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Quantitative Analyses Comparing All Major Spleen Cell Phenotypes in BB and Normal Rats: Autoimmune Imbalance and Double Negative T Cells Associated with Resistant, Prone and Diabetic Animals

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The BB rat is a model of spontaneous autoimmune diabetes. To characterize quantitatively all known immune cell subsets involved in disease pathogenesis, FACS analysis of spleen cells was performed in diabetes-prone (DP) and acutely diabetic (D) BB rats and compared with diabetes-resistant (DR) BB and normal Wistar-Furth (WF) strains. We observed increased percentages of splenic NK cells in DP and D animals compared with DR rats using an NK-specific monoclonal antibody. We found increased proportions of splenic macrophages in the T-lymphopenic DP and D rats and low macrophage contents in DR spleens compared with WF spleens. We observed that percentages of the CD4⁺CD8⁺ T cell receptor alpha/beta⁺ (double-negative) T cell subset were strikingly increased in the lymphopenic DP and D animals, compared with DR animals. We observed increased percentages of activated splenic CD5⁺ T cells expressing the IL-2 receptor and MHC class II antigen in DP and D rats compared with DR animals. Our studies suggest that (a) splenic NK cells and macrophages quantitatively appear to be involved in the pathogenesis of diabetes; (b) double-negative T cells escape from the T cell depletion process; (c) a marked increase of activated splenic T cells suggests diabetes is associated with general T cell activation processes; and (d) an altered balance among the different immune cell subsets may in part explain the pathogenesis of diabetes, since marked relative changes are observed when comparing

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the DR strain to the DP strain in both the prediabetic and diabetic stages.

Introduction

Evidence obtained from studies of spontaneous diabetes in both humans and BB rats suggests an autoimmune etiology [1, 2]. Several types of immune cells are involved in the islet inflammation that leads to beta cell destruction.

It is suggested that natural killer (NK) cells may play a role in disease pathogenesis in BB rats, since (a) the numbers of splenic and blood NK cells (OX8⁺OX19⁻) are enhanced in diabetes-prone (DP) rats [3, 4]; (b) NK cells are present in inflamed islets [5, 6]; (c) NK cells are cytolytic to islets *in vitro* [7]; (d) treatment with monoclonal antibody against CD8 protein, expressed by the majority of NK cells, reduces the incidence of diabetes [8]; and (e) administration of polyclonal antibody that binds the NK cell glycolipid AGM1 prevents autoimmune islet destruction [9]. The use of NK-specific monoclonal antibody in BB rats has not been reported; thus, quantitative definitions remain unclear.

Macrophages are apparently also involved in the development of diabetes in BB rats, since (a) macrophages are observed early in the course of islet inflammation [6]; (b) splenic macrophages kill islet cells *in vitro* during prediabetes and at diabetes onset [10]; (c) diabetes can be transferred adoptively with spleen cells containing macrophages [11]; and (d) administration of silica to DP animals prevents adoptively transferred and spontaneous disease [11–13].

In prior studies, splenic macrophage content in prediabetic DP rats was about 26% of the total spleen cell population [11], whereas it was 11% in new onset diabetic (D) BB rats [14]. Despite these morphological and functional observations, little is known *quantitatively* about the macrophage content of spleen cells in relation to other cell types in BB rat strains compared with normals.

