Rapid Publication

Superantigen-Like Effects and Incidence of Diabetes in NOD Mice

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The population of T-cells that develops in any individual can be divided into families based on sequence differences in the β-chain variable region of the T-cell receptor heterodimer. Major histocompatibility complex products and endogenous retroviral gene products have both been shown to exert powerful influences on the frequency distribution of T-cell receptor β-chain variable region families in the mouse. In most mouse strains, these repertoire modifiers appear to be fully functional early in mouse development and shape a repertoire of antigen specificities that remains essentially unchanged from the first weeks of life until old age. In NOD mice, an inbred mouse model of type I diabetes, puberty in males coincides with a β-chain variable region–specific T-cell expansion that mimics the results of exposure to exogenous superantigens in immunologically mature animals. The subsequent behavior of this subset indicates that it may play a role in the relative protection of male NOD mice from complete pancreatic β-cell destruction and overt diabetes. *Diabetes* 42:1094–98, 1993

The products of two classes of genes, one bacterial and the other viral, have been shown to interact specifically with T-cell receptors outside of the site that determines antigen specificity (1). These gene products are collectively known as superantigens to indicate that they are capable of triggering a large fraction of the mature T-cell population in a given responding individual. The high responder frequency for superantigens contrasts with the frequency of T-cells responsive to conventional MHC-restricted antigens, each of which can trigger only an extremely small fraction of mature T-cells. Viral superantigens, unequivocally identified to date only in the mouse, are associated with genes expressed by two families of retroviruses: mouse mammary tumor viruses (2), both endogenous and exogenous, and the type C retrovirus that causes the murine AIDS syndrome in susceptible strains of mice (3). Bacterial superantigens, which stimulate responses by both rodent and human T-cells, have been isolated from *Staphylococci*, *Streptococci*, and *Mycoplasma*.

T-cell responses to superantigens are mediated by T-cell receptor variable segments of the β-chain (4,5). β-chain specificity can be measured both in vivo and in vitro. In vitro, the presence of superantigens is detected by a unidirectional stimulation of T-cells in mixed lymphocyte cultures of MHC-matched individuals. Using large panels of anti-Vβ MoAbs and Vβ-specific DNA probes, several investigators have now shown that each of the recognized groups of superantigens triggers proliferation of a unique selection of TCR Vβ families (6). In vivo, the effects of superantigens have been recognized primarily by clonal elimination, during thymic maturation or after export to peripheral lymphoid organs, of T-cells bearing the same TCR Vβs that are triggered in vitro (7). The mouse TCR β-chain gene encodes 20 variable segments. Using anti-Vβ antibodies to quantify the frequency of each of the 20-Vβ families of T-cells is a very efficient way of assessing superantigen-induced shaping of the T-cell repertoire.

RESEARCH DESIGN AND METHODS

NOD/Bdc mice were derived from a breeding pair obtained in 1984 from the colony at Ehime University Medical School (Ehime, Japan) and were maintained by

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Type I diabetes, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; AIDS, acquired immunodeficiency syndrome; TCR, T-cell receptor; Vβ, β-chain variable region; TCR Vβ, T-cell receptor variable segments of the β-chain; MoAb, monoclonal antibody; Ig, immunoglobulin; IL-2, interleukin-2; SEB, *Staphylococcal enterotoxin B*.

brother-sister mating through 20 generations at the Barbara Davis Center for Childhood Diabetes (Denver, CO) under specific pathogen-free conditions. NOD/Tac mice (derived from the NOD line bred by L. Wicker and L. Peterson at Merck, Sharp, and Dohme, Rahway, NJ) were purchased as breeding triplets from Taconic Farms (Germantown, NY) and maintained under specific pathogen-free conditions in a separate room of the animal facility. All mice were serologically negative for mouse hepatic virus, mouse polio virus, pneumonia virus of mice, *Sendai* virus, and *Mycoplasma* and were cared for under a protocol approved by the University of Colorado Health Sciences Center Animal Care and Use Committee.

