Altered Thymic and Peripheral T-Lymphocyte Repertoire Preceding Onset of Diabetes in NOD Mice

DANNY ZIPRIS, ANDREW R. CROW, AND TERRY L. DELOVITCH

Insulitis occurs by 5 wk of age in all NOD mice. However, diabetes is detectable only after 3–5 mo of age and only in ~50% of females and 10% of males in our colony. Therefore, it is predictable that changes in the T-lymphocyte repertoire of diabetes-prone mice occur and predispose them to disease. We demonstrate here that an altered (with respect to control BALB/cJ mice) thymic T-lymphocyte maturation reflected by a depletion (~12%) of $CD4^+CD8^+$ T lymphocytes and a reciprocal increase in $CD4^-CD8^-$ T lymphocytes precedes the onset of diabetes. This depletion was detected only ~3 mo after insulitis and is manifested by a specific loss (~3%) of immature T lymphocytes bearing $V\beta8^{lo}$ (lo is a relative level of expression) T-lymphocyte receptor. By onset of diabetes, an even greater decrease (~35%) of $CD4^+CD8^+$ and a reciprocal increase of $CD4^-CD8^-$ T lymphocytes were apparent and accompanied by the same depletion (3%) of $V\beta8^{lo}$ T lymphocytes. Administration of cyclophosphamide (CY), which accelerates the appearance of diabetes in NOD mice, caused similar depletions of $CD4^+CD8^+$ and $V\beta8^{lo}$ thymic T lymphocytes. The same alterations in the distribution of these thymic T-lymphocyte subsets were evident even earlier in insulitis- and diabetes-free NON mice, indicating that these changes in thymic T-lymphocyte development may be necessary but not sufficient to give rise to diabetes. Despite the common genetic origin of NOD and NON mice, differences at their MHC-linked and -unlinked loci may account for their differential susceptibility to diabetes. Analyses of peripheral lymph node (LN) T lymphocytes showed a decrease (6–10%) in the frequency of the $CD4^+$ T-lymphocyte subset and a concomitant reduction (3–4%) in $CD4^+V\beta8^+$ T lymphocytes in spontaneously and CY-induced diabetic NOD mice. Interestingly, the latter reduction resulted primarily from a depletion of $CD4^+V\beta8.1^+$ LN T lymphocytes in diabetic mice and was not detectable either in prediabetic NOD mice at 16 wk of age or in nondiabetic NON mice. These data suggest that depletion of $CD4^+$ regulatory T lymphocytes and/or the rerouting of $CD4^+V\beta8.1^+$ effector T lymphocytes from the peripheral LN to the pancreas during progression to disease onset mediate the pathogenesis of diabetes. *Diabetes* 40:429–35, 1991

From the Banting and Best Department of Medical Research and the Department of Immunology, University of Toronto, Ontario, Canada.

Address correspondence to Dr. Terry L. Delovitch, Banting and Best Department of Medical Research, and the Department of Immunology, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, Canada.

Received for publication 15 May 1990 and accepted in revised form 21 November 1990.

During the development of autoimmune diabetes, insulin-secreting pancreatic islet β-cells are believed to be destroyed by autoreactive T lymphocytes (1–3). In the NOD mouse model of insulin-dependent diabetes, infiltration of T lymphocytes into the pancreas (insulitis) occurs as early as 5 wk of age in all mice (4–5). Yet, a lag period of ~3–5 mo, depending on the particular mouse colony, exists after which diabetes is detectable in only some of these mice. It is predictable then, that both before and after insulitis, changes in the T-lymphocyte repertoire of diabetes-prone NOD mice occur and predispose them to diabetes. Such changes could result from previous intrathymic positive and/or negative selection of T lymphocytes (6–9).

To test this possibility, we analyzed the pattern of thymic T-lymphocyte development and the expression of the peripheral T-lymphocyte repertoire before and at onset of diabetes in both spontaneously diabetic and NOD mice that were injected with cyclophosphamide (CY), which accelerates the appearance of diabetes (10). Our results demonstrate that abnormal thymic T-lymphocyte maturation precedes the onset of diabetes in NOD mice by at least 3 wk, and this change in T-lymphocyte development elicits an altered expression of the T-lymphocyte repertoire that persists until disease onset.

RESEARCH DESIGN AND METHODS

Breeding pairs of NOD and NON mice were originally obtained from B. Singh (Dept. of Immunology, Univ. of Alberta,

Edmonton, Canada) and E. Leiter (Jackson, Bar Harbor, ME), respectively, and were bred in our facilities (Dept. of Comparative Medicine, Univ. of Toronto, Canada). Age- and sex-matched BALB/cJ mice (obtained from our colony) were used as controls. The spontaneous incidence of diabetes in our colony reaches 50% in females and 10% in males by 6 mo of age. Mice were checked for glycosuria twice weekly (Diastix, Miles, Ontario, Canada). Mice were killed on the day of detection of hyperglycemia and used in this study. When the effects of CY (Sigma, St. Louis, MO) on thymic T-lymphocyte development and T-lymphocyte receptor (TCR) Vβ expression were studied, mice were injected with 200 μl (300 mg/kg i.p.) of the drug. Diabetes was usually detected 2–3 wk after CY injection.

