Evidence That the T Cell Repertoire of Normal Rats Contains Cells with the Potential to Cause Diabetes. Characterization of the CD4+ T Cell Subset That Inhibits This Autoimmune Potential

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Summary

Diabetes was induced in a normal nonautoimmune rat strain by rendering the animals relatively T cell deficient using a protocol of adult thymectomy and sublethal γ irradiation. All male rats and 70% of females developed an acute syndrome with severe loss of weight and hyperglycemia. Diabetes in these lymphopoenic rats was associated with extensive insulitis involving $CD4^+$ and $CD8^+$ T cells and macrophages. The $CD8^+$ T cells were essential for the development of diabetes but not insulitis. The autoimmune diabetes and insulitis were completely prevented by the injection of a particular $CD4^+$ T cell subset, isolated from healthy syngeneic donors, of the phenotype $CD45RC^{low}$ T cell receptor α/β^+ $RT6^+$ Thy-1^- OX-40^-. Cells of this protective phenotype, which make up about 5% of thoracic duct lymphocytes, were found to provide help for secondary antibody responses and produce interleukin 2 (IL-2) and IL-4, but no interferon γ, on in vitro activation. These data provide evidence for the presence of autoreactive T cells in the normal immune system of the rat and reveal that in the intact animal these cells are prevented from expressing their autoreactive potential by other T cells.

While it is well established that the clonal deletion of T cells reactive with self-antigens expressed in the thymus represents a major mechanism of self-tolerance, the processes involved in peripheral tolerance to self-antigens expressed extrathymically are less well understood. Two mechanisms have been proposed for the maintenance of self-tolerance in the periphery, one involving the functional inactivation of autoreactive T cells (T cell clonal anergy), and the other involving an active process of suppression where T cells are prevented from displaying their autoreactive potential by other antagonizing T cells. The factors involved in the establishment of T cell anergy have been extensively studied in vitro, but the role of clonal anergy to self-antigen in vivo is less clear (reviewed in reference 1).

A number of organ-specific autoimmune diseases can be induced in rodent strains that do not normally develop autoimmunity by procedures that interfere with normal T cell maturation or by rendering the animals partially T cell deficient (2–4). As described herein, thymectomy and irradiation of a nondiabetic rat strain is sufficient to induce cell-mediated autoimmune diabetes, demonstrating that autoreactive cells against certain peripheral tissues have neither been deleted nor made irreversibly anergic. The ability of a defined subset of T cells, from syngeneic healthy donors, to prevent the development of autoimmunity on transfer to these lymphopoenic animals indicates that an intact immune system contains cells with the capacity to prevent the activation of autoreactive T cells (5).

It has been observed that in response to foreign proteins and to infectious agents the humoral and cell-mediated arms of the immune response are often unequally expressed (6). Although a detailed explanation of this imbalance is still lacking, it clearly rests on the demonstration that T cells are functionally heterogeneous (7–9), and this heterogeneity is a reflection of the repertoire of cytokines produced by different cells (8, 10, 11). Studies on the factors that determine what types of cytokines are induced on exposure to antigen have shown that the nature of the APC and the presence of certain cytokines can promote the development of either Th1- or Th2-type responses (8, 12, 13). Most significantly, IL-4, a product of Th2-type T cells, inhibits the induction of Th1-type responses, suggesting that a potent Th2 reaction is likely to inhibit cell-mediated immunity (14–16). Increasing evidence for similar restricted cytokine production after antigenic stimulation in vivo (17–19) suggests that the immune system exists as a dynamic, finely regulated balance between different types of immune responses, and a similar mechanism may be involved in regulating responses to self-antigens.

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In the rat the $CD4^+$ T cell population can be divided into two functionally distinct subsets based on the expression of exon C of the leukocyte common antigen, CD45, as defined by the OX-22 mAb (20, 21). $CD45RC^{high}$ $CD4^+$ T cells have been shown to be important in cell-mediated immune responses (7, 22) and produce IL-2 and IFN-γ but little IL-4 after in vitro activation (10, 23). In contrast, the $CD45RC^{low}$ $CD4^+$ T cell population produces less IL-2 and IFN-γ but is the more potent producer of IL-4 on activation (10, 24). Consistent with this pattern of lymphokine production, the $CD45RC^{low}$ $CD4^+$ T cells provide the majority of help in secondary antibody responses (22, 25). A previous study from this laboratory (26) revealed that the transfer of separated $CD45RC^{high}$ $CD4^+$ T cells to congenic athymic nude rats led to a fatal wasting disease with severe mononuclear cell infiltrates in a variety of organs, while recipients of the $CD45RC^{low}$ $CD4^+$ T cells remained healthy. Importantly, animals receiving unfractionated $CD4^+$ T cells (a mixture of $CD45RC^{high}$ cells and $CD45RC^{low}$ cells; 2:1) also remained well, indicating that the $CD45RC^{low}$ $CD4^+$ T cells were able to regulate the pathological responses of the $CD45RC^{high}$ $CD4^+$ T cell population. Further studies on the immunoregulatory action of $CD4^+$ T cell subsets in the rat presented herein demonstrate that the development of autoimmune diabetes in immunodeficient rats can be prevented by the transfer of only the $CD45RC^{low}$ $CD4^+$ T cell subset. Detailed characterization of the protective $CD4^+$ subset has shown the phenotype to be TCR-α/β^+ $RT6^+$ $Thy\text{-}1^-$ $OX\text{-}40^-$ $CD45RC^{low}$ $CD4^+$. Cells of this phenotype provide secondary B cell help and produce IL-2 and IL-4 on in vitro activation. The data provide evidence that the maintenance of self-tolerance is in part an active T cell–mediated process, and that the regulatory cells involved may mediate this protection via the production of immunoregulatory cytokines, like IL-4 and IL-10, with the capacity to inhibit cell-mediated autoimmune reactions. A brief report on some of the work described herein has been published elsewhere (5).

