Breaking Self-tolerance in Nonobese Diabetic Mice

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Summary

Unresponsiveness to self is maintained through two mechanisms of immune regulation: thymic-negative selection and peripheral tolerance. Although thymic-negative selection is a major mechanism to eliminate self-reactive T cells, normal mice have readily detectable populations of T cells reactive to self-proteins but do not exhibit autoimmune responses. It has been postulated that autoimmune disease results from breakdown or loss of peripheral tolerance. We present data that demonstrate that peripheral tolerance or unresponsiveness to self can be broken in nonobese diabetic (NOD) mice. Immunization of NOD mice (but not of conventional mice) with self-peptides caused an immune response to the self-peptide with resultant autoproliferation of peripheral lymphocytes. Autoproliferation of self-reactive T cells in NOD mice resulted from the recognition and proliferation of the activated T cells to endogenously processed and presented self-antigens. This loss of self-tolerance demonstrated in vitro may well be the basis of NOD autoimmune disease in vivo.

A puzzle in understanding self-tolerance has been the discovery of populations of T cells reactive to self-peptides in normal mice, as well as the presence of functional self-peptide MHC complexes, in the absence of discernible autoimmune responses. Lorenz and Allen (1, 2) demonstrated functional self-hemoglobin/class II complexes in CBA/J mice. Agrawal et al. (3) subsequently found T cells spontaneously reactive to self-MHC peptides in BALB/c mice, presumably due to endogenous priming by functional MHC/MHC peptide complexes, but these cells produced no detectable autoreactivity. In a double transgenic model, Ohashi et al. (4) constructed mice with pancreatic β-cell expression of a lymphocytic choriomeningitis virus (LCMV)[1] glycoprotein, as well as CD8^{+} transgenic T cells reactive with the glycoprotein. Despite the demonstration that the LCMV glycoprotein was expressed with the appropriate MHC class I association in the pancreas and the demonstration that the cytotoxic lymphocytes bearing the transgeneic TCR were neither deleted nor anergized, the mice showed no signs of autoreactivity to the endogenous antigen. The coexistence of autoreactive T cells and functional self-peptide MHC complexes in the periphery, in the absence of autoreactivity, has been explained as peripheral unresponsiveness or tolerance. Here we present studies on breaking peripheral tolerance in nonobese diabetic (NOD) mice.

The NOD mouse is a model of autoimmune diabetes with T cell–mediated pancreatic islet inflammation (insulitis) and resultant β-cell destruction. NOD mice, however, suffer an array of coexisting spontaneous autoimmune phenomena with lymphocytic infiltration into many organs, including salivary and lacrimal glands, thyroid, parathyroid, adrenal cortex, bowel, and testes (5–7). Multiple autoantigens have been defined in NOD mice, e.g., glutamic acid decarboxylase, peripherin, carboxypeptidase H, insulin, and heat-shock proteins have been demonstrated as targets of spontaneous autoimmunity (8, 9). These data suggest that a global immune regulatory defect, rather than recognition of a single autoantigen, may be responsible for NOD autoimmune phenomenon. Here we describe a novel model of disregulation of self-reactivity in NOD mice in which immunization of NOD mice but not normal DBA/2 mice, with self-peptides produced a breakdown of tolerance and an autoproliferative response in "peripheral lymphocytes". We show that this autoproliferation is specific for self-peptides, that it does not occur in response to foreign peptides, and that it utilizes CD4^{+}, MHC class II–restricted T cells. Once activated, these T cells, unlike T cells in normal DBA/2 mice, recognize and proliferate in response to endogenously processed and presented self-antigens. This model of loss of self-tolerance in vitro may prove useful for exploring the immune regulatory defect that underlies the diffuse autoreactivity of NOD mice in vivo.

Materials and Methods

Mice. NOD mice were bred and housed in the Stanford Medical Center Department of Laboratory and Animal Medicine (DLAM). Mice were used between the ages of 8 and 12 wk (pre-

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diabetic). DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in DLAM.

Peptides. TCR peptide Vβ8.2 38–60 (DTGHGLRLIHYS-YGAGSTEKGDI) and sperm whale myoglobin (SWM) 110–121 (AIIHVLHSRHPG) were prepared and HPLC purified at the Protein and Nucleic Acid Facility, Beckman Center, at Stanford University (Stanford, CA). TCR peptide Vβ6 38–60 (DSGKGL-RLIYYSITENDLQKGDL), mouse myoglobin (MM) 110–121 (IIIEVLKKRHSG), MM 69–78 (LTALGTILKK), and ribosomal S30 peptide 75–96 (KVHGSLARAGKVRGQTPKVAKQ) were synthesized and purified at Immulogic Pharmaceutical Corp. (Palo Alto, CA).