For cell preparation, lymph node (LN; pooled axillary, inguinal, and popliteal) and thymus cell suspensions were prepared in phosphate-buffered saline containing 0.5% fetal calf serum. For monoclonal antibody (MoAb) and flow cytometric analyses, cells were double stained with phycoerythrin-conjugated anti-CD4 (GK 1.5, rat IgG_{2b};11) and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (53-6.7, rat IgG_{2a};12) MoAbs (both from Becton Dickinson, Mountain View, CA). Cells were single stained with either the anti-CD3 (145-2C11, hamster IgG;13) MoAb or one of the anti-TCR Vβ5.1 (MR9-8, hamster IgG;14), Vβ5.1 + 5.2 (MR9-4, hamster IgG;14), Vβ6 (RR4-7, rat IgG;15), Vβ8.1 + 8.2 (KJ16, rat IgG;16), and Vβ11 (RR3-15, rat IgG; 14) MoAbs, followed by further washing. TCR Vβ8.1 + 8.2 and CD3 antigens were detected by incubating the cells with FITC-labeled goat anti-rat IgG (absorbed on mouse Ig) or FITC-labeled goat anti-mouse IgG (which cross-reacts with hamster IgG; Jackson, West Grove, PA), respectively, followed by further washing. Vβ5.1$^+$, Vβ5.1 + 5.2$^+$, Vβ6$^+$, and Vβ11$^+$ T lymphocytes were detected with the respective biotinylated anti-Vβ antibodies (provided by E. Palmer, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), followed by incubation with duochrome-conjugated streptavidin (Becton Dickinson). Stained cells (10^4 cells/sample) were analyzed by flow cytometry on an EPICS V flow cytometer (Coulter, Hialeah, FL) equipped with a 3-decade logarithmic amplifier.

Parametric group data in each experiment were analyzed according to Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Depletion of CD4$^+$CD8$^+$ thymocytes in NOD and NON mice was studied. The proportions of different thymic T-lymphocyte subsets in prediabetic NOD mice (females or males) at ~16 wk of age, i.e., ~3 mo after the onset of insulitis, were similar to that of healthy BALB/cJ mice (Figs. 1 and 2). These NOD mice had a normal distribution of CD3^{hi} (where hi or lo refers to relative levels of expression present on CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ mature T lymphocytes) and CD3^{lo} (present on CD4$^+$CD8$^+$ immature T lymphocytes) thymic T lymphocytes. However, by 21 wk of age, both prediabetic female