Materials and Methods

Animals. PVG.RT1^c, PVG.RT1^u, and PVG.RT7^b strain rats were used from the specific pathogen-free unit of the Medical Research Council Cellular Immunology Unit. PVG.RT1^c and PVG.RT7^b are congenic strains that differ with respect to the allele of the leukocyte common antigen, CD45, that they express. PVG.RT1^c and PVG.RT1^u are congenic strains that differ at the MHC region.

Cells. Rat thoracic duct lymphocytes (TDL)[1] were obtained by cannulation of the duct (27). Cells were collected at 4°C overnight into flasks containing PBS and 20 U/ml heparin.

Antibodies. The mouse mAbs used in these studies were as follows: W3/25 (anti-rat CD4) (28), OX-35 (anti-rat CD4, noncompetitive with W3/25) (29), OX-22 (anti-rat exon C of CD45) (20, 21), OX-32 (anti-rat exon C of CD45, noncompetitive with OX-22) (21, 30), OX-12 (anti-rat Igκ chain) (31), OX-6 (anti-rat MHC class II) (32), OX-7 (anti-rat Thy-1.1) (33), OX-8 (anti-rat CD8) (34), OX-14 and OX-16 (both anti-rat Ig γ 2b, noncompetitive) (35), OX-21 (anti-human C3b inactivator) (36), OX-39 (anti-rat IL-2R) (37), OX-40 (against a cell surface antigen on rat $CD4^+$ T cell blasts) (37), R73 (anti-rat TCR-α/β) (38), HIS 41 (anti-rat CD45 allotype RT7^b) (39), Bu20a (antibromodeoxyuridine) (40); also, a rat mAb, P4/16 (anti-rat RT6a, PVG) (41). Biotinylated mAbs were prepared as described (42). Rabbit anti-mouse Ig (RAM-Ig), FITC-conjugated RAM Ig (RAM-FITC), and FITC-conjugated Fab fragments of RAM Ig (RAM-Fab-FITC) were used.

Isolation of T Lymphocyte Subpopulations. Rat T cell populations were negatively selected from TDL using a rosetting technique as described elsewhere (42). $CD4^+$ T cells were isolated by depletion of B cells and $CD8^+$ T cells using the mAbs OX-12, OX-8, and OX-6. $CD8^+$ T cells were obtained by depletion of B cells and $CD4^+$ T cells using the mAbs OX-12, OX-35, W3/25, and OX-6. The $CD4^+$ T cell population was further fractionated by cell sorting on a FACS II® (Becton Dickinson & Co., Mountain View, CA) on the basis of the expression of exon C of the CD45 molecule after labeling of the isolated $CD4^+$ T cells with mAbs OX-22, and OX-32, and RAM-FITC. $CD45RC^{low}$ $CD4^+$ T cells were also directly isolated by rosette depletion using the mAbs OX-22, OX-32, and OX-8. Single-positive $CD4^+$ thymocytes were isolated by depletion of $CD8^+$ and $CD45RC^+$ cells. The purity of all isolated cells was analyzed on a FACScan® (Becton Dickinson & Co.) by labeling of pre- and postdepletion samples with RAM-FITC.

Flow Cytofluorography. Dual-color flow cytofluorographic analysis of TDL was performed essentially as described (42). Briefly, 10^6 rat TDL were incubated in 50 μl of hybridoma tissue culture supernatant at 4°C for 30 min, washed with PBS containing 0.2% BSA and 10 mM NaN_3, and incubated with RAM-Fab-FITC for 30 min at 4°C. After a further wash the cells were incubated with biotinylated Ab and PE-conjugated streptavidin. After incubation with streptavidin-PE, unconjugated biotin was added at a final concentration of 3 μg/ml for 10 min to reduce cell aggregation. The rat mAb P4/16 (anti-RT6) was detected using FITC-conjugated OX-14 and OX-16 Igs.

Immunohistochemistry. Tissues were removed and either fixed in 10% formal saline and embedded in paraffin wax or frozen in a bath of solid CO_2 and *iso*-pentane. Paraffin sections (5 μm) were stained with hematoxylin and eosin. Cryostat sections (5 μm) were cut and stored with dessicant at 4°C. Staining of cells was performed by the peroxidase technique described in reference 43. Sections were fixed in ethanol, washed, and incubated with mAb for 1 h at 4°C. The bound antibody was detected by incubation at 4°C with a peroxidase-labeled RAM-Ig (Dakopatts Ltd., Copenhagen, Denmark) and 3,3′ diaminobenzidine HCl. The slides were lightly counterstained with Harris' hematoxylin.

Incorporation of Bromodeoxyuridine (BdUr). Thoracic duct–cannulated animals were infused with 0.3 mg/ml 5-bromo-2′-deoxyuridine in PBS containing 1 U/ml heparin at a rate of 2 ml/h for 18 h. TDL were collected throughout this period and cell smears made onto glass slides. Cell smears were fixed in acetone and air dried before incubation in 95% formamide for 35 min in a 67°C waterbath. The slides were removed to PBS at 4°C and stained with Bu20a (antibromodeoxyuridine) mAb by the peroxidase technique as described above.

Measurement of Serum Glucose. The glucose level in serum was detected using a quantitative enzymatic (hexokinase) glucose (HK) reagent (Sigma Diagnostics, Poole, UK).

[1] *Abbreviations used in this paper:* BdUr, bromodeoxyuridine; NOD, nonobese diabetic; RAM, rabbit anti-mouse; TDL, thoracic duct lymphocytes.