Antigen Proliferation Assays. Mice were immunized at the base of the tail with either CFA plus 10 mg/ml heat-killed *Mycobacterium tuberculosis*, H37RA (Difco Laboratories Inc., Detroit, MI) alone, or 100 μg of peptide suspended in Dulbecco's-PBS and emulsified with an equal volume of CFA plus 10 mg/ml H37RA (Difco). 6–14 d after immunization, draining inguinal LN cells were removed and single-cell suspensions were prepared. 5×10^5 cells were incubated in 96-well flat-bottom plates in either T cell media alone or with titrated doses of antigen or purified-protein derivative (PPD). T cell media consisted of RPMI 1640 supplemented with 2 mM L-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM Hepes buffer (Gibco Laboratories, Grand Island, NY), 50 mM 2-ME (Sigma Chemical Co., St. Louis MO), and 0.5% normal mouse serum. After 72 h at 37°C, 6% CO_2, cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 h later.

Naive NOD peripheral lymphocytes were obtained from the LN of naive mice, made into single-cell suspensions, and irradiated with 3,300 rads.

Anti-CD4 mAb was obtained and purified from the B cell hybridoma GK 1.5. Anti–MHC class II antibody 10.3.6 was purchased from Pharmingen (La Jolla, CA).

Minimacs Purification of CD4 Cells. Single-cell suspensions of freshly isolated LN cells were counted and incubated with anti-CD4 magnetic microbeads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C, washed, and then purified by passing through magnetic flow columns. The CD4-depleted fraction was subsequently used for reconstitution experiments (see below).

Results

DBA/2 and NOD Responses to Foreign and Self-Myoglobin Peptides. Our lab has characterized the mouse T cell immune response to the SWM peptide amino acids 110–121 in several mouse strains (10, 11). SWM 110–121 differs by five amino acids from the corresponding MM 110–121 self-peptide. After immunization, DBA/2 mice demonstrate a normal, immunogenic response to the foreign peptide SWM 110–121 (as previously reported [10, 11]). After immunization with the self-peptide MM 110–121, they showed no reactivity at tested dose levels (Fig. 1 *A*). NOD mice, when immunized with the foreign peptide SWM 110–121, showed a similar T cell proliferative response that was characteristic of a normal immune response with titratable dose–response kinetics (Fig. 1 *B*). However, NOD mice responded to immunization with the self-myoglobin peptide MM 110–121 with an unusual autoproliferation of draining LN cells in media alone, which was unaffected by the addition of the exogenous immunizing antigen at the tested doses (Fig. 1 *B*).