Although DP BB rats display a severe T lymphopenia, T lymphocytes may also be involved in islet destruction, since disease onset is (a) associated with islet T cell infiltration [5]; (b) prevented by neonatal thymectomy [15]; (c) reduced by immunosuppression therapy using glucocorticoids, anti-lymphocyte serum and/or cyclosporin-A [16, 17]; and (d) adoptively transferred with preactivated and enriched CD4⁺ T lymphocytes derived from acutely diabetic rats [18]. T cell lymphopenia includes both CD4⁺ (W3/25⁺OX19⁺) and CD8⁺ (OX8⁺OX19⁺) T cells, which are now known also to express T cell receptor alpha/beta (TCR α/β) [19, 20]. There are no reports of double-negative (CD4⁻CD8⁻) T cells in BB rat spleen or islets. The double-negative T cell content of thymus in normal adult Wistar-Furth (WF) rats is <5% [21]. It is reported that double-negative T cells expressing TCR α/β augment the production of pathogenic autoantibodies associated with lupus nephritis in both humans [22] and mice [23]. Further, double-negative T cells from murine autoimmune models of SLE, experimental allergic encephalomyelitis and diabetes have abnormally high numbers of an unusual potassium channel that is expressed in parallel with the onset of autoimmunity [24]. Despite observations that various parameters of T cell function are reduced in prediabetic and diabetic DP strain BB rats [20], activated T cells have been described as being present in inflamed

islets [25] and in thoracic duct lymphocytes of DP rats that are close to diabetes onset [26, 27]. No systematic evaluation of these T cell subsets has been reported in the BB rat model, nor has any comparison of T cell subsets to other immune cell types been presented.

Because of the involvement of multiple immune cell types and possible interactions mediated by absolute or relative changes in cell numbers in spontaneous autoimmune diabetes in the BB rat, we performed systematic fluorescence-activated cell sorter (FACS) analyses of all major spleen cell populations. We examined NK cells, macrophages, T and B lymphocytes, and T cell subsets in several animal strains, including the normal WF strain, the BB diabetes-resistant (DR) strain and the DP strain in both the prediabetic and diabetic stages. Although some of this work has been previously published, no studies have measured all subsets simultaneously, thus permitting conclusions on immune balance. Further, evaluating all subsets simultaneously permits apparent holes in repertoires to be evaluated in more depth. For example, this method encouraged our discovery of double-negative T cells in prediabetic and diabetic DP BB rat spleen since we knew that not all T cell subsets were accounted for.

Materials and methods

Animals

Adult male Wistar-Furth (WF) rats, 80–100 days of age at study were obtained from Harlan-Sprague Dawley (Indianapolis, IN, USA). Diabetes-resistant (DR) and diabetes-prone (DP) (60–120 days of age) male Wistar BB rats (RT1^u) in non-diabetic and diabetic (D) stages were kindly provided by Dr Pierre Thibert (Animal Resources Division, Sir Frederick Banting Research Centre, Ottawa, Ontario, Canada). The overall incidence of diabetes in the DP strain of BB rats is 50–60% between 60–120 days of age and <2% in the DR BB rats. Animals were defined as diabetic if urine glucose was >0.25% (Tes-Tape, Lilly, Indianapolis, IN, USA) and non-fasting blood glucose was >250 mg%. Experiments in diabetic animals were performed 4 days after diabetes onset.

Cell staining

Splenic mononuclear cells were isolated by Ficoll-Hypaque (Lymphocyte Separation Medium, Organon Teknika, Durham, NC, USA) as previously described [28], and washed twice with assay buffer containing phosphate-buffered saline (Sigma, St Louis, MO, USA), 1% BSA (Sigma) and 0.02% sodium azide (Fisher Scientific Company, Fair Lawn, NJ, USA). For staining NK cells, splenocytes were incubated for 60 min with mouse monoclonal antibody (mAb) 3.2.3 (anti-CD16) conjugated with fluorescein-isothiocyanate (FITC) [29]. For identifying macrophages, an indirect staining method was used. Briefly, the cells were incubated for 60 min with OX41 (mouse anti-rat macrophage, granulocyte and dendritic cell), washed twice with assay buffer and incubated with a 'second' antibody, FITC-conjugated goat anti-mouse IgG, that was rat cell absorbed (Caltag Laboratories, San Francisco, CA, USA) for 30 min.