Results

Protocol for the Induction of Diabetes. A protocol for the induction of diabetes in rats was developed based on the induction of thyroiditis in normal rat strains by thymectomy and irradiation as described by Penhale et al. (4). Using this protocol both diabetes and thyroiditis occurred concurrently in female PVG.RT1^c strain rats, with an incidence of diabetes ranging from 10 to 53% (44). In our studies PVG.RT1^u rats, which share the same MHC genotype (RT1^u) as the spontaneously diabetic BB rat (45) and the same non-MHC genotype as the strain studied in Penhale's thyroiditis experiments (44), were used for the induction of diabetes. PVG.RT1^u rats were thymectomized at 6 wk of age, rested for 2 wk, and then given a series of four doses of 250 rad γ irradiation 2 wk apart, a cumulative dose of 1,000 rad (5). Rats that had been treated by this protocol were termed Tx-X rats. The animals spontaneously developed disease with high incidence: 98.3% (n = 175) of male rats and 73% (n = 30) of female rats became diabetic. The onset of disease ranged from 3 to 18 wk after the last dose of irradiation (5). The rats experienced acute weight loss and hyperglycemia that proved rapidly fatal. The mean change in body weight, calculated as the difference between weight on the day of last irradiation and weight at the time of diabetes onset was a loss of 23% (n = 16) of starting body weight for diabetic animals compared with a gain of 18% (n = 10) for nondiabetic (female) rats over the same time period. Mean serum glucose levels for diabetic and nondiabetic rats were 542 ± 44 mg/dl and 188 ± 30 mg/dl, respectively.

Evidence for Cell-mediated Autoimmune Diabetes. All animals with clinical signs of diabetes had a focal lymphocytic infiltration in the islets of the pancreas (Fig. 1 *A*). Immunohistochemical analysis of frozen sections revealed extensive infiltration of T cells (Fig. 1 *C*), both CD4$^+$ (not shown) and CD8$^+$ T cells (Fig. 1 *D*), and macrophages, dendritic cells, and NK cells (data not shown). The majority of the T cells in the infiltrate expressed the IL-2R. In contrast, the pancreata of normal PVG.RT1^u rats showed no insulitis (Fig. 1 *B*). In addition, a focal thyroiditis was seen in only 1 of 24 diabetic rats examined, with circulating antithyroglobulin autoantibodies found in the serum of 3 of 36 diabetic rats. Other tissues examined included the salivary gland, small intestine, liver, lung, and kidney; no lymphocytic infiltration was observed in these organs.

To confirm that the lymphocytic infiltration was implicated in the destruction of the β cells of the pancreas, we examined the role of CD8$^+$ T cells in the development of diabetes. Using a protocol modified from Like et al. (46), known to deplete CD8$^+$ T cells in vivo, Tx-X animals were treated with either OX-8 (anti-CD8) or OX-21 mAb (isotype-matched, irrelevant antibody) from the day of the last irradiation for a 2-wk period. As illustrated in Table 1, OX-8 mAb–treated animals failed to develop diabetes in all cases, while the OX-21 mAb–treated control animals remained fully susceptible. Immunohistochemical staining of pancreata from anti-CD8-treated nondiabetic Tx-X animals revealed a periinsulitis made up of CD4$^+$ T cells and macrophages but, CD8$^+$ T cells were not detectable (data not shown). Transfer of syngeneic CD8$^+$ cells from healthy donors to CD8$^+$-depleted Tx-X rats fully restored disease susceptibility. Indeed, the provision of CD8$^+$ TCR-α/β^+ T cells alone was sufficient to mediate diabetes (Table 1). These data strongly support the view that the β cells in the Tx-X rats were destroyed by a cell-mediated immune response and are in accordance with data from the spontaneously diabetic nonobese diabetic (NOD) mouse, where CD8$^+$ T cells are essential for the pathogenesis of autoimmune diabetes (47, 48).

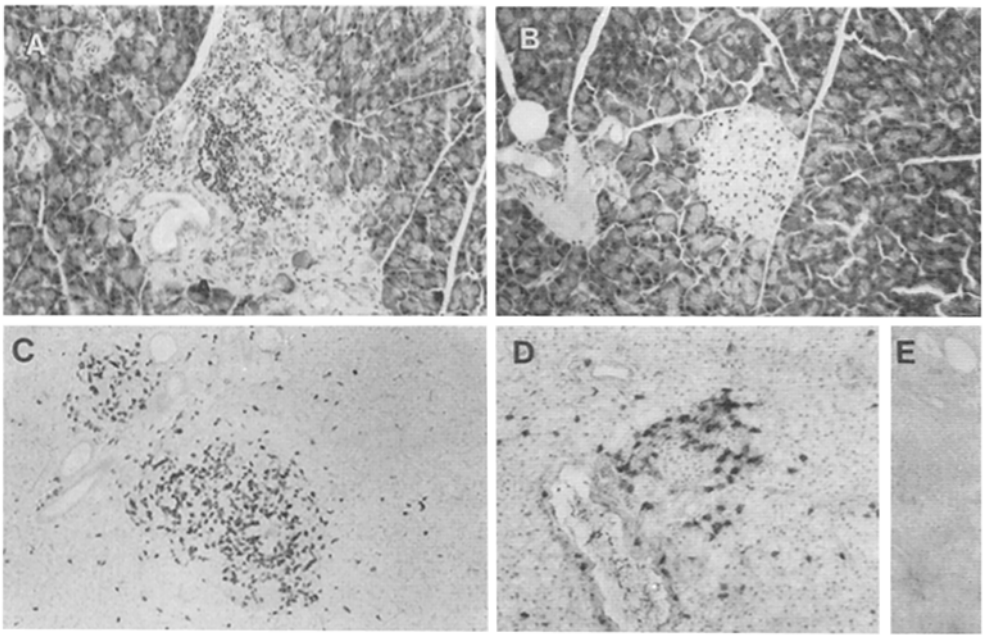

Figure 1. Immunopathology of the pancreas from diabetic Tx-X rats. Paraffin sections of pancreatic tissue from clinically diabetic Tx-X PVG.RT1^u rats (*A*) and normal nondiabetic PVG.RT1^u rats (*B*) stained with haematoxylin and eosin showing the densely stained mononuclear cell infiltrate within and around the islet of the diabetic pancreas (×200). Cryostat sections from a diabetic rat stained with an anti-rat TCR-α/β mAb (*C*) (×150), an anti-rat CD8 mAb (*D*) (×200), and with the negative control mAb OX-21 (*E*) (×200).