A

B
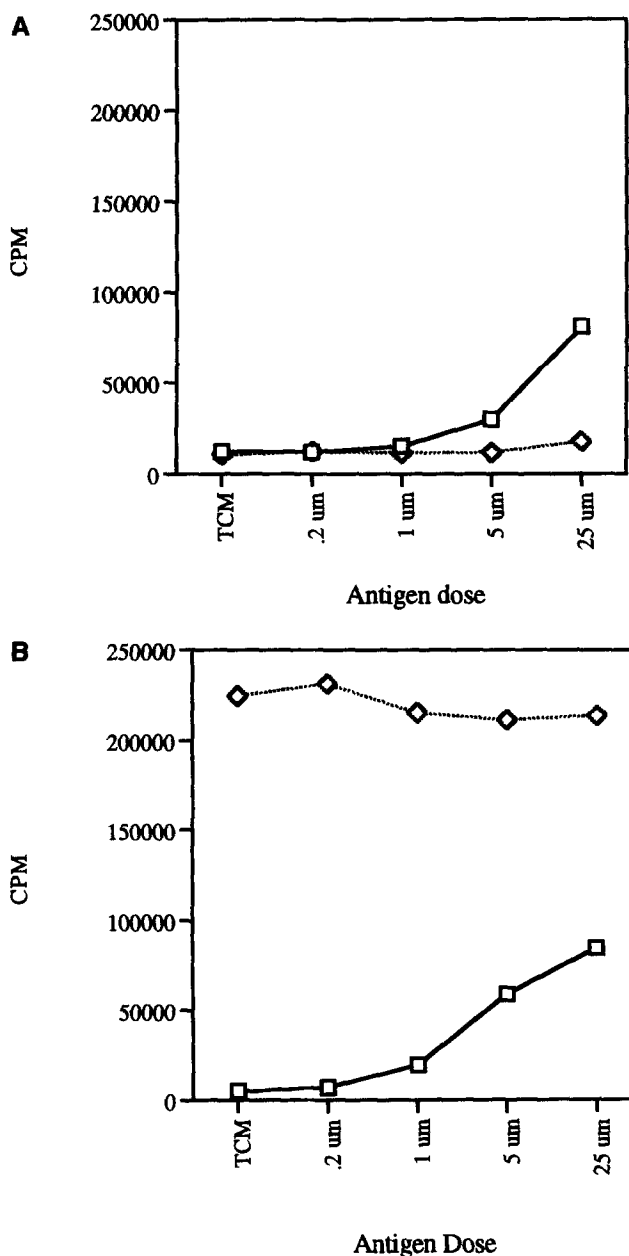
Figure 1. DBA/2 and NOD responses to foreign and self-myoglobin peptides. Groups of three 8–12-wk-old female DBA/2 (*A*) or male or female NOD (*B*) mice were immunized in the base of the tail with 5 × CFA and 100 μg of either SWM 110–121 or MM 110–121. Draining LN cells were harvested after 6–14 d and cultured in tissue culture media plus antigen in triplicate wells for 3 d before pulsing with tritiated thymidine. PPD responses were as follows: SWM-immunized NOD, 103,708; SWM-immunized DBA/2, 146,454; MM-immunized NOD, 151,534; MM-immunized DBA/2, 175,842. Each experiment was repeated three or more times. SEMs were <10% in all experiments. (*Open squares*) response of SWM-immunized animals to SWM peptide; (*diamonds*) response of MM-immunized animals to MM peptide.

Autoproliferative Response of NOD to Other Self-Peptides. We next asked whether this autoproliferative phenomenon was restricted to recognition of MM 110–121 or reflected a wider disregulation of self-reactivity in NOD mice. A sec-

ond MM peptide (68–79), two T cell receptor Vβ peptides (Vβ6 and Vβ8 amino acids 38–60), and a peptide from the widespread cellular ribosomal protein S30 all induced autoproliferation in NOD mice but not in DBA/2 mice after immunization. The presence of self-peptide in the immunizing emulsion was essential, as age-matched NOD mice immunized with CFA alone showed no autoproliferative response. Thus autoproliferation is antigen driven (Fig. 2).

MHC Class II–restricted CD4+ Cells Mediate Autoproliferation. Addition of anti-CD4 antibody, but not isotype-matched control antibodies, blocked the autoproliferation in a dose-dependent fashion (Fig. 3 *A*), demonstrating that the autoproliferation was due to the response of CD4+ cells. We demonstrated that self-antigen was presented on APC in association with NOD MHC class II by the addition of anti–class II antibody to titrated numbers of whole LN cells taken from self-peptide immunized mice (as antigen-reactive T cells) plus irradiated naive LN cells (as APCs). The anti–class II antibodies blocked autoproliferation with efficacy similar to that of anti-CD4 antibodies (Fig. 3 *B*).

Fractionation and Mixing Studies Show That Autoproliferating Cells Recognize Endogenous Antigen. Single minimacs purification of CD4+ cells from autoproliferating whole LN-cell preparations from NOD mice immunized with MM 110–121 (which in our hands produced cells that were 95%

