from *Clostridium histolyticum* (Boehringer, Mannheim), DNase type IV from bovine pancreas (Sigma Chemicals, Poole, GB) and pronase type XIV from *Streptomyces griseus* (Sigma Chemicals) were used for tissue digestion of isolated kidneys (see below).

2.3 Antibodies

MRC-OX27 (mouse anti-rat RT1.A^{c} molecules) and MRC-OX6 (mouse anti-rat RT1.B molecules) monoclonal antibodies (mAb) were from Serotec (Oxford, GB). 3A1E-12H7 (mouse anti-human CD5) mAb was purchased from Seralab (Irvine, GB). FITC-coated sheep anti-mouse Ig antibodies were purchased from Amersham International plc (Aylesbury, GB).

2.4 Rat alloreactive T cell lines

The protocol used for isolating and culturing the rat AS anti-August alloreactive T cell lines L12.4 and L12.8 has been previously described [8]. Briefly, L12.4 and L12.8 were isolated from AS spleen T cells and maintained in RPMI 1640 (Flow Labs, Irvine, GB) supplemented with 10% heat-inactivated FCS (Globepharm, Esher, GB), 2 mM L-glutamine, 1 mM sodium pyruvate, 28 mM $NaHCO_3$, 100 U/ml penicillin/streptomycin and 5×10^{-5} M 2-ME, by repetitive stimulations with irradiated August APC. August APC were isolated by incubating spleen cells for 60 min at 37 °C in plastic petri dishes. After gently washing the dishes with 5 × 5 ml of prewarmed medium, the adherent cells were harvested in PBS at 4 °C. The cells were then irradiated (30 Gy) and used as allogeneic APC to stimulate L12.4 and L12.8 cells. The lines were α,β TCR^{+}, CD4^{+}, CD8^{-} and non-cytotoxic. They were shown to be directly activated by RT1.B^{c} molecules expressed on August stimulator cells and, thus, belong to the direct pathway of sensitization. They were also shown to specifically produce IL-2.

2.5 Rat kidney cell culture

Kidneys, sterilely, taken from normal AS or August rats, were teased into small pieces (approx. 2 mm^3) in Hanks' medium (Flow Labs). The explants were incubated in a 75-cm^2 tissue culture flask (3084, Falcon, Plymouth, GB) in Hanks' medium containing 0.03% pronase, 0.01% DNase and 0.01% collagenase for 12 h at 4 °C. The suspension was then transferred into 10 ml conical tubes (Sterilin, Stone, GB) and placed on a multiaxial rotator for 30 min at 37 °C. Complete dissociation of the tissue was carried out by vigorously blowing the pieces with medium. After spinning at 500 × g for 5 min, the cell pellet was resuspended in DMEM (Flow Labs) containing 5% FCS, and dispensed in petri dishes (3003, Falcon). After overnight incubation at 37 °C, the dishes were washed twice with 5 ml of medium to remove cell debris and nonadhering cells. The plastic-adherent kidney EC were then cultured for 4–6 days in DMEM with 5% FCS; at that time the cells usually covered the whole dish. To harvest the cells, dishes were gently washed three times with 5 ml of PBS and then trypsinized for 3 min at 37 °C with Trypsin-EDTA solution (Flow Labs). EC were washed twice in PBS containing 10% FCS before being used in the assays.

RT1.B molecules and increased amount of RT1.A molecules were induced on isolated kidney EC by culturing the cells for 4 days in medium containing 50% L12.4 culture supernatant. L12.4 supernatant was harvested 3 days after stimulation of 3×10^5 L12.4 cells/ml with 1×10^5 irradiated August APC/ml as described [8]. After spinning at 500 × g for 5 min, the supernatant was filtered with 0.2 μm filters (Flow Labs) and stored as aliquots at −20 °C.