Table 1. *Role of CD8⁺ T Cells in the Development of Diabetes in Lymphopoenic Rats*

Antibody treatment*	T cell reconstitution	Incidence of diabetes
OX-8	None	0/11
OX-21	None	10/10
OX-8	CD8‡	7/7
OX-8	CD8⁺ TCR-α/β^+	2/2
None	None	8/8

* OX8 (anti-CD8) and OX21 (negative control antibody) ascites were used at a 1:5 dilution. 1 ml of antibody was administered intraperitoneally on the day of last irradiation and hence thrice weekly for 2 wk. Lymph node biopsies were taken 2 wk after cessation of antibody treatment and analyzed for the presence of any remaining CD8⁺ T cells by immunohistochemistry of cryostat sections. No CD8⁺ T cells were observed in the lymph nodes of OX-8-treated Tx-X rats (data not shown).
‡ 10^7 CD8⁺ cells isolated by rosette depletion (purity, 98%) were injected intravenously 2 wk after cessation of antibody treatment. CD8⁺ TCR-α/β^+ T cells were isolated by cell sorting (purity, 99.1%) and 10^7 cells injected as above.

Table 2. *Prevention of Diabetes on Injection of Syngeneic CD4⁺ T Cells from Normal Donors*

Phenotype of cells injected	No. of cells injected intravenously	Incidence of diabetes
Unfractionated TDL	3×10^7	3/6
CD4⁺ T cells	1×10^7	3/6
CD8⁺ T cells	5×10^6	6/6
CD45RC^{high} CD4⁺ T cells	5×10^6	2/2* 2/2
CD45RC^{low} CD4⁺ T cells	5×10^6	0/6 0/10‡
None	–	32/32

CD4⁺ and CD8⁺ T cells subsets were negatively selected from TDL by rosette depletion as outlined in Materials and Methods. The purity of isolated cells was >96%. The number of separated CD4⁺ and CD8⁺ T cells injected was proportional to the number represented in the inoculum of unfractionated TDL. CD4⁺ T cells isolated by rosette depletion (98.2% pure) were subdivided by fluorescent cell sorting into CD45RC^{high} and CD45RC^{low} subsets (98.5 and 96.2% pure, respectively).
* Six of eight rats developed a lethal wasting disease 2 wk after CD45RC^{high} CD4⁺ T cell reconstitution. The rats were not diabetic but displayed diffuse lung pathology on histological examination. The two remaining animals in the group survived >3 wk postreconstitution and developed diabetes with hyperglycaemia. In a second experiment, two of two rats developed diabetes.
‡ Six animals were reconstituted with CD45RC^{low} CD4⁺ cells obtained by cell sorting. In a second experiment CD45RC^{low} CD4⁺ cells were isolated directly from TDL by rosette depletion (purity, 97%), and 10 animals were reconstituted.

The ability of CD8⁺ TCR-α/β^+ T cells alone to restore diabetes in Tx-X rats pretreated with anti-CD8 mAb suggests that, unlike the spontaneously diabetic BB rat (46), CD8⁺ NK cells are not essential for disease in this diabetes model.

Prevention of Diabetes by the Transfer of Syngeneic CD4⁺ T Cells from Healthy Donors. Previous studies have shown that autoimmunity in rodents rendered lymphopoenic by experimental manipulation (49, 50), or genetically lymphopoenic as the BB rat (51), and autoimmune diabetes in the NOD mouse (52, 53) can be prevented by the transfer of syngeneic CD4⁺ T cells from normal nonautoimmune donors. To examine the role of different CD4⁺ T cell subsets in the prevention of autoimmune diabetes, Tx-X rats were injected with TDL from syngeneic healthy donors on the day of the last dose of irradiation. All Tx-X rats used in the reconstitution studies were male with the expected diabetic incidence of 98–100%. As shown in Table 2, the CD4⁺ T cell fraction of TDL at a dose of 10^7 cells was capable of reducing the incidence of diabetes to 50%. Consistent with the requirement of CD8⁺ T cells for induction of disease, transfer of CD8⁺ T cells did not prevent diabetes (Table 2). However the transfer of 5×10^6 CD45RC^{low} CD4⁺ T cells completely inhibited the development of diabetes and insulitis (Table 2). The reconstituted rats were monitored for a 20-wk period after cell transfer; neither diabetes nor insulitis developed during this time. The transfer of CD45RC^{low} CD4⁺ T cells appears to mediate a sustained suppression of autoimmune diabetes. In contrast, CD45RC^{high} CD4⁺ T cells did not protect against diabetes (Table 2) but induced a lethal wasting disease, with severe leukocytic infiltrates in the lung, similar to that seen in nude rat recipients of this cell type (26).