A

Anti-CD4

B

Antigen Dose
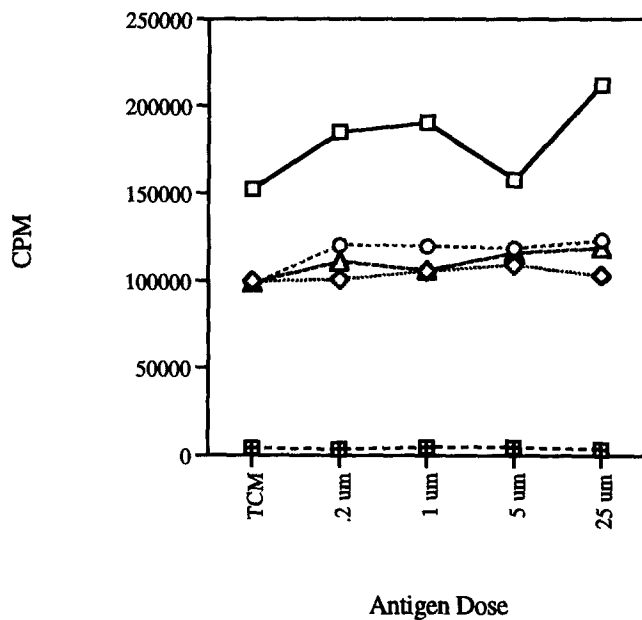
Figure 2. Autoproliferative response of NOD to other self-peptides. Groups of three 8–12-wk-old NOD mice (five different experiments are shown) were immunized with either 100 μg peptide in 5 × CFA, or 5 × CFA alone, in the base of the tail. PPD responses were as follows: Vβ8.2 38–60, 286,228 (*open triangles*); Vβ6 38–60, 163,940 (*open circles*); MM 69–78, 224,261 (*open squares*); ribosomal S30 75–96, 186,173 (*open diamonds*); CFA alone, 246,162 (*crossed squares*). Each experiment was repeated three or more times. The CFA and MM 69–79 experiments used male or female mice; the S30 and TCR peptide experiments used female NOD mice.
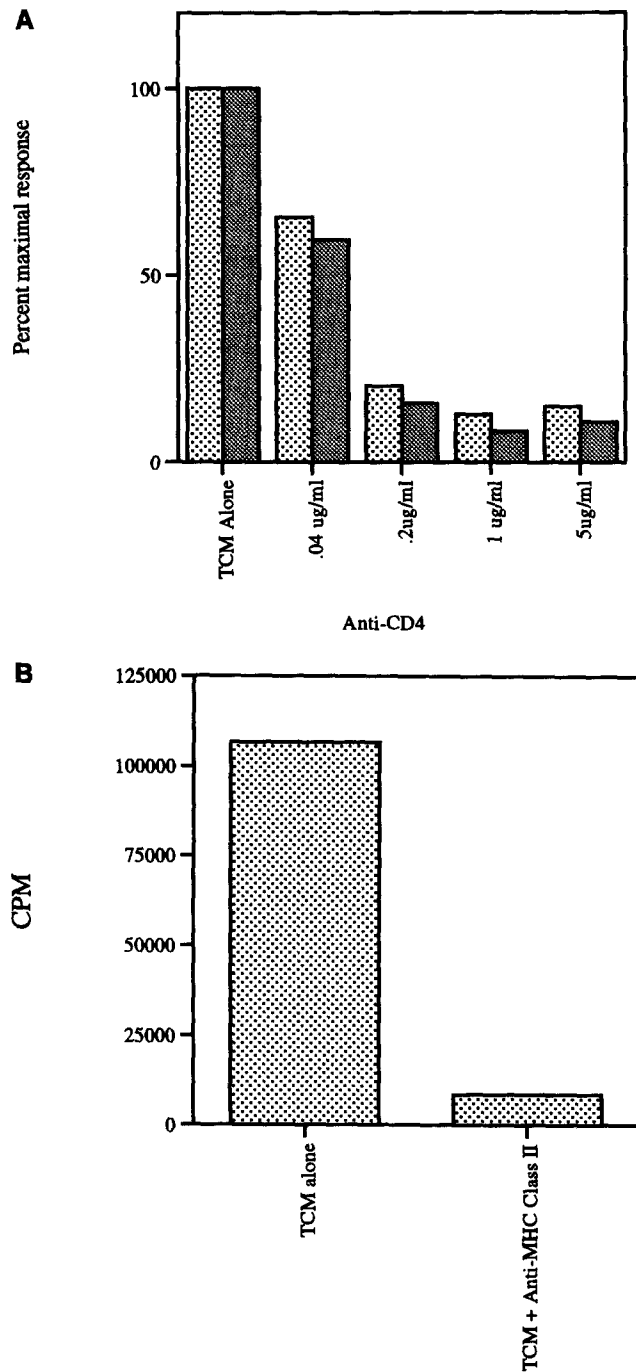
Figure 3. Effect of anti-CD4 or anti–MHC class II antibodies on the autoproliferative response. (*A*) Effect of anti-CD4 on autoproliferating cells. Coarse stippling shows the S30 response, fine stippling, TCRP Vβ8.2 38–60 response. The proliferation in media alone was 240,537 cpm in the S30 experiment; 476,836 cpm in the TCRP Vβ8.2 38–60 experiment. (*B*) Effect of anti–MHC class II antibody on the autoproliferative response. 5.0×10^5 MM 110–121–immunized LN cells were cultured with 5.0×10^5 irradiated naive NOD LN cells in media alone or media plus 10 μg/ml of antibody 10.3.6 (cross-reactive with I-A^{g7}). Each experiment was repeated three or more times.