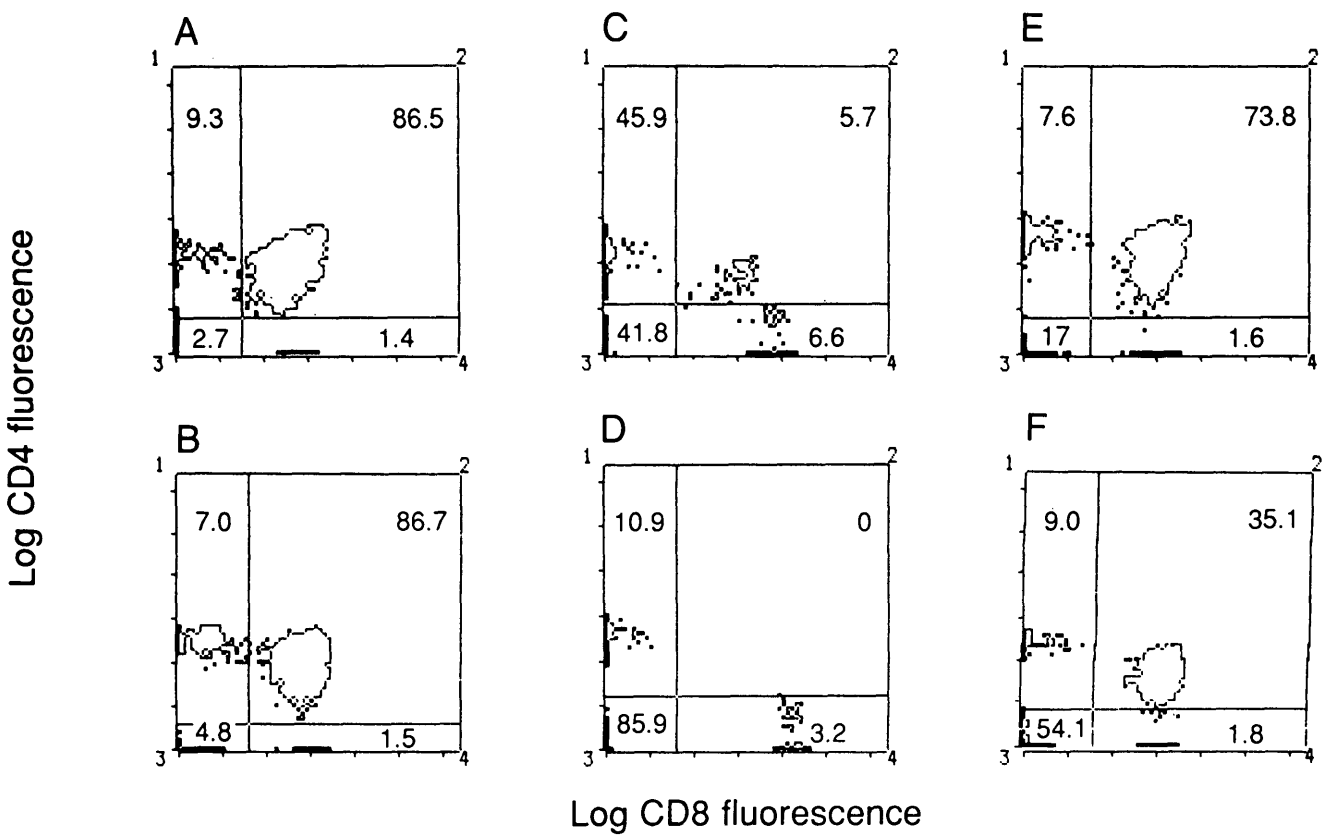

FIG. 1. Depletion of CD4$^+$CD8$^+$ thymocytes in NOD and NON mice. Thymocytes were stained with anti-CD4 and anti-CD8 monoclonal antibodies and analyzed by flow cytometry (see Table 1). *A*: untreated 16-wk-old BALB/cJ mouse; *B*: prediabetic 16-wk-old female NOD mouse; *C*: 16-wk-old female BALB/cJ mouse; and *D*: 16-wk-old female NOD mouse were administered cyclophosphamide 5 days previously. *E*: 10-wk-old female NON mouse; *F*: spontaneously diabetic 6-mo-old NOD mouse. Percentages of T lymphocytes in quadrants of each histogram are indicated.

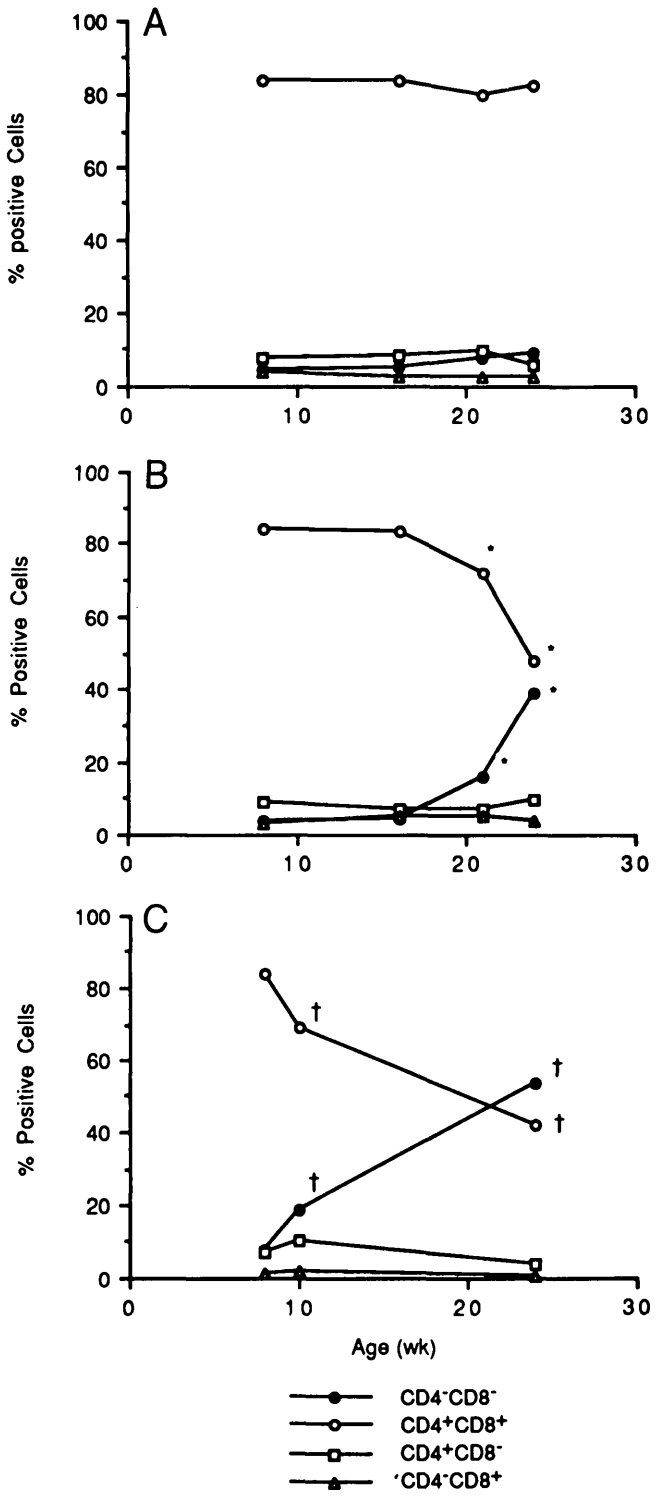

FIG. 2. Age-dependent changes of thymocyte subset distributions before and at onset of diabetes in control BALB/cJ (*A*) and NON (*C*) mice, and NOD mice (*B*). Thymocytes were stained with anti-CD4 and anti-CD8 monoclonal antibodies and analyzed by flow cytometry. *Points*, mean value per group size of mice as shown in Table 1. SE was always 5–15%. *$P < 0.05$ vs. 16-wk-old NOD mice. †$P < 0.05$ vs. 8-wk-old NON mice.

and male NOD mice displayed a 30% reduction in their number of thymocytes, an 11.7% increase in $CD4^-CD8^-$ T lymphocytes, and a reciprocal decrease in $CD4^+CD8^+$ T lymphocytes compared with 16-wk-old prediabetic mice (Figs. 1 and 2). Furthermore, although the percentages of their $CD3^{hi}$ T lymphocytes were normal, those of their $CD3^{lo}$ T lymphocytes were diminished considerably, reflecting the extensive depletion of $CD4^+CD8^+CD3^{lo}$ T lymphocytes. Even more profound changes in thymic T-lymphocyte frequencies were observed at the onset of diabetes in NOD mice at 6–7 mo of age, probably due to an advanced stage of cell depletion. As expected, control BALB/cJ mice between 4 and 7 mo of age displayed none of the progressive T-lymphocyte subset changes identified above for NOD mice (not shown). In contrast, abnormal T-lymphocyte frequencies ($CD4^-CD8^-$, 18.9%; $CD4^+CD8^+$, 69%) and a drastic reduction in the number of thymocytes were seen in 10-wk-old insulitis- and diabetes-free NON mice (Figs. 1 and 2). The reduced thymocyte number in NON mice was observed even neonatally beginning at 1 wk of age.