The protective effect of CD45RC^{low} CD4⁺ T cells was highly dependent on cell dose; 5×10^6 CD45RC^{low} CD4⁺ T cells were capable of protection, while the transfer of 2.5×10^6 of the same cells was insufficient (Fig. 2). This critical dependency on cell dose may be explained by the degree of chimerism observed on transfer of CD45RC^{low} CD4⁺ T cells to the lymphopoenic rats. Two congenic rat strains expressing different allotypes of CD45 were used to study the expansion of CD45RC^{low} CD4⁺ T cells on transfer to Tx-X rats; 5×10^6 CD45RC^{low} CD4⁺ T cells, isolated from healthy PVG.RT7^b strain rats (RT7^b or LCA 1.2 allotype), were used to reconstitute Tx-X PVG.RT1^c strain rats (RT7^a or LCA 1.1 allotype). Fig. 3 illustrates the proportion of donor and host CD4⁺ T cells in the Tx-X recipient 5 wk after cell transfer. As shown, the inoculum of CD45RC^{low} CD4⁺ donor cells gave rise to 50% of the total CD4⁺ T cells in the Tx-X recipient (Fig. 3 *C*). In the 18-h period when the TDL were collected, the number of cells recovered was $\sim 10^8$; of these, $7–8 \times 10^6$ were CD4⁺ T cells of donor origin. If the number of the donor cells recovered is proportional to the number of cells injected, then transfer of 2.5×10^6 cells would generate a 1:2 donor-to-host CD4⁺ T cell ratio; from the dose-response curve (Fig. 2), this appears insufficient to suppress diabetes induction.

To investigate the role of the thymus in the maintenance

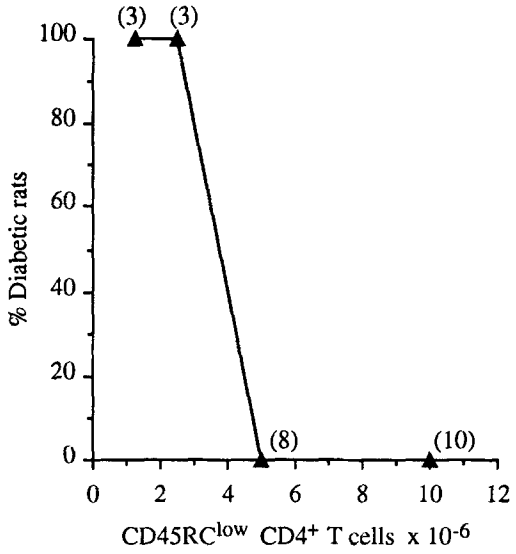

Figure 2. Dose-response curve for the suppression of diabetes by CD45RC^{low} CD4$^+$ T cells. Numbers in parentheses represent the number of animals in each group. CD45RC^{low} CD4$^+$ T cells were negatively selected from TDL by rosette depletion; the purity of isolated cells was 97%. Cells were injected intravenously into Tx-X rats on the day of the last dose of γ irradiation.

Table 3. *The CD45RC^{low} CD4$^+$ T Cell Population That Protects against Diabetes Is Long Lived in the Periphery*

Phenotype of cells injected	No. of cells injected intravenously	Incidence of diabetes
CD45RC^{low} CD4$^+$ TDL	5 × 10^6	0/4
CD45RC^{low} CD4$^+$ Tx-TDL*	5 × 10^6	0/5
CD45RC^{low} CD4$^+$ thymocytes	5 × 10^6	2/10
None	-	6/6

T cell subsets were negatively selected from TDL or thymus by rosette depletion, with the purity of isolated cells >97%.
* Tx-TDL were obtained from healthy rats thymectomized 10 wk before cannulation.

of peripheral CD4$^+$ T cells with the capacity to prevent autoimmune disease, the protective effect of CD4$^+$ T cells from long-term thymectomized adult donors was analyzed. As shown in Table 3, CD45RC^{low} CD4$^+$ T cells from long-term thymectomized donors were at least as potent in preventing diabetes as similar cells from normal donors. Therefore, the protective CD4$^+$ T cell subset is long lived in the periphery and its regulatory effect appears not to be dependent on continued replenishment by the thymus. As described for the prevention of other autoimmune diseases (52, 54, 55), CD45RC^{low} CD4$^+$CD8$^-$ mature thymocytes also gave partial protection from diabetes similar to the protection given by unseparated peripheral CD4$^+$ T cells. Whether the thymocytes themselves have the capacity to suppress autoimmunity or require a maturation event occurring on release into the periphery of the Tx-X host is not known (see Discussion).

Changes in CD4$^+$ T Cell Phenotype on Reconstitution of Tx-X Rats with CD45RC^{low} CD4$^+$ T Cells. The protocol for the induction of diabetes results in severe lymphopoenia. The percentages of T and B lymphocytes in TDL of prediabetic animals shown in Table 4 represent in real terms a threefold decrease in B cell number and a 12-fold decrease in T cell number 5 wk after thymectomy and irradiation. The rate of lymphocyte turnover in Tx-X rats, as measured in vivo by the kinetics of incorporation of the nucleic acid analogue