For direct, two-color staining of T lymphocyte subsets and activated T cells, splenic mononuclear cells were incubated for 60 min with mouse monoclonal antibodies R-7.3-FITC (anti-TCR α/β), OX39-FITC (anti-IL-2R) or OX6-FITC (anti-RT1B MHC class II monomorphic). After incubation, cells were washed twice with assay buffer and incubated with phycoerythrin (PE) conjugated OX19 (anti-CD5) or W3/25-PE (anti-CD4) for 60 min or with OX8-PE (anti-CD8) for 30 min. For measuring double-negative cells, cells were incubated first with R-7.3-FITC mAb for 60 min, washed twice with assay buffer and incubated with W3/25-PE for 60 min and with OX8-PE for 30 min. For staining B lymphocytes, cells were incubated for 30 min with OX12-PE (anti-rat κ chain). All incubations were performed on ice, in the dark, using 10^6 spleen mononuclear cells, and all mAb (Serotec, Oxford, UK) were used at saturating concentrations. After the last incubation with antibody, cells were washed twice with assay buffer and fixed in 1% paraformaldehyde. For direct staining, the background was determined by the incubation of splenocytes in assay buffer without mAb or by incubations with purified isotypic mouse polyclonal IgG that was conjugated with FITC or PE. For indirect staining, background staining was determined using cells incubated with the 'second' antibody conjugated with FITC.

Flow cytometric analysis

Stained and fixed splenocytes were analysed with a fluorescence-activated cell sorter (FACScan, Becton Dickinson, San Jose, CA, USA) using a C30 software program (Becton Dickinson). Ten thousand cells were collected, red blood cells and granulocytes were gated out, and the results for mononuclear cells were displayed in either contour graphs or histograms.

Statistical analysis

All data presented are mean $\pm$ SEM, and data among the four animal groups are compared using one-way ANOVA.

Results

To determine whether diabetes was associated with abnormal spleen cell populations, normal WF, DR BB, and non-diabetic and diabetic DP BB rat strains were studied using FACS. As shown in Figure 1, we observed more than a two-fold increase of NK cells in non-diabetic and diabetic DP rats compared with WF and DR strains using the NK-specific mAb 3.2.3, which was significant at $P < 0.025$ among groups. We compared percentages of NK cells using mAb 3.2.3. to percentages of OX8⁺ TCR α/β ⁻ cells in all four animal groups. The results were similar in the normal WF strain: $3.4 \pm 0.8\%$ using 3.2.3. mAb compared with $3.6 \pm 0.4\%$ of OX8⁺ TCR α/β ⁻ cells (P =not significant). In the BB strains, the 3.2.3. method gave slightly lower results than the OX8⁺ TCR α/β ⁻ method. In the non-diabetic group, the percentage of splenic NK cells stained by 3.2.3. mAb was $9.3\% \pm 1.1\%$ compared with $11 \pm 1.4\%$ of OX8⁺ TCR α/β ⁻ cells: In the diabetic group these values were $7.8 \pm 2.3\%$ and $13 \pm 1.7\%$ (P =NS) respectively and in the DR group they were $3.4 \pm 0.3\%$ compared with $5.1 \pm 0.4\%$ ($P < 0.05$), respectively.

Figure 1. FACS analysis of rat spleen cells in Wistar-Furth (WF, $n=4$), diabetes resistant (DR, $n=5$), diabetes prone (DP, $n=5$) and diabetic (D, $n=5$) rats. The bars represent mean percentages of positively stained cells; the error represents SEM. B cells were estimated using OX-12, T cells using R-7.3, macrophages using OX41 and NK cells using 3.2.3. * Significant difference in comparison with DR rats; **significant difference in comparison with WF rats. A: $P<0.001$, B: $P<0.01$, C: $P<0.05$. □ WF; ▨ DR; ▧ DP; ▩ D.

Since there has been no systematic analysis of splenic macrophage content in comparison with other predominant splenic cell populations using specific staining procedures in WF and in different BB rat strains, we also studied macrophage levels. An increase of greater than two- or three-fold in the mean percentage of splenic macrophages in non-diabetic and diabetic DP animals was observed compared with the DR strain (Figure 1). The difference among groups was significant at $P<0.001$. Splenocytes from DR animals showed a 50% decrease of macrophage content compared with WF rats.