Flow cytometry. The following antibodies were used: CD4 (GK1.5, F. Fitch, Chicago, IL); CD8 (53–6.72 sp, ATCC); Vβ8 family and Vβ8.2 (F23.1 and F23.2, M. Bevan, La Jolla, CA); Vβ8.1 + Vβ8.2 (KJ16–133 sp, P. Marrack, Denver, CO); Vβ14 (R14–2, D. Raulet, Boston, MA); Vβ5 family and Vβ9 (MR9–4 and MR10–2, O. Kanagawa, St. Louis, MO); 187.1 (anti-mouse Ig, L. Kappa, Chapel Hill, NC). Biotin and fluorescein conjugation of ammonium sulfate precipitates from ascites or culture supernatant were performed by standard methods. Cells were analyzed simultaneously for expression of CD4 or CD8 (fluorescein-conjugated antibodies) and TCR Vβ (biotinylated antibodies and Streptavidin-phycoerythrin, Fisher, Pittsburgh, PA) as described previously (8). A minimum of 25,000 cells was analyzed for each measurement of subset size among peripheral T-cells; 5000 cells were analyzed for phenotyping of T-cell pools expanded in vitro. Analyses were performed on an EPICS C cytofluorograph using a 5-W argon laser at 488 nm and standard optics. Cells were gated using forward and 90°C light scatter, and calculations were made using standard software supplied by Coulter, Hialeah, FL.

Determination of Vβ8.3 subset. Because no specific MoAb is available that binds exclusively to Vβ8.3 polypeptide, the fraction of T-cells bearing this Vβ was determined by subtraction of the Vβ8.1 plus Bβ8.2 population, which binds KJ16–133, from the total Vβ8 subset, which binds F23.1. The mean frequency of the Vβ8.3 population among T-cells in mature male NOD mice is ~7.6% (25% of all $V\beta8^+$ T-cells). Simultaneous testing of peripheral T-cells from different sources (spleen, lymph node, and peripheral blood) showed no significant differences in the frequencies of these TCR Vβ subsets. After proliferation induced by monoclonal F23.1 in vitro, in the presence of IL-2, virtually all T-cells expressed $V\beta8^+$ T-cell receptors, and $V\beta8.3^+$ T-cells represented ~30% of the T-cell pool.

RESULTS

Mature, nondiabetic NOD mice maintain an extremely stable T-cell repertoire. Using MoAbs recognizing 12 of the ~20 TCR Vβ families, we documented little variability in frequency of TCR Vβ subsets, similar to what is seen in non–diabetes-prone mouse strains (9). Figure 1*A* shows that the levels of four representative Vβ subsets were remarkably stable from weaning until maturity, whereas

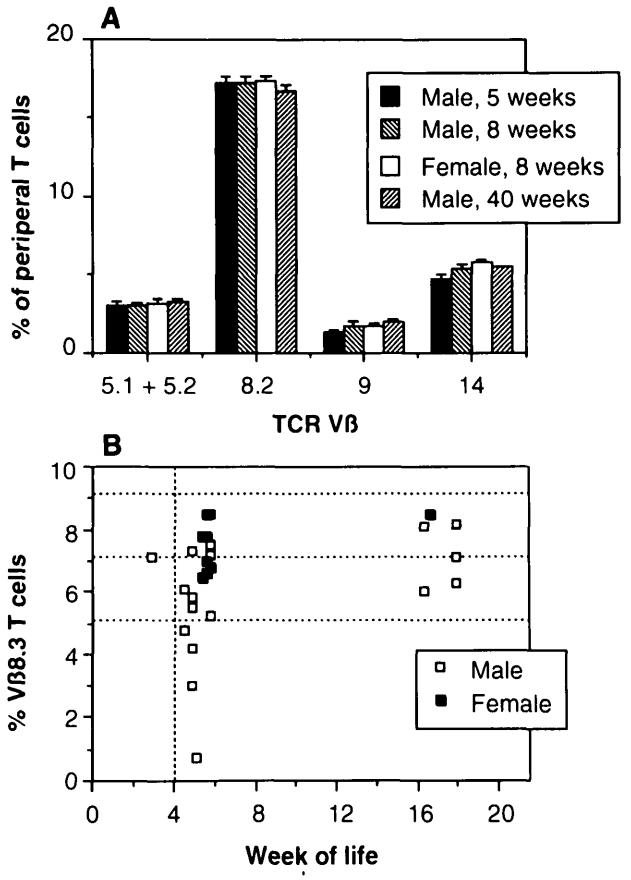

FIG. 1. Significant variability is seen in the levels of $V\beta8.3^+$ T-cells, but not among those of other T-cell Vβ subsets. *A*: two-color flow cytometry was used to determine the fraction of splenic T-cells in each Vβ subset as the fraction of the total $CD4^+ + CD8^+$ population. Bars represent means ± SE of three (40-wk-old males) or four (all other groups) determinations from individual mice. Vβ subsets were randomly chosen from among those for which MoAbs were available. *B*: splenic T-cells from male or female NOD mice were analyzed for expression of Vβ8.3. (Horizontal – – –), means ± 2 SD for mice >16 wk of age; (vertical – – –), 4 wk of age.

other experiments documented consistent levels into old age (>10 mo, data not shown).