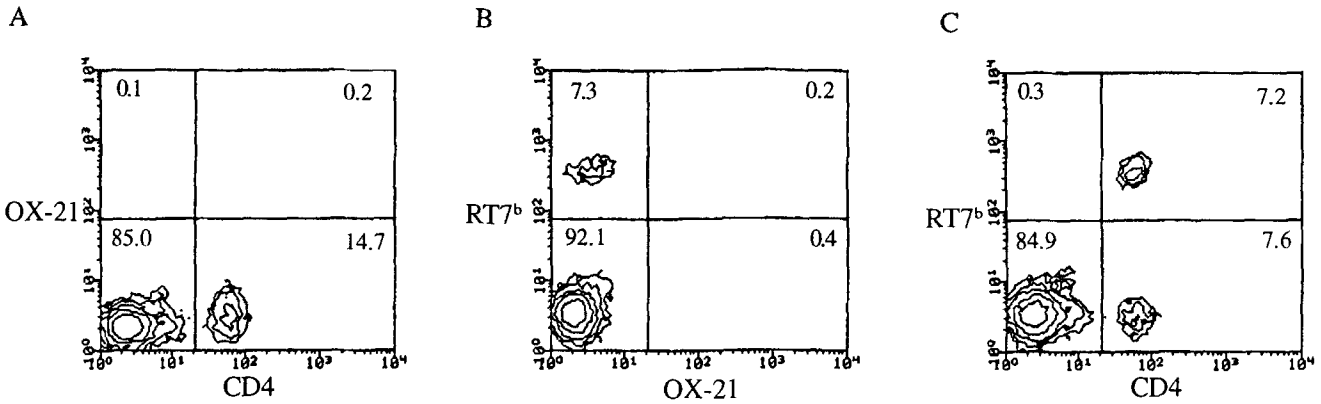

Figure 3. Expansion of CD45RC^{low} CD4$^+$ cells on transfer to the Tx-X host. Dual-color immunofluorescence staining of TDL collected from PVG.RT1^c Tx-X rats 5 wk after reconstitution with CD45RC^{low} CD4$^+$ T cells from congenic PVG.RT7^b rats, which expressed a different CD45 allele (RT7^b or LCA 1.2) to the PVG host (RT7^a or LCA 1.1 allotype). (*A*) Cells labeled with anti-rat CD4 mAb (W3/25) and the negative control mAb OX-21 showing the total percentage of CD4$^+$ T cells after reconstitution. (*B*) Donor-derived cells labeled with the anti-rat RT7^b allele mAb, HIS 41, and the negative control mAb revealing the total percentage of donor cells 5 wk posttransfer. (*C*) Labeling with anti-rat CD4 mAb and the anti-rat RT7^b mAb showing that the donor cells have expanded within 5 wk of transfer to represent 50% of all CD4$^+$ T cells in the reconstituted nondiabetic Tx-X host. Note that all the donor cells have remained CD4$^+$.

BdUr, was rapid, with 20% of cells incorporating the label in 18 h compared with a 1% incorporation over the same time period for normal rats. The persistence of lymphopoenia in the Tx-X rats in the face of this proliferative activity implies that many of the dividing cells died, both in prediabetic and in protected animals. On further phenotypic analysis, the majority of $CD4^+$ and $CD8^+$ T cells from prediabetic animals were large and the $CD4^+$ T cells expressed the activation antigens IL-2R and OX-40 (rat $CD4^+$ T cell blast antigen [37]) and high levels of MHC class II (Table 4). That the majority of both $CD4^+$ (Table 4) and $CD8^+$ T cells were $CD45RC^{low}$ is also consistent with the cells being activated, as the expression of the OX-22 epitope is lost on activation in vitro (7). Further, a significant proportion of the B cell population were blasts. On culturing lymph nodes in vitro from these animals, it was found that there was a high level of cell proliferation in the mesenteric nodes but not the cervical lymph nodes (Els Meussen, personal communication), suggesting that lymphocyte activation was driven predominantly by antigens in the gut. As Table 4 also illustrates, on transfer of $CD45RC^{low}$ $CD4^+$ T cells, the number of activated IL-$2R^+$ OX-40^+ $CD4^+$ T cells in the Tx-X host was reduced by 50%, indicating a general dampening down of the highly activated state of lymphocytes in prediabetic Tx-X rats.

Phenotypic and Functional Heterogeneity within the CD45-RC^{low} $CD4^+$ T Cell Population. The $CD45RC^{low}$ $CD4^+$ T cell population can be further subdivided by the expression of Thy-1, RT6, IL-2R, and OX-40 antigens (5). The subsets defined by the differential expression of these antigens represent $CD4^+$ T cells in different stages of maturation (56) and activation (37). The phenotypic differences were found to be accompanied by differences in the ability to suppress the development of diabetes in lymphopoenic rats (Table 5). Depletion of $RT6^+$ cells left a $CD45RC^{low}$ $CD4^+$ T cell subpopulation that was incapable of protecting against diabetes (Table 5). In an independent experiment it was shown that the depletion of $RT6^+$ cells left a mixed population that contained mainly activated cells (OX-40^+ and/or IL-$2R^+$) together with some Thy-1^+ cells representing recent thymic migrants (56) and some null cells of undefined phenotype. The complementary depletion of the activated IL-$2R^+$ and OX-40^+ cells resulted in a $CD45RC^{low}$ $CD4^+$ T cell inoculum containing four subsets: $RT6^+$Thy-1^-, $RT6^+$Thy-1^+, $RT6^-$Thy-1^+, and $RT6^-$Thy-1^- (null cells). Cells within this population retained the capacity to suppress the development of autoimmune diabetes, and removal of the Thy-1^+ cells also left the protective effect of the $CD45RC^{low}$ $CD4^+$ T cell population intact. Thus, the $CD45RC^{low}$ $CD4^+$ T cell subpopulation that mediated protection was TCR-α/β^+ $RT6^+$ Thy-1^- IL-$2R^-$ OX-40^-. These data do not exclude the possibility that some protective $RT6^+$ cells expressed Thy-1, IL-2R, or OX-40; however, activated T cells downregulate RT6 antigen expression (57), and cells expressing both RT6 and IL-2R/OX-40 are infrequent in rat TDL (data not shown). Consistent with the established functional activities of unfractionated $CD45RC^{low}$ $CD4^+$ T cells (7, 10), the subpopulation of these cells that prevented diabetes was shown to mediate help for secondary antibody responses and to produce IL-2 protein and mRNA for IL-4, but no IFN-γ protein, on in vitro activation (data not shown).