Severe T lymphopenia was observed in non-diabetic and diabetic DP rats, whereas the percentage of TCR α/β^+ cells was similar in WF and DR strains (Figure 1). The differences among groups were significant at $P<0.001$. The mean percentage of B lymphocytes was similar in the four animal groups (Figure 1) i.e., there were no differences among the groups. Thus, in the non-diabetic and diabetic DP rat strain, B lymphocytes were the predominant cell type, followed by macrophages, T cells and NK cells. In the normal WF and DR BB strains, T lymphocytes were predominant, followed by B cells, macrophages and NK cells. To determine whether we detected all the different cell types present in the spleen cell preparations, we added the percentage of T and B lymphocytes, macrophages and NK cells in each animal group. The totals are 102 ± 4 for each strain.

We estimated the severity of T lymphopenia using T cell subsets defined by double staining. Significantly ($P<0.05$) decreased levels of CD4⁺ and CD8⁺ T cells (TCR α/β^+) were found in non-diabetic ($13 \pm 1.9\%$ and $3.8 \pm 1.0\%$, respectively, of total

Figure 2. FACS analysis of T cell subsets in rat spleens in same strains and number of animals as shown in Figure 1 except double negative cells were studied in 10 non-diabetic and diabetic DP BB rats. The mean percentages, errors and symbols are as used in Figure 1. CD4⁺ T cells were estimated using W3/25⁺, R-7.3⁺ T cells, CD8⁺ T cells using OX8⁺, R-7.3⁺ T cells, double negative or CD4⁻CD8⁻ T cells using W3/25⁻, OX8⁻, R-7.3⁺ T cells and CD5⁻ T cells using OX19⁻, R-7.3⁺ T cells. Since all statistics in this figure use the DR rat as the comparison animal, no statistical connotations are represented over the DR bars. By ANOVA, the difference between groups is $P < 0.001$. *Significant difference in comparison with DR rats; **significant difference in comparison with WF rats. A: $P < 0.001$. □ WF, ▤ DR, ▨ DP, ▧ D.

splenic mononuclear cells) and diabetic DP rats ($11 \pm 1.0\%$ and $2.8 \pm 0.9\%$) compared with WF ($33 \pm 1.9\%$, $17 \pm 1.4\%$) and DR strains ($35 \pm 3.3\%$, $11 \pm 2.2\%$). When CD4⁺ TCR α/β ⁺ T cells are expressed as a percentage of the total TCR α/β ⁺ T cells, we observed $58 \pm 1.0\%$ in WF, $70 \pm 2.1\%$ in DR ($P < 0.01$ vs WF), $59 \pm 8.5\%$ in non-diabetic and $57 \pm 2.2\%$ in diabetic DP BB rats ($P < 0.01$ vs DR). When CD8⁺ TCR α/β ⁺ T cells are expressed as a percentage of total TCR α/β ⁺ T cells, we observed $30 \pm 1.0\%$ in WF, $22 \pm 2.7\%$ in DR ($P < 0.05$ vs WF), $16 \pm 3.0\%$ in non-diabetic ($P < 0.01$ vs WF) and $15 \pm 4.0\%$ diabetic DP BB rats ($P < 0.01$ vs WF). Splenic double-negative and CD5⁻ T cell percentages, expressed as a percentage of total mononuclear cells, were more than doubled in non-diabetic animals ($20 \pm 1.5\%$, $12 \pm 2.8\%$ respectively), diabetic DP animals ($7.8 \pm 2.0\%$, $10.7 \pm 1.8\%$) and WF rats ($8.8 \pm 1.1\%$, $11.4 \pm 1.3\%$) compared with the DR animals ($3.7 \pm 0.4\%$, $4.1 \pm 0.3\%$, $P < 0.05$). If double-negative TCR α/β ⁺ T cells are expressed as a percentage of total TCR α/β ⁺ T cells, we observed 2- to 4-fold increases among non-diabetic and diabetic DP BB rats compared with WF or DR strains (Figure 2), a difference that is significant at the $P < 0.001$ level. Similar changes were observed for splenic CD5⁻ TCR α/β ⁺ T cells in WF, DR, non-diabetic and diabetic DP rats (Figure 2). Figure 3 shows how double-negative T cells were analysed by contour graphs in the four animal groups.