On this background of stability, the behavior of one Vβ subset was remarkable. We first noted that male NOD mice tested at ~35 days of age had extremely variable levels of the Vβ8.3 T-cell subset (Fig. 1*B*). In this age-group, 4 of 12 males had a frequency of $V\beta8.3^+$ T-cells ≤2 SD below the mean established for older animals. This variability was not seen in male mice tested at 4 mo, nor in female mice at either age. We then followed levels of $V\beta8.3^+/CD4^+$ and $V\beta8.3^+/CD8^+$ T-cells by repeated blood sampling from 5 wk to 4 mo of life in 5 NOD males from a single litter. The $V\beta8.3^+/CD4^+$ subset showed minor variability in frequency at the ages tested in these mice (Fig. 2*A*). Within the CD8 subset, however, 2 mice had significantly lower levels of $V\beta8.3^+$ cells at 5 wk (≥2 SD below the mean at 4 mo), and 4 of 5 were below this limit at 11 wk (Fig. 2*B*).

One possible reason for such a drop in circulating $V\beta8.3^+/CD8^+$ cells is selective sequestration. Activation of T-cells has been shown to result in changes both in their migration patterns in vivo and adherence to vascular

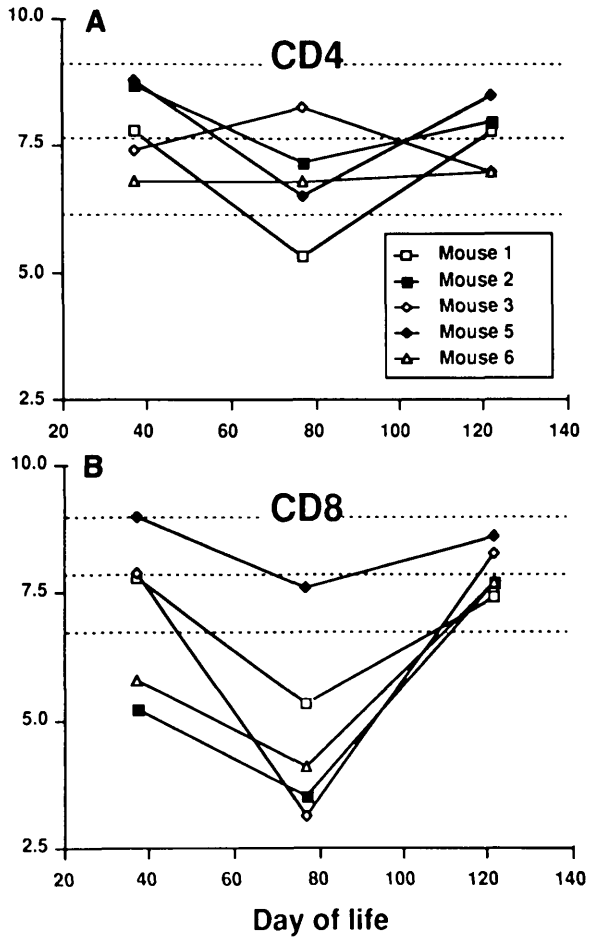

FIG. 2. Circulating Vβ8.3$^+$/CD8$^+$ T-cells fall to low levels in young NOD mice. Using two-parameter analysis (13), peripheral blood from 5 male NOD littermates was sampled at 37, 77, and 122 days of age, and the percentages of T-cells bearing Vβ8.3 from the CD4 (*A*) and CD8 (*B*) subsets were determined. (– – –), means ± 2 SD for each population of Vβ8.3$^+$ T-cells at 122 days of age.

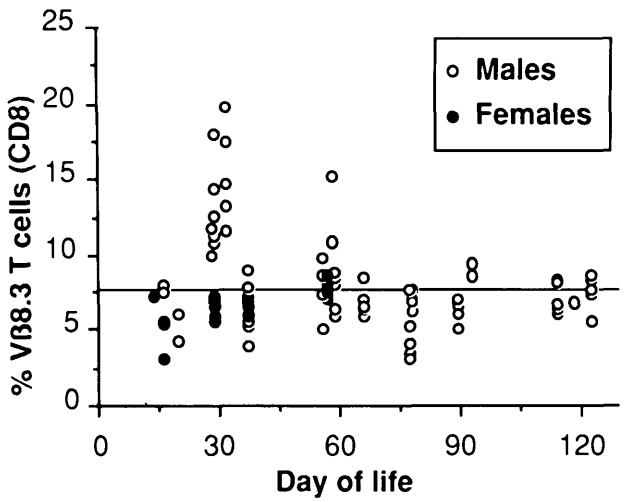

FIG. 3. NOD male mice, but not female mice, have a large expansion of Vβ8.3/CD8 T-cells during the 4th wk of life. The frequencies of Vβ8.3/CD8 T-cells in individual male and female mice from 14 to 120 days of age were determined by two-parameter cytofluorograph analyses of peripheral blood (mice >21 days of age) or spleen cell preparations (mice ≤21 days of age). (———), mean for mice ≥114 days of age.