TCR Vβ expression was studied in NOD and NON thymocytes. The altered T-lymphocyte subset distribution in prediabetic and diabetic NOD mice was accompanied by an interesting progressive change in their thymic T-lymphocyte repertoire. The $V\beta8^+$ T-lymphocyte subpopulation analyzed here and in all subsequent experiments included both $V\beta8.1^+$ and $V\beta8.2^+$ T lymphocytes as detected by reactivity with the KJ16 MoAb. Compared with NOD mice at 16 wk of age, NOD mice analyzed at 3 wk before onset, at onset, and after onset of diabetes all showed a 2–3% loss of Vβ8- but not Vβ6- or Vβ11-bearing T lymphocytes (Table 1). Although this reduction in $V\beta8^+$ thymocytes was low, it is significant and has been observed in >20 diabetic mice assayed. This loss occurred only in the $V\beta8^{lo}$ (from 5.7 to 3.2%) and not $V\beta8^{hi}$ T-lymphocyte subset, consistent with the notion that immature $CD3^{lo}V\beta8^{lo}$ T lymphocytes are depleted in prediabetic (21-wk-old) and diabetic NOD mice. The percentage of $V\beta8^+$ T lymphocytes in NON mice was reduced compared with BALB/cJ mice but did not differ significantly from that of NOD mice. A similar percentage of $V\beta6^+$ T lymphocytes was noted in NOD and NON mice.

The effect of CY administration on thymic T-lymphocyte development in NOD mice was then studied. Because we noted that $CD4^+CD8^+$ T lymphocytes are extensively depleted in the thymus of prediabetic NOD mice, we examined whether the accelerated development of diabetes elicited by CY administration is mediated by the direct action of CY on these $CD4^+CD8^+$ rapidly dividing precursor T lymphocytes. We reasoned that if CY administration in prediabetic NOD mice elicits diabetes in 100% of injected mice within 2–3 wk by the same mechanism that we observed in spontaneously diabetic NOD mice, this would considerably facilitate our attempts to study changes in the T-lymphocyte repertoire during the rapid onset of disease. Thymuses from 12-wk-old NOD mice, injected 5 days previously with a single dose (300 mg/kg) of CY, were acutely depleted of total thymocytes and of $CD4^+CD8^+CD3^{lo}$ and $V\beta8^{lo}$ T lymphocytes in particular (Fig. 1; Table 2), confirming the notion that CY exerts its toxic effect primarily on rapidly dividing precursor T lymphocytes (17). Compared with untreated control NOD mice, the depletions were offset by notable increases in the $CD4^-CD8^-$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $V\beta8^{hi}$ T-lymphocyte subsets. After 2–3 wk of CY administration, at the time of onset of diabetes in NOD mice, the T-lymphocyte subset distributions in CY-administered diabetic NOD mice (Table

TABLE 1
T-lymphocyte–receptor Vβ expression on thymocytes from BALB/cJ (control), NOD (diabetes-susceptible), and NON (diabetes-resistant) mice

Phenotype	16-wk-old BALB/cJ (%)	NOD (%)			10-wk-old NON (%)
		Prediabetic		Diabetic	
		16 wk old	21 wk old	≥24 wk old	
$CD3^{hi}$	7.5 ± 0.5	8.7 ± 0.6	8.8 ± 1.0	9.0 ± 1.3	9.9 ± 1.0
$CD3^{lo}$	29.9 ± 0.5	19.6 ± 2.6	10.5 ± 4.1*	12.9 ± 3.5*	24.6 ± 2.0
Vβ6	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
Vβ8	9.0 ± 3.6	8.7 ± 0.5	5.3 ± 0.9*	5.4 ± 1.3*	6.7 ± 0.8
Vβ11	0.3 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	0.7 ± 0.1
Thymocytes/mouse ($\times 10^{-6}$)	114 ± 13	123 ± 17	87 ± 14	28 ± 5*	30 ± 3*

Values are means ± SE. Values for $V\beta8^+$ T lymphocytes include $V\beta8.1^+$ and $V\beta8.2^+$ T lymphocytes. The results shown are expressed as a percentage of the total thymocytes. Hi and lo, relative levels of expression. Thymocyte suspensions were prepared from prediabetic (21-wk-old males and females, n = 10; 16-wk-old prediabetic, n = 11), spontaneously diabetic (24-wk-old females and 36-wk-old males, n = 10), and control noninjected BALB/cJ (n = 5) or NON (n = 10) mice.
*$P < 0.05$ vs. 16-wk-old NOD mice.

2) mirrored those of the spontaneously diabetic NOD mice (Table 1). Although similar, although not as dramatic, changes in the various thymic T-lymphocyte subsets were found in BALB/cJ mice after 5 days of CY administration, these changes were only transient because, after 2–3 wk of CY administration, the proportions of BALB/cJ thymic T-lymphocyte subsets returned to normal (Table 2). Thus, CY administration resulted in a persistent redistribution of thymic T-lymphocyte subsets in prediabetic NOD mice and not in age- and sex-matched control BALB/cJ mice. The effects of CY seen in treated NOD mice paralleled those observed in spontaneously diabetic NOD mice.