To determine levels of activated splenic T cells, IL-2 receptor (IL-2R) and MHC class II antigen expression were assessed on T cell subsets (Figure 4). IL-2 receptor

Figure 3. A representative histogram graph of two-color FACS analysis of splenic double negative TCR α/β^+ T cells from each of the four animal groups: D = diabetes prone diabetic, DP = diabetes prone non-diabetic, WF = Wistar Furth strain, and DR = diabetes resistant strain. The spleen cells were stained with R-7.3-FITC mAb (anti-TCR α/β), W3/25-PE (anti-CD4) and OX8-PE (anti-CD8). Only those cells staining for TCR α/β^+ are depicted. The vertical line separates the double positive T cells (to left) from CD8 $^+$ CD4 $^+$ (double positive) TCR α/β^+ T-cells (to right). See text and Figure 2 for statistical assessment.

and MHC class II expression among CD5 $^+$ T cells were more than 1.5 times higher in non-diabetic and diabetic DP strain rats compared with WF or DR strains, and the differences among groups for both monoclonal antibodies were significant at the $P < 0.001$ level. DR rats had significantly lower percentages of activated T cells (IL-2R expression) compared with WF animals. We observed similar results when comparing MHC class II antigen expression of DR rat splenic T cells with WF rats.

To estimate whether the data observed in the non-diabetic and diabetic DP strain were associated with absolute mononuclear cell count abnormalities or relative percentage changes, the mean absolute cell counts were calculated, reflecting the total number of cells in a particular splenic mononuclear subset. As shown in Table 1, absolute numbers of non-diabetic and diabetic DP rats were more than 50% decreased for total T cells, 80–90% decreased for CD4 $^+$ and CD8 $^+$ T cells, and more than 50% decreased for activated T cells when compared with WF and DR rats. Despite the altered percentage distributions, there were no significant differences in absolute numbers of NK cells, macrophages, double-negative T cells, and CD5 $^-$ T cells in non-diabetic and diabetic DP rats compared with DR animals. Within

Figure 4. FACS analysis of activated T cells in rat spleen in same strains and numbers of animals as shown in Figure 1. The mean percentages, error bars and symbols are as used in Figure 1. See methods for reagents used. □ WF; ▨ DR, ▩ DP, ▤ D.

Table 1. Absolute numbers of spleen cells in Wistar-Furth (WF), diabetes resistant (DR), prediabetic diabetes prone (DP) and diabetic (D) BB rats

Animal groups	WF (n=4)	DR (n=5)	DP (n=5)	D (n=5)	ANOVA comparison among groups
B cell	24 ± 4.7 ^f	54 ± 14	35 ± 12	26 ± 10	NS
T cell	36 ± 7.8	74 ± 16	17 ± 5.4	14 ± 5.4	<0.005
			a ^d , b ^c	a ^d , b ^c	
Macrophage	11 ± 3.0	14 ± 5.1	16 ± 3.5	19 ± 5.8	NS
NK cell	2.2 ± 0.6	5.0 ± 0.9	6.0 ± 0.9	3.6 ± 0.8	<0.05
		b ^c	b ^c		
CD4 ⁺ T cell	21 ± 4.4	51 ± 9.3	8.9 ± 2.1	7.2 ± 2.5	<0.001
		b ^c	a ^d , b ^c	a ^d , b ^c	
CD8 ⁺ T cell	11 ± 3.0	18 ± 5.0	3.3 ± 1.5	1.6 ± 0.6	<0.01
			a ^d , b ^c	a ^d , b ^c	
CD4 ⁺ CD8 ⁺ T cell	6.0 ± 1.2	5.6 ± 1.3	6.5 ± 2.5	6.3 ± 3.0	NS
			n=10	n=10	
CD5 ⁺ T cell	7.0 ± 1.2	5.9 ± 1.1	11 ± 4.0	7.9 ± 3.3	NS
IL-2R ⁺ T cell	8.6 ± 1.9	15 ± 4.8	3.2 ± 0.6	3.9 ± 1.6	<0.05
			a ^d , b ^c		
MHC Class II ⁺ T cell	3.7 ± 0.9	5.7 ± 1.3	2.1 ± 0.7	2.2 ± 0.9	NS