2.6 Immunofluorescence

To assess the expression of MHC class I and class II molecules on cultured kidney EC, cells were harvested from culture and incubated for 45 min at 4 °C with MRC-OX27 or MRC-OX6 mAb (undiluted supernatants). Cells incubated with 3A1E-12H7 were used as negative controls. After two washes with RPMI supplemented with 5% FCS, cells were incubated with FITC-sheep anti-mouse Ig antibodies (1/10 dilution) for 35 min at 4 °C. Cells were then washed once and fixed in 1% paraformaldehyde solution in PBS. Positive cells (percent) were determined with an Epics Profile (Coulter Electronics, Luton, GB).

2.7 Proliferation assay

August-specific proliferation was assayed in U-bottom 96-well micro-plates (Flow Labs) in RPMI 1640 supplemented as above. Responder cells (50×10^3/well) were mixed with either irradiated (30 Gy) August splenic APC, or irradiated (50 Gy) AS or August kidney EC harvested from 4–6 day cultures. The plates were incubated for 24, 48 or 72 h at 37 °C in 5% CO_2-atmosphere. After pulsing with [^{3}H] dThd (1 μCi/well) (Amersham International plc) for 12 h, the plates were harvested and radioisotope incorporation was counted with an LKB 1219 liquid scintillation counter (Pharmacia LKB, Milton Keynes, GB).

2.8 Mixed lymphocyte-kidney cell reaction

L12.4 and L12.8 cells (50×10^3/well) were incubated with cultured kidney EC (25×10^3/well) overnight at 37 °C in U-bottom 96-well microplates in RPMI 1640 supplemented as above. Irradiated August APC (20×10^3/well) were then added to each well and the plates were cultured for further 24, 48 or 72 h. [^{3}H] dThd (1 μCi) was added to each well for

the last 12 h of the culture, and incorporation was assessed as above.

2.9 CTLL assay

Culture supernatants were tested for the presence of IL-2 using the IL-2-dependent cell line CTLL-2 [12]. CTLL-2 cells were maintained in culture in medium containing 5 U/ml human rIL-2. For testing, the cells were harvested, washed three times and starved for 12 h in IL-2-free medium. The cells were then washed twice further and plated in flat 96-well plates (Flow, Irvine, GB) at 5×10^3 cells/well in 50 ml medium. Each well then received 100 µl of the culture supernatant to be tested. Twelve hours later, 1 µCi of [^{3}H] dThd was added to each well. After a further 8-h period of culture, [^{3}H] dThd incorporation was assessed as described above.

2.10 Kidney transplantation

AS recipients were given a 5-Gy dose of γ-radiation from a ^{135}Cobalt source (National Institute for Medical Research, Mill Hill, GB). After 24 h they were orthotopically transplanted with left kidneys from August donors. Experimental animals received various numbers of alloreactive T cells, intravenously in 0.5 ml PBS within 4 h following transplantation. Contralateral nephrectomy was performed 7 days later. The functional state of the allografts was assessed by determining blood urea nitrogen of the transplanted animals at regular intervals by a colorimetric urease method. The assay kit was supplied by Sigma Chemicals.

The depletion of donor passenger leukocytes from August or (AS × August)F_1 kidneys was carried out by "parking" for >50 days the kidneys into primary AS recipients, as described [13]. Protection of the grafts against rejection during its temporary residence into the primary AS hosts was achieved by giving cyclosporin A (Sandoz, Basel, Switzerland) to the recipients (10 mg/kg/day for 10 days after transplantation). Only grafts with a satisfactory renal function (blood urea nitrogen <13 mmol/l) were used as PCD allografts and retransplanted into secondary AS recipients for rejection studies as described above.