TCR Vβ expression was studied in LN cells from CY-administered and noninjected NOD mice. It has been suggested that a loss of certain T lymphocytes (e.g., regulatory T lymphocytes) in the periphery of spontaneously and CY-administered diabetic NOD mice would allow potentially autoreactive T lymphocytes to attack the pancreas and cause diabetes (5). Therefore, we investigated whether any changes in TCR expression are detectable in the peripheral LN T-lymphocyte subpopulations of such mice relative to prediabetic NOD, NON, and control BALB/cJ mice. Relative to prediabetic 16-wk-old mice, both spontaneous and CY-administered diabetic mice had a net reduction (~6–10%) in their $CD4^+$ LN T lymphocytes and a concomitant net increase (~5–6%) in their $CD8^+$ LN T lymphocytes (Table 3). Significant decreases of $CD4^+V\beta8^+$ T lymphocytes were found in spontaneously and CY-administered diabetic NOD mice. In addition, prediabetic and diabetic NOD mice all possessed the same frequency (~7–8%) of $CD8^+V\beta8^+$ T lymphocytes, and this frequency was reduced considerably in NON mice. The distribution of $CD4^+V\beta11^+$ T lymphocytes was virtually the same in prediabetic and diabetic NOD mice. After normalization for the number of $CD3^+$ LN T lymphocytes in various mice, we found a reduction (~3%) in $V\beta8^+$ T lymphocytes in spontaneously diabetic NOD mice relative to 16-wk-old prediabetic NOD mice (Table 3). A similar decrease (~4%) in this T-lymphocyte subpopulation was observed in CY-administered diabetic mice. The finding that the frequency of $V\beta8^+$ T lymphocytes is reduced in both sets

TABLE 2
Thymocyte subset distribution in 12- to 16-wk-old cyclophosphamide-injected NOD (diabetes susceptible) mice

Phenotype	Noninjected (%)	Cyclophosphamide injected (300 mg/kg)			
		BALB/cJ (%)		NOD (%)	
		5 day	2–3 wk	5 day	2–3 wk
$CD4^-CD8^-$	4.3 ± 0.6	49.8 ± 8.1	7.7 ± 1.9	79.8 ± 3.4*	27.7 ± 4.8*
$CD4^+CD8^+$	83.4 ± 1.9	13.5 ± 5.1	81.2 ± 2.6	0.7 ± 0.2*	58.4 ± 6.3*
$CD4^+CD8^-$	7.2 ± 0.7	21.1 ± 4.5	7.4 ± 1.2	13.2 ± 2.2*	9.4 ± 2.7
$CD4^-CD8^+$	5.1 ± 2.0	15.6 ± 3.7	3.7 ± 0.7	6.3 ± 1.0	4.5 ± 1.3
$CD3^{hi}$	8.7 ± 0.6	NT	NT	18.6 ± 4.1	7.6 ± 2.1
$CD3^{lo}$	19.6 ± 2.6	NT	NT	0.0 ± 0.0*	14.6 ± 1.0*
$V\beta8^{hi}$	2.0 ± 0.1	NT	NT	12.4 ± 2.0*	1.7 ± 0.8
$V\beta8^{lo}$	6.7 ± 0.7	NT	NT	2.2 ± 0.5*	4.7 ± 0.7*
Thymocytes/mouse ($\times 10^{-6}$)	123 ± 17	9 ± 1	57.0 ± 9.8	4 ± 1*	32 ± 5*

Values are means ± SE. Hi and lo, relative levels of expression. The results are expressed as a percentage of the total thymocytes. Thymocyte suspensions were prepared from 12- to 16-wk-old female and male NOD and BALB/cJ mice that were (n = 5) or were not (n = 11) injected either 5 days or 2–3 wk previously with 300 mg/kg i.p. cyclophosphamide in phosphate-buffered saline. Cells were stained and analyzed by flow cytometry as in Table 1. The $V\beta8^+$ T lymphocytes were separated into the $V\beta8^{lo}$ and $V\beta8^{hi}$ subpopulations. NT, not tested.
*$P < 0.001$ vs. 12- to 16-wk-old NOD mice.

TABLE 3
T-lymphocyte–receptor Vβ expression by lymph node T lymphocytes in BALB/cJ, NOD (diabetes-susceptible), and NON (diabetes-resistant) mice

Phenotype	16-wk-old BALB/cJ (%)	NOD (%)			10-wk-old NON (%)
		16-wk-old prediabetic	≥24-wk-old spontaneously diabetic	14- to 18-wk-old cyclophosphamide-injected diabetic	
$CD4^+$	70.0 ± 1.4	73.5 ± 1.9	67.5 ± 3.8*	63.2 ± 2.1*	79.5 ± 12.9
$CD8^+$	23.5 ± 1.6	25.0 ± 1.3*	30.8 ± 1.3	29.4 ± 1.3*	15.8 ± 1.6*
$CD4^+V\beta8^+$	12.1 ± 0.9	13.9 ± 0.4	11.5 ± 0.5*	10.9 ± 0.1*	14.8 ± 2.4
$CD8^+V\beta8^+$	7.2 ± 0.4	8.2 ± 0.4	8.0 ± 1.0	7.2 ± 0.3	4.9 ± 0.2*
$CD4^+V\beta11^+$	1.1 ± 0.4	5.0 ± 0.2	4.4 ± 0.2	3.8 ± 0.4	11.1 ± 1.9
$CD8^+V\beta11^+$	0.3 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	2.4 ± 0.1	1.2 ± 0.3
Vβ5.1	0.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.3	1.2 ± 0.2	1.3 ± 0.0
Vβ5.2	0.9 ± 0.0	1.3 ± 0.5	1.3 ± 0.2	1.6 ± 0.3	1.3 ± 0.0
Vβ6	8.8 ± 0.5	8.2 ± 0.7	8.7 ± 0.5	9.1 ± 0.4	4.5 ± 0.9