a, Significant difference in comparison with DR rats; b, significant difference in comparison with WF rats. ^cP < 0.001; ^dP < 0.01; ^eP < 0.05; ^fValues are means ± SEM of absolute numbers (× 10⁶) of mononuclear subpopulations per spleen.

the time points examined i.e., BB rats 80–100 days old, we noted no T cell subset changes when comparing data from non-diabetic and diabetic DP animals. Metabolic data from these animals have been collected but these data are reported elsewhere (submitted).

Discussion

Several recognition, regulatory and effector systems (e.g. macrophages, T and B lymphocytes, NK cells and lymphokines) appear to be involved in the pathogenesis of spontaneous diabetes in BB rats. Examination of the relative changes in specific mononuclear subsets could be useful in furthering our understanding of the pathogenesis. Our data confirm normal levels of B lymphocytes, T lymphopenia and high percentages of macrophage and NK cells. In addition we observed high mean percentages of double-negative T cells and splenic activated T cells in non-diabetic and diabetic DP animals compared with the DR strain. We observed significant alterations of immune cell balance in the spleens among the different non-diabetic and diabetic DP rats, especially when compared with the DR BB rat strain. Finally, since our data on the major cell types are consistent with other data from different BB rat colonies, it appears that splenocyte populations are relatively similar from colony to colony.

Several observations suggest NK cells are involved in the islet destruction. NK cells are often characterized as $\text{OX8}^+\text{OX19}^-$ cells, yet not all NK cells express CD8 (OX8^+) [8]. The reported percentage of splenic $\text{OX8}^+\text{OX19}^-$ cells is 6–8% in non-diabetic and diabetic DP animals [3, 4]. In non-diabetic DP rats, 5% of thoracic duct lymphocytes express CD8 and one-third of the CD8^+ cells express the T cell receptor α/β ; thus, the $\text{CD8}^+\text{TCR } \alpha/\beta^-$ cells could also be considered to be NK cells [30]. We used an NK-specific mAb which binds all rat NK cells [29]. We confirm the relatively high percentage of NK cells previously reported to be present in prediabetic and diabetic DP rat spleens. We have also found a marked increase of NK cells, up to 40% of the total mononuclear cells, in inflamed islets derived from non-diabetic and diabetic DP rats [31]. This observation suggests that inflamed islets are more enriched for NK cells in the later stages of prediabetes and at disease onset when compared with the cell populations in the spleen. These data, coupled with cytolytic data [7], suggest that NK cells are a key mononuclear subset perhaps involved in the non-specific destruction of islets in the later stages of autoimmune insulinitis in the BB rat.

The macrophage has a central role in autoimmunity, including antigen presentation, lymphokine production and effector activity [2, 10]. In this study, a reciprocal relationship between splenic macrophages and T cells was observed. The lymphopenic non-diabetic and diabetic DP animals all had increased levels of macrophages compared with normal controls, while DR rats (non-lymphopenic) had low levels of macrophages compared with normal animals. Although elevated macrophage levels could be a 'compensatory' response to T lymphopenia, the magnitude of the increase and the fact that a 'compensatory' elevation among B lymphocytes was absent suggest other possible mechanisms. The origin of T cell depletion in DP BB rats is unclear but is genetically linked [2]. Several studies suggest a post-thymic maturation defect, since (a) histologically the thymus of DP BB rats is normal [32];