3 Results

3.1 Rat alloreactive CD4$^+$ T cells activated through the direct pathway of sensitization do not reject PCD kidney allografts

Previous studies have shown that the AS anti-August alloreactive CD4$^+$ T cell line L12.4, when adoptively transferred to irradiated AS recipients, was capable of rejecting transplanted August kidneys, provided passenger leukocytes of August genotype were present within the allografts [8]. In the present study, we wished to confirm these findings with a different AS anti-August alloreactive T cell line, L12.8. Like L12.4, L12.8 is CD4$^+$, non-cytotoxic, and is specific for RT1.B^c products presented by August splenic stimulators (data not shown). However, although L12.4 has been shown to be Vβ10 and Vβ8.5 TcR$^-$ and to contain 90–95% Vβ8.2 TcR$^+$ cells, L12.8 has cells expressing Vβ10 and Vβ8.5 TcR, but does not appear to be Vβ8.2 TcR$^+$ (Alam et al., manuscript in preparation).

AS rats, after being given 5 Gy irradiation, were transplanted with normal August kidneys or with PCD August kidneys. At completion of the transplant, the recipients were injected intravenously with L12.8 cells. The survival and the function of the allografts are presented in Table 1. The control animals, having received normal August grafts but no cells, enjoyed long-term survival with only a moderate rise in serum urea on day 10 that was followed by spontaneous recovery. When the recipients were injected with L12.8, normal August allografts underwent acute rejection with high levels of serum urea nitrogen on day 10. All the animals in these groups died within 11 days. As few as 1×10^6 L12.8 cells were sufficient to cause the acute rejection of the August kidneys. By contrast, PCD August kindeys did not sustain acute rejection after retransplantation into the irradiated secondary AS recipients that received L12.8 cells. Even injecting a dose of 10×10^6 L12.8

Table 1. The direct-pathway-sensitized alloreactive CD4$^+$ T cell line, L12.8, causes rejection of normal August kidney allografts but does not reject retransplanted (ReTx) passenger leukocyte-depleted August kidney allografts

Cells injected at the time of Tx	Allograft	No. of animals(n)	Serum urea level (mmol/l)					Median survival time (days)
			day 7[a]	day 10	day 14	day 21	day 28	
–	August Tx[b]	5	8.4 ± 0.9	37.2 ± 18.7	21.4 ± 4.8	16.1 ± 2.4	11.6 ± 2.3	>50
10×10^6 L12.8	August Tx	4	8.1 ± 0.8	95.9 ± 13.2	–	–	–	11
4×10^6 L12.8	August Tx	3	11.4 ± 1.3	83.2 ± 22.3	–	–	–	10
2×10^6 L12.8	August Tx	3	11.2 ± 0.3	ND[d]	–	–	–	11
1×10^6 L12.8	August Tx	2	10.4 ± 0.1	ND	–	–	–	11
10×10^6 L12.8	August ReTx[c]	4	9.6 ± 0.2	34.8 ± 27.4	21.4 ± 3.4	15.8 ± 2.0	17.8 ± 6.3	>50

a) day after transplantation (Tx).
b) AS recipients were irradiated (5 Gy) from a Co source. After 24 h, they were transplanted with normal August kidney allografts.
c) AS recipients were irradiated (5 Gy) from a Co source. After 24 h, they were transplanted with PCD August kidney allografts.
d) ND; Not determined, rats clinically uremic.

Table 2. The direct-pathway-sensitized alloreactive CD4+ T cell line, L12.4, fails to cause rejection of passenger leukocyte-depleted (ASxAugust)F_1 kidney allgorafts retransplanted into non-irradiated AS recipients

Cells injected at the time of Tx	Allograft	No. of animals(n)	Serum urea level (mmol/l) day 7[a)]	day 10	day 14	day 21	day 28	Median survival time (days)
–	(ASxAugust)F_1 ReTx[b)]	4	6.5 ± 0.9	11.1 ± 6.8	11.2 ± 3.0	12.4 ± 4.8	11.0 ± 2.5	> 50
10×10^6 L12.4	(ASxAugust)F_1 ReTx	4	9.2 ± 2.5	11.7 ± 2.3	11.1 ± 2.0	10.8 ± 1.5	11.1 ± 2.4	> 50

a) Day after transplantation.
b) AS recipients were transplanted with PCD (ASxAugust)F_1 kidney allografts.

cells did not affect the survival of the retransplanted allografts (median survival time > 50 days).