endothelium in vitro. These changes correlate with alterations in the level of expression or the adhesion function of T-cell surface molecules such as LCAM-1 (MEL-14), CD44 (Pgp1), LFA-1, and several other members of the integrin family (10–14). We therefore considered the possibility that a Vβ-specific activation before 35 days of age might alter systemic circulation of this lymphocyte population and lead to its sequestration in a site of inflammation. For both males and females, Vβ8.3/CD4 T-cell frequency was stable and within 2 SD of the mean found in mice ≥120 days of age (data not shown). For CD8 T-cells, younger males (16–20 days of age) fell within or below the range found in older mice, but males from 3 of 3 litters tested between 28 and 30 days of age had Vβ8.3$^+$ T-cell frequencies >2 SD over the mean for older animals (range 9.9–19.8% of all CD8$^+$ cells) (Fig. 3). Although the Vβ8.3/CD8 population decreased rapidly in size in mice from 2 of the litters, for mice in 1 of 3 litters, the Vβ8.3/CD8 T-cell frequency remained elevated for a prolonged period (>60 days). No comparable increase was observed in the Vβ8.3/CD8 subset in female NOD mice.

The autoimmune diabetes of NOD mice results from a T-cell–dependent pancreatic infiltrate that selectively destroys insulin-producing β-cells. Neonatal NOD mice have normal pancreatic architecture without evidence of infiltrating mononuclear cells. Between 4 and 12 wk of age, NOD mice of both sexes develop increasingly severe pancreatic infiltration consisting largely of T-cells. In our colony, ~50% of animals have early signs of perivascular mononuclear cell infiltration between 4.5 and 5.5 wk. By 12 wk, virtually all animals examined have shown extensive perivascular infiltration, with evidence of early islet invasion in some. Although diabetes rarely appears before 14 wk of age, by 30 wk, 25% of males and 56% of females have become overtly diabetic (M.M., unpublished observations). Clearly, the transient expansion of the Vβ8.3/CD8 subset observed in male mice slightly precedes detectable mononuclear cell infiltration, whereas the transient decrease that follows coincides with expansion of these infiltrates. Because Vβ8.3 subset activation occurs selectively in male mice, the lower incidence of complete β-cell destruction and overt diabetes in males suggests that these cells might play a regulatory or inhibitory role in the diabetogenic process.

To test the hypothesis that activated Vβ8.3$^+$ T-cells can play an inhibitory role in diabetes, we postulated that transfer of activated Vβ8.3$^+$ T-cells into female NOD mice would decrease the incidence of overt disease. Splenic and lymph node T-cells from diabetic female NOD mice were expanded using antibodies reactive with Vβ8$^+$ T-cell receptors. The activated cells were transferred as a single dose of 10^7 cells to otherwise unmanipulated NOD females during the 2nd to 3rd wk of life. By 30 wk of age, 7 of 16 (44%) untreated females and 8 of 18 (44%) receiving expanded cells from the Vβ8.1 and Vβ8.2 subsets alone had become diabetic (Fig. 4*A*). However, transfer of expanded T-cell populations con-