CD4 positive by FACS® analysis) showed that the CD4-enriched autoproliferating T cells had a markedly decreased proliferation when compared with unfractionated LN cells, consistent with the removal of the APC fraction from these CD4-enriched cells (Fig. 4 A). Reconstitution of the CD4-enriched cells with the CD4 depleted portion, which carries the majority of the APC fraction, restored autoproliferation (Fig. 4 A).

The demonstration that CD4 cells from immunized NOD mice recognized endogenously processed and presented self-antigen was achieved by the addition of CD4+ minimacs-enriched cells (from animals immunized with self-

peptide) to single-cell suspensions of naive irradiated NOD LN cells as APCs (Fig. 4 B). Vigorous autoproliferation resulted from the addition of the self-peptide–primed CD4+ T cells to unprimed APCs. Priming the NOD mice with self-peptide was essential, as addition of CD4+ cells from control NOD mice to irradiated naive LN cells did not produce autoproliferation.

These data strongly support our hypothesis that autoproliferation is mediated by antigen-specific CD4+ cells responding to endogenously processed self-peptides presented by MHC class II on APCs.

Discussion

We have developed a novel model to study the loss of self-tolerance in NOD mice in vitro, which may reflect critical mechanisms underlying NOD autoimmunity in vivo. Data presented above demonstrate that NOD mice immunized with self-peptides show an autoproliferative response to endogenously processed and presented self-antigens in vitro after immunization with self-peptides, which has not been demonstrated in any other mouse strain. NOD autoreactive T cells persist and expand in the periphery in an exaggerated and poorly regulated fashion as the result of a critical immune regulatory defect. An animal model that resembles the NOD global defect in self-reactivity is one developed by Sakaguchi and Sakaguchi (12) in which neonatal cyclosporin A treatment and/or thymectomy of otherwise normal mice allowed expression of multiorgan autoimmunity, presumably as a result of loss of immune regulation. The cyclosporin A model may have direct relevance for understanding NOD autoimmunity, since it has been reported that cyclosporin A treatment can worsen autoimmunity in NOD mice (13). The idea of a pathophysiologically important immune regulatory defect in NOD mice is further supported by the studies of Dardenne et al. (14), which showed that early thymectomy increased the incidence of diabetes in NOD female mice, whereas later thymectomy had no effect on disease incidence. This finding was explained by the postulated loss of regulatory T cells. Our lab, as well as Boitard et al. (15, 16), has shown that transfer of CD4-enriched prediabetic spleen cells can significantly delay the onset of diabetes induced by adoptive transfer of diabetic spleen cells, which supports the existence of a regulatory T cell subset.