The ability of direct pathway CD4+ T cells to reject kidney allografts depleted of passenger cells was also tested in a retransplantation model where the second AS recipients did not receive γ-irradiation treatment. The experimental system involved transplanting PCD (AS × August)F_1 kidney allografts into normal AS recipients. Under these conditions, retransplanted F_1 allografts are fully accepted by their second hosts (Table 2; [14]). This contrasts with the fate of normal (AS × August)F_1 primary kidney allografts that are always rejected within 11 days [14]. In attempts to restore the rejection of retransplanted PCD F_1 grafts, L12.4 cells were injected perioperatively into the AS recipients. Table 2 displays the results of the experiment. None of the retransplanted F_1 grafts underwent rejection.

3.2 Tubular EC isolated from August kidneys fail to stimulate a proliferative response from AS anti-August alloreactive T cells

EC of PCD kidney allografts are known to express donor MHC class II alloantigens at the cell surface [8–10] and, therefore, represent a potential stimulus for direct pathway recipient's CD4+ T cells. However, the above observation that donor PCD kidney allografts are not rejected by direct pathway alloreactive CD4+ T cells, suggests that, at least *in vivo*, the EC fail to stimulate these cells. To investigate this defect, EC were isolated by enzymic digestion of whole August kidneys and cultured in minimum essential medium. The cells were then tested for their capacity to stimulate an *in vitro* proliferative response from the direct pathway-sensitised AS anti-August alloreactive T cell lines, L12.4 and L12.8.

After 4–6 days in DMEM supplemented with 5% FCS, the EC were usually confluent. Immunolabeling with an anti-keratin antibody revealed that a majority of these cells

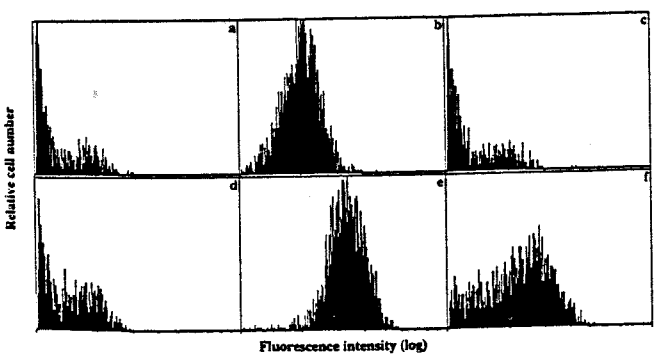

Figure 1. Fluorocytometric analysis of rat EC from August kidneys, after 5 days of culture in DMEM, for the cell surface expression of RT1.A and RT1.B products. EC were cultured in medium alone (a, b, c) or in medium supplemented with 50% of L12.4 culture supernatant (d, e, f). After 5 days, the cells were harvested and labeled with 3A1E-12H7 (mouse anti-human CD7) (a, d), MRC-OX27 (mouse anti-rat RT1.A[c)] (b, e) or MRC-OX6 (mouse anti-rat RT1.B) (c, f) mAb, and FITC-sheep anti-mouse Ig antibodies.