Values are means ± SE. Values are normalized and expressed as a percentage of the mean number of $CD3^+$ lymph node T lymphocytes in each mouse strain analyzed. The percentage of $CD3^+$ lymph node T lymphocytes was ~84% in BALB/cJ (n = 5), prediabetic (n = 11), and spontaneously diabetic (n = 10) NOD mice; and 90% in cyclophosphamide-injected diabetic (n = 5) NOD mice and 43% in NON (n = 10) mice. Cells were stained and analyzed for CD3 and T-lymphocyte–receptor Vβ expression as in Table 1.
*$P < 0.05$ vs. 16-wk-old NOD mice.

of diabetic mice is striking because the percentages of LN T lymphocytes in these mice bearing $V\beta5.1^+$, $V\beta5.2^+$, $V\beta6^+$, and $V\beta11^+$ T lymphocytes were essentially the same as those obtained in prediabetic NOD mice. Compared with prediabetic and diabetic NOD mice, 16-wk-old BALB/cJ and 10-wk-old NON mice were not depleted of any $V\beta8^+$ T lymphocytes.

Although NON and BALB/cJ mice both express I-E molecules, $V\beta5.1^+$ and $V\beta11^+$ LN T lymphocytes were depleted in BALB/cJ but not NON mice (Table 3). The percentage of $V\beta5.2^+$ LN T lymphocytes in NON mice was approximately the same as that in BALB/cJ mice, and that of their $V\beta5.1^+$ and $V\beta11^+$ LN T lymphocytes was increased approximately six- and ninefold, respectively, in relation to BALB/cJ mice. The percentage of $V\beta6^+$ T lymphocytes was equivalent in all of the mice examined.

DISCUSSION

We examined the development of the T-lymphocyte repertoire in autoimmune NOD mice and compared it with that of insulitis- and diabetes-free NON mice. Our studies demonstrate that an altered pattern of thymic T-lymphocyte maturation is detectable at an average age of 21 wk in prediabetic male and female NOD mice. This alteration correlates with a change in the developing T-lymphocyte repertoire that is manifested in part by a rather significant reduction in the proportion of $CD4^+CD8^+$ thymocytes and the intrathymic depletion of immature $CD3^{lo}V\beta8^{lo}$ T lymphocytes. These two events precede the onset of diabetes by a minimum of ~3 wk and are evident in both spontaneously and CY-accelerated diabetic mice. Although the mechanism of this altered thymic T-lymphocyte development in prediabetic mice is not yet known, it is possible that a defect in a bone marrow–derived thymic stromal cell(s) causes the change in thymocyte maturation. This possibility is supported by findings that the expression of NOD diabetogenic alleles in bone marrow–derived progenitor cells is sufficient for the development of diabetes (18). In addition, it was shown that a defect in function of bone marrow–derived antigen-presenting cells in the BB rat has an important role in inducing peripheral lymphocyte abnormalities and diabetes (19). Moreover, an aberrant thymic maturation of $CD8^+$ T lymphocytes that may give rise to the expression of functionally and phenotypically abnormal T lymphocytes was also identified in the BB rat (20).

It may also be argued that the change in thymic T-lymphocyte maturation noted in NOD mice results from diabetes-associated stress and dehydration. However, this is unlikely because similar changes were detected in diabetes-resistant NON mice. Furthermore, despite the fact that only ~50% of females and 10% of males develop diabetes in our NOD mouse colony, abnormal thymic T-lymphocyte development was evident in all female and male NOD mice at 21 wk of age.

The defect in intrathymic T-lymphocyte development in NON mice is even more severe than that noted in prediabetic and diabetic NOD mice. Moreover, this defect arises at a younger age in NON mice than in prediabetic NOD mice and leads to a marked decrease in the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes in their LNs (data not shown). This might preclude the maturation of autoreactive T lymphocytes in an NON thymus that are requisite for migration to the pancreas and the development of diabetes. Our findings are consistent with previous reports that demonstrated T-lymphocyte abnormalities (T lymphocytopenia and unresponsiveness to concanavalin A) in the periphery of NON mice (21). Because NOD and NON mice were derived from the same ICR founder strain (4), our observations indicate that common genetic factors may cause a defect in thymic T-lymphocyte development in these two strains. In contrast to NOD mice, NON mice express I-E and normal I-A (i.e., Asp 57^+ Aβ-chain) MHC class II antigens (22); thus, neither lack of I-E expression nor the unique I-A (i.e., Asp 57^- Aβ-chain) antigens present in NOD mice are responsible for this defect. Nonetheless, these MHC differences might cause the antigen-MHC specificities of $CD4^+V\beta8^+$ and

$CD8^+V\beta8^+$ T lymphocytes to differ between NON and NOD mice, and these thymus-dependent differences could give rise to diabetes in NOD but not NON mice. Additional genetic differences between NON and NOD mice at the MHC-unlinked insulin-dependent diabetic 2 and 3 loci (21) may also account for the differential susceptibility of these mice to diabetes.