(b) thymus allografts fail to improve lymphopenia or prevent DP spontaneous diabetes [33]; and (c) BB T lymphocytes have a shorter life span outside the thymus than T cells derived from PVG and Louvain rat strains [34]. On the other hand, a significant deletion of mature $CD4^-CD8^+ TCR \alpha/\beta^+$ thymocytes has been described in DP BB rats [35]. Previous observations that T cell-mediated IL-2 production and T cell proliferation in response to Con-A can be suppressed by activated macrophages *in vitro* [36, 37], coupled with our data demonstrating reciprocal macrophage/T cell balance, suggest that T cell lymphopenia may be mediated by macrophages in pre-diabetic and diabetic DP rats, although our results do not directly evaluate this possibility. Regardless of whether the lymphopenic process starts in the thymus, the process may continue at the post-thymic level in peripheral lymphoid tissue. We have also found about 5% macrophages in inflamed islets using FACS analysis of islet-infiltrating immune cells [31]. These macrophages may present the putative beta cell antigen [38] and/or may be directly cytolytic against β cells [10, 39].

Despite T cell lymphopenia, T cells are present in islets and may be critical to β cell destruction in the BB rat. Thus we have further analysed splenic T cell subsets. The degree of $CD8^+$ T cell depression is controversial; some studies report almost total absence of $CD8^+$ T cells ($OX8^+OX19^+$) [4, 32], while others report that approximately 30% of $CD8^+$ cells express $TCR \alpha/\beta$ ($OX8^+R-7.3^+$) in lymphatic drainage [30]. This difference may be due to different antibodies used in these studies i.e., anti- $CD5$ vs anti- $TCR \alpha/\beta$. In our study we confirmed that T lymphopenia included both the $CD4^+$ and $CD8^+$ T cells and, as in the R-7.3 ($TCR \alpha/\beta$) study in thoracic duct lymphocytes, we also detected about 2–4% splenic $CD8^+$ T cells in non-diabetic and diabetic DP rats. Contrary to $CD4^+$ and $CD8^+$ T cells, the $CD4^-CD8^- TCR \alpha/\beta^+$ T cell subset (double-negative) percentages were not decreased in non-diabetic and diabetic DP animals. As shown in Figure 2, double negative T cells were markedly increased in both groups of DP rats compared with DR or WF. This percentage data is augmented, since both WF and DR rats are not lymphopenic; thus there is a relative increase shown in the DP rats. Since the total number of splenic T cells is markedly less in DP rats, and since the absolute double negative T-cell numbers were similar in all four groups of rats, there is a marked shift in the balance of T cells present in DP rats. We observed the same phenomenon among $CD5^- TCR \alpha/\beta^+$ T cells. Since it has been reported that double-negative T cells do not express 'bright' $CD5$ [22], we suggest that $CD4^-CD8^- TCR \alpha/\beta^+$ T cells and $CD5^- TCR \alpha/\beta^+$ T cells may represent the same T cell subset. Since absolute numbers of double-negative $TCR \alpha/\beta^+$ T cells and $CD5^- TCR \alpha/\beta^+$ T cells were similar in all four animal groups, the ratios of double-negative T cells to total T cells are markedly altered among the groups. These data also suggest that double-negative T cells are not depleted, e.g. $CD4^+$ and $CD8^+$ T cells in the DP strain of BB rat. These data further suggest that if these cells are pathogenic in this model, as suggested by the potassium channel abnormalities and islet infiltration (see below), it is the immunoregulatory balance abnormality that is important. Since the majority of $CD4^+$ or $CD8^+$ T cells are $CD5^+$, we suggest that the T cell lymphopenia of the DP BB rat is primarily related to the depression of $CD5^+ TCR \alpha/\beta^+$ cells rather than the $CD5^- TCR \alpha/\beta^+$ cells.