We have shown that NOD mice retain the capacity to respond normally to foreign peptides while demonstrating an abnormal response to self-peptides both in vitro and in vivo. Presumably the response to the foreign peptide is regulated over time by the disappearance of the foreign antigen, whereas after priming with exogenously supplied self-peptide antigen, the autoproliferating self-reactive T cells are driven by the continuous presence of endogenously processed and presented self-peptides. The presentation of functional self-peptide/MHC II complexes in vivo has been clearly demonstrated by Lorenz and Allen (1), who showed that hemoglobin-specific T cell hybridomas from CE/J mice recognize hemoglobin peptide on naive hemoglobin allotype–dissim-
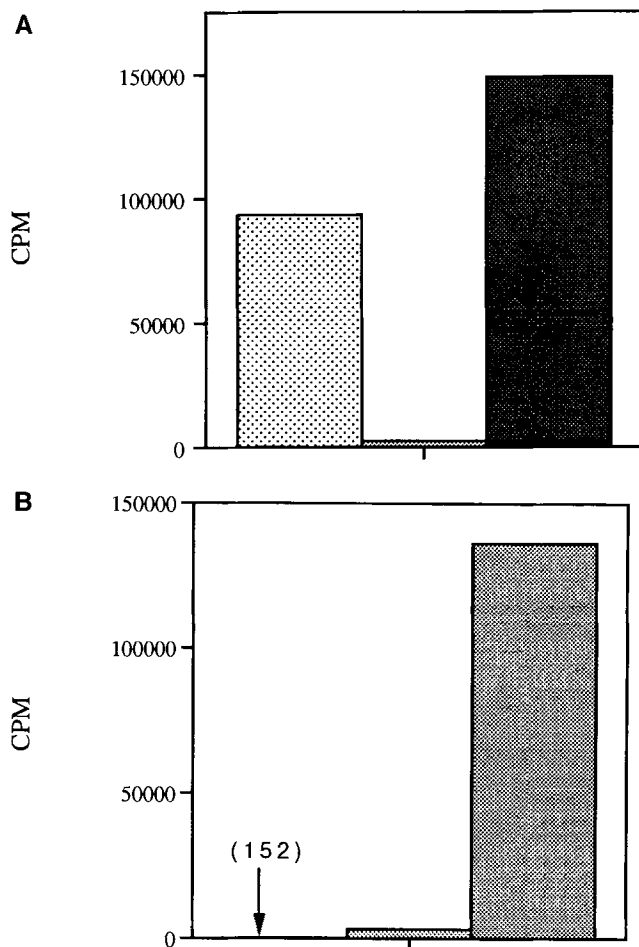
Figure 4. Fractionation and mixing studies of autoproliferating cells: recognition of endogenous self-antigen. (A) LN cells from NOD mice immunized with self-peptides were incubated with anti-CD4 magnetic microbeads for 15 min at 4°C, washed, and enriched by passing through magnetic flow columns. The CD4(−) fraction was used for reconstitution. (First bar) MM 110–121–immunized unfractionated LN cells; (middle bar) MM 110–121–immunized CD4+ cells alone; (third bar) MM 110–121 CD4+ cells reconstituted with CD4−cells. (B) In a separate experiment, self-peptide (MM 110–121) immunized, minimacs enriched CD4+ cells were mixed with irradiated naive NOD LN cells as APCs. (First bar) Irradiated naive NOD LN cells alone; (second bar) CD4+ MM 110–121–immunized cells alone; (third bar) mixing of naive irradiated cells with MM 110–121–immunized CD4+ cells. Each experiment was repeated at least three times.

ilar CBA/J APCs. Their studies demonstrated that self-hemoglobin peptide was present and functional on APCs with a wide tissue distribution. CBA/J mice, however, lacked a proliferative response to self-hemoglobin, despite the demonstration of abundant and functional expression of processed hemoglobin antigen appropriately presented by APC (1, 2). T cells that recognize self-peptides are demonstrably present in other normal mouse strains (e.g., response of DBA/2 and [PLJ/SJL]F1 mice to self-TCR peptides), as shown in our lab (17); thus, not all autoreactive T cells are clonally deleted. T cells from naive BALB/c mice spontaneously recognize self-MHC II peptides, presumably due to endogenous priming by functional MHC/MHC peptide complexes, yet these mice demonstrate neither autoimmune sequelae nor autoproliferation in vitro after immunization with self-peptide (3). Double-transgene models demonstrate that self-reactive T cells can persist in the periphery (at supraphysiologic levels), in the presence of their target self-antigen, without discernible autoreactivity (4). Thus, the limited or regulated recognition of self in normal CBA/J, BALB/c, and (PLJ/SJL)F1 mice, or in double transgenic mice, is in marked contrast to our findings of an exaggerated response to immunization with self-peptide seen in NOD mice. We postulate that immunization of NOD mice with self-peptides in CFA may mimic a pathogenic event in vivo (e.g., an immune response to an infectious process), which allows cell destruction, determinant spreading, and the unveiling of cryptic self-epitopes (18) with resultant activation of T cells that recognize and proliferate in response to endogenously processed self-antigen. An analogy may be drawn to the Ohashi (4) model, in which infection with live LCMV resulted in the irreversible breakdown of self-tolerance to the viral glycoprotein expressed as self in the pancreatic islets. This process is manifest in NOD mice as autoproliferative cells in vitro and as tissue-infiltrating self-reactive lymphocytes in vivo. The initial event, whether induced by CFA plus peptide or as the immune response to injury, may break self-tolerance in an irreversible fashion in NOD mice. Why NOD mice possess such an apparently large and diverse population of autoreactive cells is a matter of conjecture. One interesting possibility is that the processes of thymic negative selection are impaired due to the recently demonstrated poor peptide binding characterisitics of the NOD MHC class II Ia^{g7} molecules (19), which might allow the escape of high affinity self-reactive T cells in NOD mice compared with conventional mice. Future studies on autoproliferation in NOD mice may demonstrate that this phenomenon reflects a major defect in the regulation of peripheral self-tolerance, incomplete thymic negative selection, or a combination of both defects, and may further illustrate how autoreactive T cells, in the setting of defective immune regulation, could initiate and perpetuate autoimmune disease.

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