Table 4. *Phenotypic Changes on Reconstitution of Tx-X Rats with $CD45RC^{low}$ $CD4^+$ T Cells*

	Percent positive cells		
TDL	Normal	Prediabetic	$CD45RC^{low}$ $CD4^+$ T cell reconstituted
B cells	47 ± 2	73 ± 4	82 ± 3
$CD4^+$ T cells	33 ± 2	15 ± 4	14 ± 4
$CD8^+$ T cells	18 ± 2	9 ± 3	7 ± 2
$CD4^+$ T cells			
$CD45RC^{high}$	70 ± 6	7 ± 1	24 ± 7
IL-$2R^+$	8 ± 4	41 ± 8	22 ± 5
OX-40^+	12 ± 6	56 ± 4	24 ± 4
MHC class II^{high}	5 ± 1	40 ± 5	32 ± 2

TDL were collected from $CD45RC^{low}$ $CD4^+$ T cell–reconstituted Tx-X rats and prediabetic Tx-X rats 5 wk after the last dose of irradiation. Normal TDL were from age- and sex-matched control PVG.$RT1^u$ rats. The figures represent the mean percentage of positively labeled cells in whole TDL or the $CD4^+$ T cell fraction thereof from four animals in each group. TDL output in 18 h was an average 10^8 cells from both prediabetic and reconstituted animals compared with an average 3.5×10^8 cells from normal rats.

Table 5. *Functional Heterogeneity within the $CD45RC^{low}$ $CD4^+$ Cell Subset in the Ability to Suppress Diabetes*

Phenotype of $CD4^+$ cells injected	No. of cells injected intravenously	Incidence of diabetes
Unfractionated $CD45RC^{low}$	5×10^6*	0/18
$CD45RC^{low}$ TCR-α/β^-	5×10^6*	3/3
$CD45RC^{low}$ TCR-α/β^-	2×10^5‡	2/2
$CD45RC^{low}$ TCR-α/β^+	5×10^6‡	0/2
$CD45RC^{low}$ Thy-1^-	5×10^6*	0/7
$CD45RC^{low}$ $RT6^-$	5×10^6*	3/4
$CD45RC^{low}$ $RT6^-$	3×10^6‡	2/2
$CD45RC^{low}$ $RT6^+$	3×10^6‡	0/3
$CD45RC^{low}$ OX-40^- IL-2-R^-	5×10^6*	1/7
None	–	36/36

* Cells were negatively selected from TDL by rosette depletion (purity, >96%).

‡ $CD45RC^{low}$ $CD4^+$ T cells were negatively selected from TDL by rosette depletion and further subdivided by fluorescent cell sorting. The purity of sorted populations was always >98%.

Discussion

The most direct interpretation of the results presented in this paper is that autoreactive T cells are to be found in animals that show no tendency to develop autoimmune disease, and that these autoimmune T cells are normally inhibited in some way from expressing their autoimmune potential by other T cells. These conclusions, if correct, indicate that self-tolerance does not rest solely on the deletion from the T cell repertoire of autoreactive cells but depends, in part, on some homeostatic mechanism in which the immune system plays a crucial regulatory role. There are other data that support this conclusion (49, 50, 52, 53) but alternative explanations can be proposed for the present results. The protocol for the induction of diabetes results in rats that are relatively T cell deficient, and it may be argued that these animals are rendered prone to some diabetogenic infection. Infection alone cannot account for the diabetes because $CD8^+$ T cells are required for disease to occur but, in principle, the $CD8^+$ T cells could be killing virus-infected β cells of the pancreas. If this is so then one may ask why such a protocol leads specifically to the infection of the same cells as those affected by autoimmunity in the BB rat and nonlymphopoenic individuals, such as NOD mice and human diabetics. Using the same protocol as described here, Penhale et al. (44) have shown that in a different rat strain both thyroiditis and diabetes can occur in the same individual, and we have observed the same effect in a few of our own Tx-X rats; these two endocrinopathies arise spontaneously in humans and are considered to be autoimmune in origin. However, until the etiology of these diseases in humans and experimental animals is understood the role that infection plays in them will remain undefined. Attempts to identify environmental factors that account for the <40% concordance for diabetes in monozygotic twins have not yielded a clear result, and studies in experimental animals have produced conflicting data on the effects of intercurrent infection on the incidence of autoimmune disease (reviewed in reference 58). It may well be that infection does play a part in these autoimmune diseases, and does so by perturbing the homeostatic mechanism rather than by any direct pathogenic effect. Our own data, while by no means conclusive on this point, are not at variance with this possibility. A high frequency of T cells recovered from the thoracic duct of diabetes-susceptible Tx-X rats expressed activation markers and were in cell cycle. Given the high mitotic activity of lymphocytes from the mesenteric nodes (but not the cervical nodes) of Tx-X animals, these T cells probably originate in the gut-associated lymphoid tissue, suggesting that the antigenic stimulus for proliferation comes from the gut, from either dietary or gut flora antigens. It has been observed by Penhale and Young (59) that the incidence of thyroiditis in Tx-X rats reared under specific pathogen-free conditions was lower than that in conventionally reared animals but could be augmented by the transfer of intestinal material from the latter. Furthermore, it has been shown that splenocytes from a diabetes-resistant BB rat subline, if depleted of $RT6^+CD4^+$ T cells and activated in vitro with Staphylococcal enterotoxins, are able to transfer diabetes to young diabetes-prone BB rats, indicating that bacterial products can activate diabetogenic T cells (60).