The depletion of $CD4^+V\beta8^+$ T lymphocytes from the periphery of spontaneously diabetic NOD mice is compatible with the report that splenic $CD4^+$ T lymphocytes, when obtained from young but not older prediabetic mice, display suppressorlike function because they inhibit the transfer of diabetes to irradiated NOD recipients (23). Evidence for the presence of such regulatory $CD4^+$ T lymphocytes in diabetic NOD mice was also provided by Reich et al. (24). They proposed that the development of diabetes depends on the balance between effector and regulatory T lymphocytes. Thus, a reduction in such regulatory $CD4^+$ peripheral T lymphocytes could enhance the activity of autoreactive T lymphocytes bearing either $V\beta8^+$ or other TCR Vβ-chains (e.g., Vβ5.1 and Vβ5.2, [25]) and elicit the onset of disease.

Injection of mice with CY inactivates rapidly dividing precursor T-lymphocytes and abrogates the function of peripheral suppressor T-lymphocytes (17). CY administration also accelerates the development of diabetes in female and male prediabetic NOD mice within 2–3 wk after administration (5,10). In addition, spleen cells from CY-administered mice can transfer diabetes to syngeneic irradiated NOD recipients (26), and administration of anti-Vβ8 (Vβ8.1 + 8.2 + 8.3) into CY-administered NOD mice can prevent diabetes (27). CY does not appear to act directly on insulin-secreting pancreatic islet β-cells (26). In agreement with these reported effects of CY administration are our observations that CY causes marked changes in the T-lymphocyte repertoire of NOD mice by inactivating a substantial portion of $CD4^+CD8^+$ thymic T lymphocytes and by reducing the percentage of $CD4^+V\beta8^+$ T lymphocytes in the thymus and periphery. In fact, the reduction in the latter T-lymphocyte subset is due to a decline in the frequency from 6 to 2% of $V\beta8.1^+$ T lymphocytes in peripheral LN as estimated by the differential reactivity of these cells with the F23.2 (anti-Vβ8.2) and KJ16 (anti-Vβ8.1 + 8.2) MoAbs (unpublished observations). It is possible that certain regulatory T lymphocytes, i.e., $V\beta8.1^+$ T lymphocytes, are eliminated in the thymus and that this leads to a change in the ratio of effector to regulatory T lymphocytes in the periphery. The selective depletion of $CD4^+V\beta8.1^+$ T lymphocytes by CY in the periphery of NOD mice is similar to the recent findings of Eto et al. (28). They showed that the mechanism by which CY induces tolerance to Mls-1^a in Mls-1^b mice involves rapid and selective elimination of $CD4^+V\beta6^+$ but not $CD8^+V\beta6$ T^+ lymphocytes. Furthermore, they proposed that CY destroys Mls-1^a–reactive T lymphocytes that proliferate in the periphery of the tolerance-induced mice. By analogy, regulatory $CD4^+V\beta8.1^+$ T lymphocytes that proliferate in the periphery of NOD mice could be depleted after CY administration. Thus, the ability of CY administration to overcome immunosuppression (17,26–31) and give rise to disease may be a consequence of its capacity to both alter the pattern of thymic T-lymphocyte maturation and deplete subsets of peripheral $CD4^+$ regulatory T lymphocytes.

Finally, although we found that $CD4^+V\beta8^+$ T lymphocytes, and mainly $CD4^+V\beta8.1^+$ T lymphocytes, are depleted from peripheral LN in prediabetic and diabetic NOD mice, this may not be a true depletion per se because it is possible that some of these T lymphocytes infiltrated the pancreas of these mice and consequently escaped detection in the LN. Evidence supporting this possibility is provided by the observations that the percentage of $V\beta8^+$ T lymphocytes is increased considerably in the pancreas of diabetic NOD mice, and the incidence of insulitis can be decreased approximately two- to threefold by prolonged pretreatment of neonatal NOD mice with the F23.1 anti-Vβ8 MoAb (Vβ8.1 + 8.2 + 8.3) (32). If this increase in $V\beta8^+$ pancreatic T lymphocytes is due to an increase in $CD4^+V\beta8.1^+$ T lymphocytes, this would implicate the latter T-lymphocyte subset in the pathogenesis of diabetes in NOD mice. Further experimentation is required to test this possibility.

ACKNOWLEDGMENTS

This work was supported by a grant from the Canadian Diabetes Association. D.Z. was supported by a Canadian Diabetes Association postdoctoral fellowship.

We thank Dr. E. Palmer for providing biotinylated anti-Vβ monoclonal antibodies. Special thanks is extended to C.A. Smith for expertise with fluorescence-activated cell-sorter analyses.