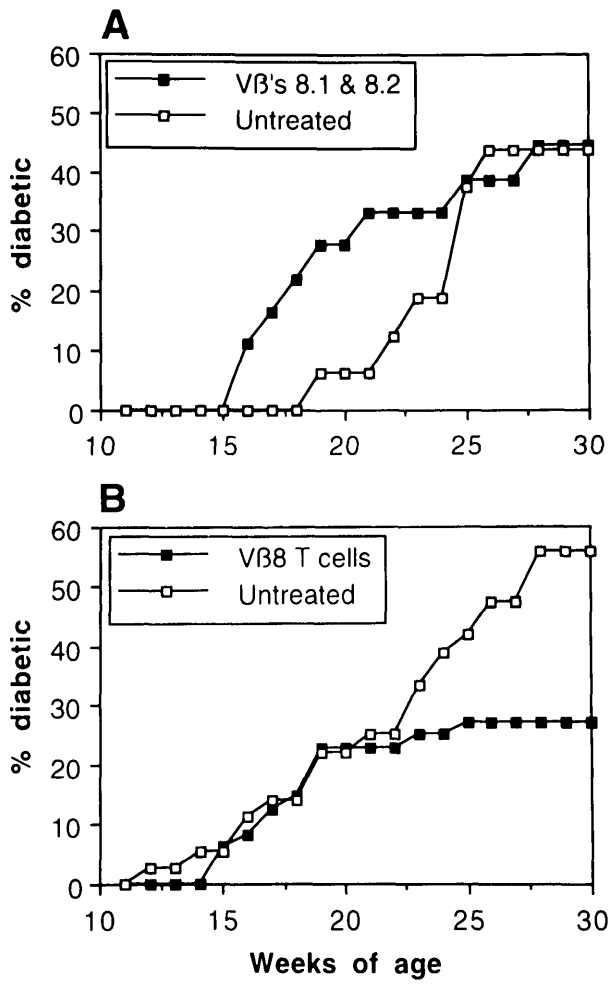

FIG. 4. Activated Vβ8⁺ T-cells inhibit development of overt diabetes. Pooled lymph node and splenic T-cells from newly diabetic female NOD mice were activated for 72 h by an anti-Vβ MoAb (KJ16–133 [*A*]; F23.1 [*B*]) in the presence of IL-2 before intraperitoneal injection. Diabetes was detected by glycosuria, and pancreatic β-cell loss was confirmed histologically after euthanasia following at least 7 days of a confirmed insulin requirement.

taining Vβ8.3⁺ cells decreased the incidence of diabetes in recipients. Only 13 of 48 (27%) females receiving activated T-cells from all three Vβ8 families (~30% Vβ8.3⁺) were diabetic by 30 wk (Fig. 4*B*) compared with 20 of 36 (56%) of their untreated female littermates ($\chi^2 = 5.85$, $P < 0.025$). These results support the hypothesis that activation of the Vβ8.3 T-cell subset in male NOD mice is related to their lower incidence of overt diabetes.

DISCUSSION

Although early reports emphasized the clonal deletion or clonal inactivation of peripheral T-cells after exposure in vivo to superantigens, two groups have recently shown that superantigens can induce clonal expansion in vivo. MacDonald et al. (15) showed that the number of T-cells expressing Vβ8 increased dramatically in the spleens of adult mice within 2 days after injection of SEB. This early expansion was accompanied by an increase in the proliferative response to SEB in vitro. By 7 days after injection, however, the frequency of Vβ8⁺ T-cells in spleen had decreased to levels below baseline, and no response to SEB could be detected in vitro. This phase of the response was followed by a slow return to baseline of Vβ8⁺ T-cell frequency and a partial recovery of the response in vitro to SEB by 30 days after the initial exposure. Hoffmann et al. (16) have established that the same pattern of expansion with hyperresponsiveness occurs in adult mice on first exposure to the endogenous viral superantigen encoded by mouse mammary tumor virus 7 (Mls-1a).

Therefore, the expansion in vivo of the Vβ8.3/CD8 subset in NOD mice is consistent with new exposure to an unidentified superantigen at ~4 wk of age. Two pieces of evidence suggest that this reflects a developmentally regulated expression of an endogenous superantigen. First, the early expansion of Vβ8.3⁺ T-cells has been noted to occur in males from two separate lines of NOD mice at identical ages. After documenting this phenomenon in NOD mice from the colony in Denver, we found expansion and loss of Vβ8.3⁺ T-cells in male NOD mice obtained from the colony at Taconic Farms. Second, only male NOD mice have shown evidence of a significant transient increase or decrease in Vβ8.3⁺ T-cells. If the expansion resulted from exposure to an exogenous pathogen, it seems likely that females would also show signs of its transmission. However, this response is unique in that the expansion and loss of Vβ8.3⁺ T-cells is seen only in the CD8 subset. One of the characteristics of other known superantigens, both bacterial and viral, is their requirement for MHC class II molecules in stimulation of T-cell responses. This is reflected by a more dramatic response from the CD4 subset, presumably because of the enhancement of class II/TCR interactions by the adhesion functions of CD4. Superantigen effects dependent on either MHC class I or CD8 molecules have not been reported, implying that the Vβ8.3 superantigen in NOD mice, which affects primarily CD8 T-cells, may represent a new class of superantigens.

The identity of the Vβ8.3-specific superantigen is unknown. Because steroid hormones, including androgens, are known to regulate mouse mammary tumor virus proviral transcriptional activity (17), work is currently underway to assess the possibility that androgen-mediated induction of mouse mammary tumor virus genes may be responsible for the sex-related differences in diabetes incidence in NOD mice.

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