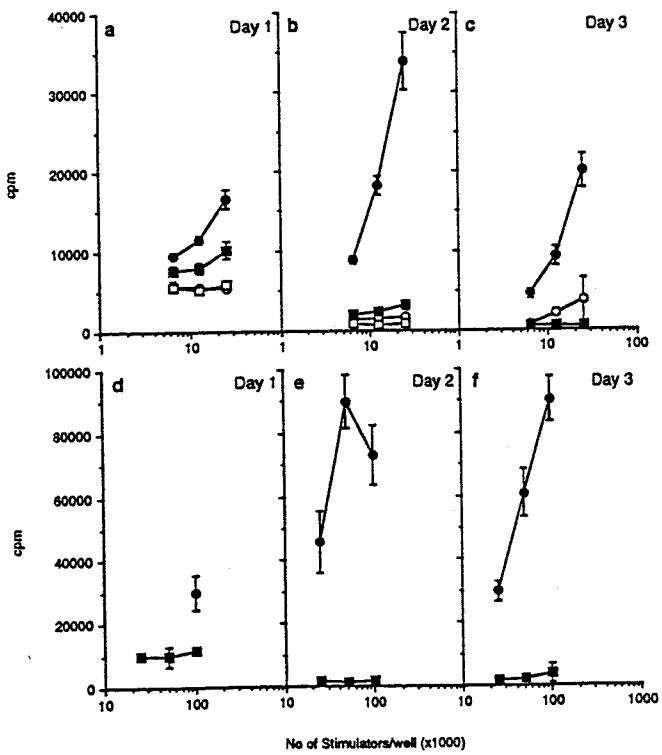

Figure 2. Comparison of the proliferative responses given by the AS anti-August alloreactive CD4+ T cell lines L12.4 and L12.8 when stimulated by August splenic APC or by cultured August EC expressing RT1.B[c)] products. L12.8 (a, b, c) and L12.4 (d, e, f) (50 × 10^3 cells/well) were stimulated in standard *in vitro* proliferation assays (see Sect. 2) with irradiated AS (○) or August (●) splenic APC, or with irradiated AS (□) or August (■) RT1.B+ EC. Responders were harvested after 1, 2 or 3 days of culture, and [^{3}H] dThd incorporated during the last 8 h of culture was counted. Results are expressed as mean cpm of quinteplicate. For each point, SD is indicated by vertical bars.

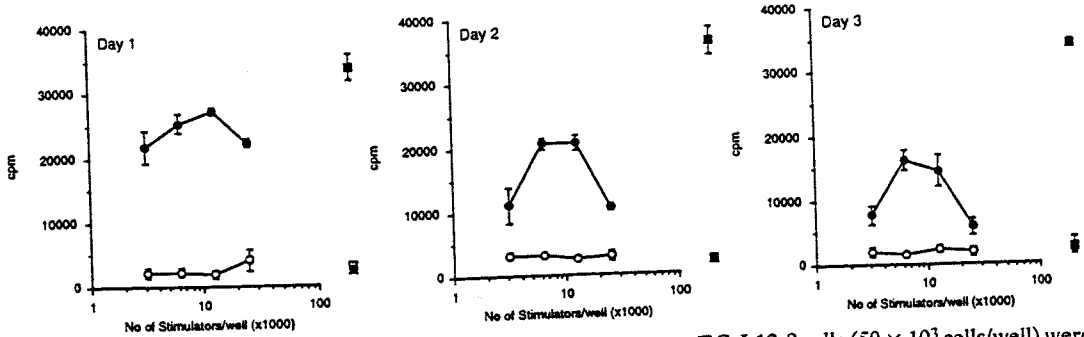

Figure 3. IL-2 secretion by L12.8 cells stimulated with August kidney EC. L12.8 cells (50 × 10^3 cells/well) were stimulated with AS (○) or August (●) EC expressing RT1.B products in culture microplates. After 1, 2 or 3 days, 100 µl of culture supernatant were taken from each well and assayed for IL-2 content with CTLL-2 cells (see Sect. 2). Controls included supernatants taken from cultures of L12.8 cells alone (▲), or stimulated by AS (□) or August (■) splenic APC (25 × 10^3 cells/well). Results are expressed as mean cpm of quinteplicate. For each point, SD is indicated by vertical bars.

contained intracytoplasmic filaments of cytokeratin, a general feature of epithelial cells (data not shown). Fluorescence-activated flow cytometry studies showed that cultured EC did not express MHC class II products (Fig. 1). However, culturing the cells in the presence of L12.4 culture supernatant induced MHC class II antigens at the cell surface (Fig. 1). Percentage of positive cells ranged from 63% to 91%. As shown in Fig. 2, RT1.B^{c+} August EC were able to stimulate weak specific proliferation in L12.4 and L12.8 on day 1, but no response was detectable on days 2 or 3. This was in sharp contrast with the responses generated by August splenic APC, where a strong peak of [^{3}H] dThd incorporation was observed on day 2.