Very little is known about the function of double-negative T cells. Most express $TCR \gamma/\delta$ in normal animals, but we did not study this population since these cells are

primarily found in epithelial tissues [40]. Double-negative TCR α/β^+ cells, which represent a smaller proportion of the total double-negative T cell subset, have been implicated in the pathogenesis of several autoimmune diseases including diabetes [22–24]. Supporting this contention, we recently reported markedly increased levels of double negative T cells in both the spleen and islets of prediabetic and diabetic NOD mice [41]. We have also made identical observations in pre-diabetic and diabetic BB rat islets [31]. Thus, combining the results reported in this paper with our other results in BB rats and NOD mice, there is a consensus that double negative T cells represent the second most prominent T-cell infiltrating islets *in situ* in two spontaneously diabetic animal models. Functional studies using double-negative T cells from prediabetic and diabetic BB rats would help in the understanding of their role in the pathogenesis of autoimmune diabetes. Preliminary functional data strongly emphasize the cytolytic potential of overall islet infiltrative cells for killing islet target cells compared with splenic effector cells [31, 41]. Thus the overall balance in *in situ* islet cells is cytolytic. How the double negative T cell fits into this cytolytic scheme is unclear at present.

Activated T cells are reported to be present in inflamed islets, and treatment with IL-2R antibody, combined with a subtherapeutic dose of cyclosporin A, reverses diabetes and enhances pancreatic insulin content in diabetic BB rats [25]. Increased incidence of Ia antigen-bearing T lymphocytes has been reported in the thoracic duct, blood and lymph nodes of diabetic BB rats [26, 27]. In these studies T cells were determined with W3/13, a mAb which also binds NK cells. In our experiments T cells were measured using TCR α/β . We observed an increased level of T cell activation in the spleen of non-diabetic and diabetic DP rats compared with DR BB and WF strains, using percentages of cells expressing both IL-2R and class II antigens. In addition, DR rats showed a lower level of splenic T cell activation compared with normal WF animals. Although it is difficult to reconcile T cell lymphopenia with T cell-mediated autoimmune diabetes, our data suggest that non-depleted T cell subsets in the lymphopenic animal and/or activated T cell subsets are present in sufficient quantity to result in disease pathogenesis. The spleen T cell percentages are relatively constant during these later stages of prediabetes (80–100 days of age) and diabetes when compared with islet inflammation, where T cells markedly (four-fold) increase after diabetes onset [31].

Except for CD4⁺ and CD8⁺ T lymphocytes and activated T cells, there were no significant differences among absolute numbers of NK cells, macrophages or double-negative T cells in non-diabetic and diabetic DP animals compared with DR BB rats. However, the markedly elevated percentages and ratios of NK cells, macrophages and double-negative T cells when comparing the DP and DR strains may reflect an immune balance abnormality leading to autoreactivity and diabetes [42]. The balance problem in autoreactivity is emphasized by studies in which athymic, non-diabetes prone rat strains develop diabetes when injected with splenocytes obtained from RT6-depleted non-diabetes prone rats [43]. Since depletion of RT6⁺ T cells induces diabetes in the DR BB strain, this population may have a suppressor function, and relatively small changes in circulating absolute numbers or significant relative percentage changes, may lead to clinical autoimmune disease [43, 44].

In summary, (a) our data suggest that splenic NK cells, measured with specific mAb, are increased in prediabetic and diabetic DP rats; (b) the markedly elevated

macrophage levels suggest that macrophages may be directly responsible for T lymphopenia; (c) a population of CD8⁺ T cells may be present in the prediabetic and diabetic DP spleens; (d) double-negative and CD5⁻ T cell percentages are increased in prediabetic and diabetic DP animals; (e) splenic T cells show a high level of activation in prediabetic and diabetic DP strain rats; and (f) autoimmune diabetes may reflect an immune balance abnormality with a relative shift of cell subset dominance toward double-negative or activated T cells, NK cells and macrophages.

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