As the data presented in this paper show, $CD4^+$ T cells, of the phenotype $CD45RC^{low}$ TCR-α/β^+ $RT6^+$ Thy-1^- OX-40^-, when transferred from healthy, syngeneic donors into Tx-X rats, were able to prevent the autoimmune T cells from causing diabetes. Cells of this phenotype have been shown to produce IL-2 and IL-4 but not IFN-γ, although we cannot say that individual cells make both of the named lymphokines since our studies have not been carried out at the single cell level. In addition, preliminary studies have shown that mRNA for IL-10 was detectable from the $CD45RC^{low}$ but not the $CD45RC^{high}$ $CD4^+$ T cell subset after in vitro activation (A. Beyers and D. Fowell, unpublished results). Consistent with these results, $RT6^+$ $CD4^+$ cells have also been implicated in the protection from diabetes in the BB rat (61), though these cells have not been extensively characterized. Both IL-4 and IL-10 have been shown to inhibit cell-mediated immunity in vivo, partly by the downregulation of the production of IFN-γ (15, 16, 62). Given the importance of IFN-γ in the induction of diabetes in other rodent models (63, 64), the protective T cells that we have characterized may inhibit the development of diabetes by producing these inhibitory lymphokines. An observation compatible with this interpretation of our data is the demonstration that in vivo administration of rIL-4 facilitates remission of collagen-induced cell-mediated autoimmune arthritis (65). It is also notable that some susceptibility genes for diabetes in the NOD mouse have been mapped to the regions encoding certain cytokines, including IL-4 and IL-2 (66). Combining these data with those presented herein suggests that insulin-dependent diabetes mellitus develops spontaneously in individuals who, in addition to possessing the appropriate MHC alleles, suffer a degree of immune dysregulation through the inheritance of a particular set of alleles for polymorphic genes involved in the regulation of cytokine expression. That such variation in cytokine expression can occur is illustrated by the finding of different mouse strains whose T cell responses are characteristically either Th1- or Th2-like (67). Work is in progress to establish the changes in cytokine synthesis produced in vivo by the injection of the protective $CD45RC^{low}$ $CD4^+$ T cells.

A striking feature of the prevention of diabetes by the injection of $CD45RC^{low}$ $CD4^+$ T cells was the change in surface phenotype of T cells recovered from the thoracic duct lymph of Tx-X rats (Table 4). The percentage of $CD4^+$ T cells that expressed activation markers was reduced to about half of that found in prediabetic animals, while there was a marked increase in $CD45RC^{high}$ $CD4^+$ cells. It appears that injection of the $CD45RC^{low}$ $CD4^+$ T cells produces a $CD4^+$ T cell subset distribution that more closely approximates that of a normal rat. However, there were significant differences between the $CD45RC^{high}$ $CD4^+$ cells in reconstituted Tx-X rats and the $CD45RC^{high}$ $CD4^+$ cells of normal rats. Preliminary results in PVG.$RT1^c$ rats using RT7 allotype congenic strains indicate that the majority of

$CD45RC^{high}$ $CD4^+$ T cells in reconstituted Tx-X rats are of donor origin and are larger than the cells of this phenotype found in normal rats. They represent, therefore, a population that must be relatively rare in normal T cell development, and their function is not known at present. Although the BdUr data indicated a high frequency of proliferating cells in prediabetic and reconstituted rats, these animals remained relatively lymphopoenic compared with normal animals in which far fewer cells were in cell cycle. It is apparent that in Tx-X rats there is a high rate of lymphocyte death although the cause of this is not known; this question merits further study.

Finally, we note that $CD4^+CD8^-$ thymocytes were also able to inhibit diabetes in our Tx-X rats. Thymocytes of this phenotype in mice have been shown to produce IL-4 and IL-10 (and some IFN-γ) on activation (68), suggesting that they resemble, at least to some degree, peripheral $CD4^+$ T cells of the Th2 type. This resemblance may explain why thymocytes were protective against cell-mediated autoimmune diabetes in our experiments, and it is a notable finding in this context that mature $CD4^+$ thymocytes from spontaneously diabetic NOD mice are incapable of producing IL-4 on in vitro activation by TCR crosslinking (69). The Th2-like properties of mature thymocytes from normal animals suggest that recent thymic migrants may also have the same characteristics. If so the question arises (68) as to whether they retain this cytokine repertoire if they encounter antigen before they mature into naive T cells that produce only IL-2 on primary activation (8, 9). Such a mechanism would serve to generate cells with the ability to suppress cell-mediated autoimmune disease if the relevant antigen was already present in the periphery when the $CD4^+$ T cells left the thymus. It is recognized that the $CD45RC^{low}$ compartment of peripheral $CD4^+$ T cells contains at least three cell types: recent thymic migrants that are downregulating their expression of Thy-1 and upregulating RT6 as they mature towards naive cells that are $RT6^+CD45RC^{high}$ (56); $RT6^+$ $CD45RC^{low}$ memory cells that have derived from mature naive $CD45RC^{high}$ precursors and that mediate secondary helper activity for B cells (25; and D. Fowell unpublished results); and activated T cells that have downregulated the expression of RT6 but express IL-2R, OX-40 antigen, and Thy-1 (37, 56, 57). In principle the $RT6^+Thy\text{-}1^-OX\text{-}40^-$ cells that protect against diabetes could be either recent thymic migrants in a late stage of maturation towards $RT6^+$ $CD45RC^{high}$ naive cells or mature resting T cells that are progeny of cells that have already encountered antigen. However, as $CD45RC^{low}$ $CD4^+$ T cells from rats that had been thymectomized 10 wk earlier were able to protect against diabetes, it is evident that recent thymic migrants play no essential role in the protection, and it seems that the protective cells in the periphery are not naive. It is evident that this conclusion does not exclude the possibility that the protective cells encountered their specific antigen at an earlier time when they were recent thymic migrants. This hypothesis presupposes that the protective T cell is specific either for the autoantigen or for some extrinsic antigen that evokes a tolerance-breaking response. Our data have no bearing on this point, but studies of induced tolerance to alloantigens in rats have provided evidence for an active $CD4^+$ T cell–mediated tolerogenic mechanism that appears to be alloantigen specific (70). The involvement of $CD4^+$ T cells in induced tolerance raises the possibility that therapeutic protocols may allow the acceptance of allografts by evoking essentially the same mechanism as that mediating self-tolerance.

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