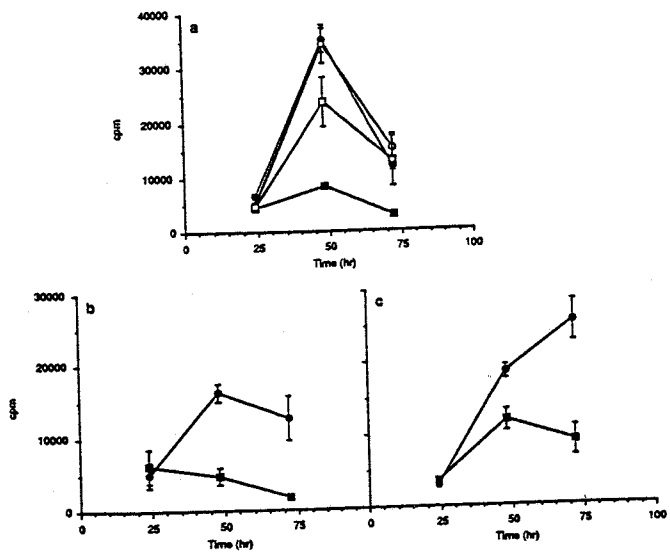

Figure 4. Effect of pre-exposure to RT1.B^{c+} EC on the ability of L12.4 and L12.8 cells to respond to August spleen APC. L12.4 (a) and L12.8 (b, c) cells (50 × 10^3/well) were preincubated with irradiated RT1.B^{l+} (●) or RT1.B^{l-} (○) AS EC or RT1.B^{c+} (■) or RT1.B^{c-} (□) August EC (25 × 10^3/well) for 16 h. Irradiated August spleen APC (20 × 10^3) were then added to each well. Responders were harvested after 1, 2 or 3 days of culture, and [^{3}H] dThd incorporated during the last 8 h of culture was counted. In c, rIL-2 (2 U/ml) was added together with the irradiated August APC. Data in b and c were obtained from the same experiment. Results are expressed as mean cpm of quinteplicate. For each point, SD is indicated by vertical bars.

3.3 RT1.B^{c+} EC isolated from August kidneys stimulate a weak, but specific, production of IL-2 from AS anti-August alloreactive T cells

The capacity of RT1.B^{c+} August EC to stimulate specific IL-2 production from direct pathway alloreactive T cells was also examined. The IL-2 content of supernatants collected from cultures of L12.8 cells stimulated by August EC was determined with the IL-2 dependent cell line, CTLL-2. As seen in Fig. 3, L12.8 cells specifically secreted IL-2 in response to irradiated RT1.B^{c+} August EC, but not when exposed to MHC class II-positive AS EC. However, the quantity of [^{3}H] dThd taken by CTLL-2 cells in the assay was moderate in comparison to the amount incorporated when supernatants from cultures of L12.8 cells stimulated with irradiated August splenic APC, were tested (Fig. 3). Moreover, the quantity of IL-2 produced by L12.8 stimulated by August APC could support a maximum proliferation of CTLL-2 cells (as assessed by titrating with rIL-2, data not shown) for at least 3 days (Fig. 3). L12.8 cells stimulated by August EC, however, did not produce enough IL-2 for the CTLL-2 cells to reach their plateau of maximum proliferation at any time during the assay (Fig. 3). Instead, it appeared that the amount of IL-2 produced fell steadily, as assessed by the lower rates of [^{3}H] dThd incorporation seen on day 2 and 3.