REFERENCES

1. Rossini AA, Mordes JP, Like AA: Immunology of insulin-dependent diabetes mellitus. *Annu Rev Immunol* 3:289–320, 1985
2. Wicker LS, Miller BJ, Chai A, Terada M, Mullen Y: Expression of genetically determined diabetes and insulitis in the nonobese diabetic (NOD) mouse at the level of bone-marrow derived cells: transfer of diabetes to nondiabetic (NOD × B10) F1 mice with bone-marrow cells from NOD mice. *J Exp Med* 167:1801–10, 1988
3. Miller BJ, Appel MC, O'Neil JJ, Wicker LS: Both the $Lyt\text{-}2^+$ and $L3T4^+$ T-cell subsets are required for the transfer of diabetes in nonobese diabetic mice. *J Immunol* 140:52–58, 1988
4. Makino S, Kunimoto K, Muraoka Y, Muzushima Y, Katagiri K, Tochino Y: Breeding of a non-obese, diabetic strain of mice. *Exp Anim* 29:1–13, 1980
5. Bach JF: Mechanisms of autoimmunity in insulin-dependent diabetes mellitus. *Clin Exp Immunol* 72:1–8, 1988
6. Kappler JW, Roehm N, Marrack P: T-cell tolerance by clonal elimination in the thymus. *Cell* 49:273–80, 1987
7. MacDonald HR, Schneider R, Lees RK, Howe C, Acha-Orbea H, Festenstein H, Zinkernagel RM, Hengartner H: T-cell receptor Vβ use predicts reactivity and tolerance to *Mls*^{a}-encoded antigens. *Nature* (Lond) 332:40–45, 1988
8. Teh HS, Kisielow P, Scott B, Kishi H, Uematsu Y, Bluthmann H, von Boehmer H: Thymic major histocompatibility complex antigens and the T-cell receptor determine the CD4/CD8 phenotype of T-cells. *Nature* (Lond) 335:229–33, 1988
9. Scott B, Bluthmann H, Teh HS, von Boehmer H: The generation of mature T-cells requires interaction of the T-cell receptor with major histocompatibility antigens. *Nature* (Lond) 338:591–93, 1989
10. Harada M, Makino S: Promotion of spontaneous diabetes in nonobese diabetes-prone mice by cyclophosphamide. *Diabetologia* 27:604–606, 1984
11. Dialynas DP, Quan ZS, Wall KA, Pierres A, Quintans J, Loken MR, Pierres M, Fitch FW: Characterization of the murine T-cell surface molecule, designated L3T4, identified by monoclonal antibody G.K. 1.5: similarity of L3T4 to the human Leu3/T4 molecule. *J Immunol* 131:2445–51, 1983
12. Ledbetter JA, Rouse RV, Mickler HS, Herzenberg LA: T-cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. *J Exp Med* 152:280–95, 1980
13. Bluestone JA, Pardoll D, Sharrow SO, Fowlkes BJ: Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature* (Lond) 326:82–84, 1987
14. Bill J, Kanagawa O, Woodland DL, Palmer E: The MHC molecule I-E is necessary but not sufficient for the clonal deletion of Vβ11-bearing T-cells. *J Exp Med* 169:1405–19, 1989
15. Kanagawa O, Palmer E, Bill J: The T-cell receptor Vβ6 domain imparts

reactivity to the Mls-1^a antigen. *Cell Immunol* 119:412–26, 1989
16. Roehm N, Herron L, Cambier J, DiGuisto D, Haskins K, Kappler J, Marrack P: The major histocompatibility complex restricted antigen receptor on T cells: distribution on thymus and peripheral T cells. *Cell* 38:577–84, 1984
17. Turk JL, Parker D: Effect of cyclophosphamide on immunological control mechanisms. *Immunol Rev* 65:99–113, 1982
18. Serreze DV, Leiter EH, Worthen SM, Shultz LD: NOD marrow stem cells adoptively transfer diabetes to resistant (NOD × NON)F1 mice. *Diabetes* 37:252–55, 1988
19. Georgiou HM, Bellgrau D: Thymus transplantation and disease prevention in the diabetes-prone Bio-Breeding rat. *J Immunol* 142:3400–405, 1989
20. Plamondon C, Kottis V, Brideau C, Metroz-Dayer M-D, Poussier P: Abnormal thymocyte maturation in spontaneously diabetic BB rats involves the deletion of CD4$^-$CD8$^+$ cells. *J Immunol* 144:923–28, 1990
21. Prochazka ME, Leiter EH, Serreze DV, Coleman DL: Three recessive loci required for insulin-dependent diabetes in non-obese diabetic mice. *Science* 237:286–89, 1987
22. Acha-Orbea H, McDevitt HO: The first external domain of the nonobese diabetic mouse class II I-A chain is unique. *Proc Natl Acad Sci USA* 84:2435–39, 1987
23. Boitard R, Yasunami R, Dardenne M, Bach JF: T-cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J Exp Med* 169:1669–80, 1989
24. Reich E-P, Scaringe D, Yagi J, Sherwin RS, Janeway CA Jr: Prevention of diabetes in NOD mice by injection of autoreactive T-lymphocytes. *Diabetes* 38:1647–51, 1989
25. Reich EP, Sherwin RS, Kanagawa O, Janeway CA Jr: An explanation for the protective effect of the MHC class II I-E molecule in murine diabetes. *Nature* (Lond) 341:326–28, 1989
26. Yasunami R, Bach JF: Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in NOD mice. *Eur J Immunol* 18:481–84, 1988
27. Bacelj A, Charlton B, Mandel TE: Prevention of cyclophosphamide-induced diabetes by anti-$V_{\beta 8}$ T-lymphocyte–receptor monoclonal antibody therapy in NOD/Wehi mice. *Diabetes* 38:1492–95, 1989
28. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nomoto K: Intrathymic clonal deletion of Vβ6$^+$ T-cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J Exp Med* 171:97–113, 1990
29. Mitsuoka A, Morikawa S, Baba M, Harada TJ: Cyclophosphamide eliminates suppressor T-cells in age-associated central regulation of delayed hypersensitivity in mice. *J Exp Med* 149:1018–28, 1979
30. Lando Z, Teitelbaum D, Arnon R: Induction of experimental allergic encephalomyelitis genetically resistant strains of mice. *Nature* (Lond) 287:551–52, 1980
31. Charlton B, Bacelj A, Slattery RM, Mandel TE: Cyclophosphamide-induced diabetes in NOD/WEHI mice: evidence for suppression in spontaneous autoimmune diabetes mellitus. *Diabetes* 38:441–47, 1989
32. Fukuda M, Horio F, Kubo R, Hattori M: Monoclonal antibody F23.1 against T-cells bearing V beta 8 TcR elements can prevent the development of insulitis in NOD mice (Abstract). *Diabetes* 38 (Suppl. 2):12A, 1989