3.4 Exposure to RT1.B^{c+} EC isolated from August kidneys induces a state of specific non-responsiveness in the direct-pathway alloreactive AS anti-August $CD4^+$ T cells

To investigate whether L12.4 and L12.8 had developed non-responsiveness, or anergy, as a consequence of co-culture with August EC, they were incubated overnight in microculture plates in the presence of irradiated EC. Following this, irradiated August spleen APC were added to each well and the proliferation of the responder cells was then assessed by measuring [^{3}H] dThd uptake. Control cultures of L12.4 and L12.8 were incubated overnight with AS strain EC. As shown in Fig. 4a, L12.4 cells that had been previously exposed to AS EC mounted a substantial proliferation response. Cells pretreated with RT1.B^c-non-expressing August EC also proliferated well to August APC, while cells that were pre-exposed to RT1.B^{c+} August EC only gave a weak proliferation, indicating that this latter

pretreatment induced a functional inactivation of the cells. The reduction in response observed was ~sevenfold relative to cells exposed to AS EC. This inactivation required exposure to EC that expressed RT1.B^c products as RT1.B^{c-} August EC and AS EC were unable to induce unresponsiveness. Similar results were obtained with L12.8 cells (Fig. 4b). Adding rIL-2 together with the irradiated August spleen APC, however, restored the capacity of L12.8 cells preincubated with RT1.B^{c+} EC to generate a proliferative response (Fig. 4c), suggesting that pre-exposure to RT1.B^{c+} EC prevented L12.8 cells from secreting sufficient IL-2 to induce a proliferative response.

Taken together, the above data indicate that, once expressing class II MHC antigens, EC have the capacity to anergize alloreactive CD4$^+$ T cells, *i.e.* to make them refractory to subsequent alloantigen challenge.

4 Discussion

The absence of costimulation in T cell responses to antigens is known to lead to the lack of clonal expansion and to the development of a state of specific non-responsiveness that makes the T cells refractory to subsequent stimulations by antigen/costimulator complexes [15, 16]. The inactivation of T cells by failure to provide costimulation suggest that, for most tissues, not expressing costimulatory ligands would ensure protection against autoimmune self reactivity. In tune with this concept is the present observation that, unless they contain donor "passenger" leukocytes, kidney allografts are not susceptible to rejection by direct pathway alloreactive CD4$^+$ T cells despite expressing specific TcR ligands. This would imply that PCD allografts do not provide the costimulatory signal required for T cell activation, therefore protecting themselves against potentially reactive T cells. Our finding that, *in vitro,* allogeneic class II-positive kidney EC have the capacity to induce specific non-responsiveness in direct pathway alloreactive CD4$^+$ T cells, suggests that in PCD allografts, TcR engagement by EC, putatively lacking accessory ligands, would prevent direct pathway T cells from rejecting the grafts. Most importantly, this would imply that the depletion of donor hemopoietic "passenger" cells from allografts, that occurs in situations where pharmaceutical immunosuppression is applied to prevent early acute rejection, would lead to a gradual induction of specific immune tolerance among direct pathway CD4$^+$ T cells.

Induction of immune non-responsiveness by non-hemopoietic cells expressing MHC class II products is not new. Human and mouse keratinocytes [17, 18], and mouse pancreatic β cells [19, 20], have been reported to induce a specific inactivation state similar to what we observed in our system using rat kidney cells; *i.e.* pre-exposure to MHC class II products associated with antigen at the surface of these non-hemopoietic cells gave a negative signal to the T cells, rendering these unable to respond to subsequent antigenic challenges. More recently, MHC-class II-positive human T cells were also shown to be able to induce anergy among other T cells [21].

Wuthrich et al. [22], and later Hagerty and Allen [23], showed that EC from the renal proximal tubules can stimulate *in vitro* MHC class II-restricted responses against soluble antigens from specific T-T hybridoma clones. These findings contradict our observations. In our system, isolated tubular EC were unable to stimulate a specific proliferative response from alloreactive CD4$^+$ T cells, even after up-regulation of cell surface MHC class II-expression, confirming the results of Bishop et al. [24] using cultured human kidney tubular EC. To conciliate the two types of observation, one could propose the ability of renal tubular EC to modulate their state of immunogenicity. This modulation could reflect the capacity of EC to regulate the expression of the requisite accessory signal needed for full T cell activation. A possible factor that may influence the immunogenicity of renal EC is the exposure to different cytokines that in turn would induce or inhibit second signal expression. A simpler alternative hypothesis to explain these conflicting results is that requirement(s) for activation might be different for normal CD4$^+$ T cells and T-T hybridoma clones.

Anergic T cells have been described to have a greatly reduced capacity to produce IL-2 in response to antigen stimulation [16]. Recently, it was reported that anergic T cells have a defect in antigen-induced transcription of the IL-2 gene [25]. Analysis of the gene promoter revealed that the transcription factor AP-1 was specifically down-regulated. Addition of exogenous IL-2 in the system, however, restored both antigen resposiveness and AP-1 activity. In our experiments, the addition of rIL-2 together with allogeneic spleen APC also restored the capacity of non-responsive direct-pathway alloreactive CD4$^+$ T cells to mount a proliferative response, suggesting that the phenomenon we observed was, indeed, anergy. According to these observations, one might also suggest that, *in vivo,* the production of IL-2 would restore the capacity of direct-pathway alloreactive CD4$^+$ T cells to cause rejection of PCD kidney allografts. This idea is supported by the recent finding that, in double transgenic mice expressing the allogeneic class I molecule H-2K^b on the β-cells of the islets of Langerhans, and a H-2K^b-specific antigen receptor on T lymphocytes, autoimmune destruction of the islet cells does not occur unless IL-2 is locally produced [26].

One argument that has been raised in discussion is that PCD kidney allografts may contain sufficient, suppressive T cells to negate the graft-rejecting properties of L12.4 and L12.8. According to this proposition, it could be unnecessary to suggest that RT1.B^{c+} EC have induced anergy. Two observations make us think that this explanation is unlikely. The first is that the experiments reported here show that anergy is induced *in vitro* without participation of a suppressive T lymphocyte population. The second is that retransplantation of August PCD kidneys into unirradiated naive AS recipients is followed by rejection [2], indicating that the number of suppressive T cells that may be transferred with the retransplanted kidney must be small. Considering that less than 10^6 L12.8 cells have been shown in our exeriments to cause acute rejection of normal August kidneys in the irradiated naive AS recipients, and 10^7 L12.8 cells fail to reject PCD August kidneys, it would be necessary to postulate an extraordinary effectiveness of the suppressive population transferred in the PCD kidney.

The last issue to be discussed arises from the observation that non-irradiated AS recipients fail to reject retransplanted heterozygous (AS × August)F$_1$ PCSD kidney

allografts [14] and this study. This contrats with the fate of retransplanted homozygous August kidneys that have, in non-irradiated AS recipients, a median survival of 22 days [2]. In the F_1 transplants, both AS and August MHC class II are presumably expressed by the epithelium of the renal tubules. Moreover, because of the shared haplotype, the F_1 kidney EC have the ability to express AS MHC class II molecules associated with endogenously-synthesized peptides of August genotype, and, thus, can be recognized by indirect-pathway alloreactive $CD4^+$ T cells. According to our *in vitro* observation that class II-positive kidney EC can anergize specifically alloreactive $CD4^+$ T cells, one can suggest the hypothesis that retransplanted F_1 allografts have the capacity to inactivate alloreactive $CD4^+$ T cells of either direct- or indirect-sensitization pathways, and, therefore, to protect themselves against rejections mediated by both types of $CD4^+$ T cells.

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