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Prevention of autoimmune insulitis in nonobese diabetic mice by expression of major histocompatibility complex class I L^d molecules

(H-2 antigen/insulin-dependent diabetes mellitus/transgenic mice)

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ABSTRACT **Nonobese diabetic (NOD) mice spontaneously develop a T-cell-mediated autoimmune disease that is similar in many respects to insulin-dependent diabetes mellitus in humans. NOD mice were shown to express major histocompatibility complex class I K^d and D^b antigens. To examine the possible involvement of major histocompatibility complex class I molecules in the development of autoimmune insulitis, we attempted to express a different type of class I molecule in NOD mice by crossing C57BL/6 mice transgenic for the class I L^d gene with NOD mice. The backcross progeny expressed the L^d antigen on the peripheral blood lymphocytes at a level comparable with that of the BALB/c mice. The cell surface expression of endogenous class I and class II antigens on the peripheral blood lymphocytes was not affected. Analysis of these mice revealed that the expression of the class I L^d antigen significantly reduced the incidence of insulitis at 20 weeks of age. *In situ* hybridization of a biotinylated probe on mouse chromosomes showed that the L^d transgene was located in the E area of chromosome 6 with which no genetic linkage to insulin-dependent diabetes mellitus was demonstrated. These results suggest that the NOD-type class I molecules are involved in the development of insulitis in NOD mice.**

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that is characterized by the infiltration of lymphocytes into the islets of Langerhans of the pancreas (insulitis) followed by destruction of insulin-secreting β cells, leading to overt diabetes (1, 2). The susceptibility to IDDM has been shown to be associated with certain alleles of the major histocompatibility complex (MHC) class II genes, *HLA-DQA1* and *HLA-DQB1* (3–7). The nonobese diabetic (NOD) mouse spontaneously develops IDDM with remarkable similarities to the human disorder (8, 9). Experimental crosses between NOD and other strains of mice revealed that there are several susceptibility genes necessary for disease onset in NOD mice (10–15). One of these, *Idd-1*, has been linked to MHC on chromosome 17 (10–13). Three other genes (*Idd-3*, *Idd-4*, and *Idd-5*) have been mapped to chromosomes 3, 11, and 1, respectively, by linkage studies using polymorphic DNA markers (14, 15).

The MHC class II molecules of NOD mouse have two distinctive features: one is the lack of the I-E molecule (10), and the other is the unique presence of the I-A^{g7} molecule (10, 16). Previously, we reported that the development of insulitis and diabetes is completely prevented by I-E expression in E^d_α transgenic NOD mice (17–19). We and others also demonstrated by using I-A transgenic NOD mice that the unique presence of the I-A^{g7} molecule is indispensable for the development of insulitis (20–22).

Several lines of evidence suggest that class I molecules also participate in the disease development in NOD mice. First, not only CD4-positive but also CD8-positive T cells are present in the islet infiltrates. Second, both CD4-positive and CD8-positive T cells are required for the induction of diabetes in transfer experiments (23, 24), and the development of cyclophosphamide-induced diabetes can be prevented by anti-CD8 antibody (25). Similarly, the anti-H-2K^d antibody can prevent spontaneous and cyclophosphamide-induced diabetes (26). Ohashi *et al.* (27) and Oldstone *et al.* (28) have developed viral antigen transgenic mice in which viral infection triggers destruction of β cells mediated by an anti-viral cytotoxic T-cell response, leading to diabetes. Furthermore, Faustman *et al.* (29) showed faulty expression of class I molecules in the NOD mouse and humans with IDDM and suggested that low-level expression of class I molecules causes a defect in antigen presentation and impairs self-tolerance, which leads to autoimmune disease. All these data suggest that NOD-type MHC class I molecules are also related to susceptibility to insulitis and diabetes in NOD mice.

The class I molecules of NOD mice are K^d and D^b as identified by conventional allo-antisera and monoclonal antibodies (mAb) (10). To determine whether the class I molecules are involved in the development of insulitis, we examined the effects of the expression of another type of class I molecule on the incidence of insulitis in NOD mice. The L^d antigen is one of the most extensively characterized murine class I molecules, and it was expressed in NOD mice by crossing them with L^d-expressing transgenic mice (B6-L^d), which we produced previously (J.M., unpublished results). Here we report that the transgenic expression of L^d molecules significantly reduced the incidence of insulitis in NOD mice.

MATERIALS AND METHODS

Construction of Ac-L^d Plasmid DNA. To replace the promoter of the murine *H-2L^d* gene, the L^d gene fragment including the entire coding sequence was excised from pL^d4 (30) by partial digestion with *Bam*HI and was linked to the chicken β-actin promoter in place of the *lacZ* gene in pAc-lacZ (31), resulting in pAc-L^d plasmid (32). pAc-L^d allowed high levels of cell surface expression of the L^d antigen upon introduction into murine L cells. pAc-L^d was digested with *Xho* I and *Eco*RI, and the resulting ≈6-kilobase β-actin

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Abbreviations: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; mAb, monoclonal antibody.

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promoter–L^d (Ac-L^d) gene fragment was purified and used for microinjection.

Production of Transgenic Mice and Introduction of the *Ac-L*^{d} Gene into NOD Mice. Several hundred molecules of the *Ac-L*^{d} fragment were microinjected into fertilized eggs of C57BL/6 (B6) mice as described (33). Two lines of transgenic mice (B6-L^d) were obtained. One line of B6-L^d, in which a single copy of the transgene had been integrated, was crossed with NOD mice to obtain the F_1 generation. (B6-L^d × NOD)F_1 progeny expressing the L^d molecule on the surface of peripheral blood lymphocytes (PBLs) were selected and were backcrossed with NOD mice to produce backcross progeny.

Immunofluorescence Analysis of PBLs. Approximately 1 × 10^6 PBLs were purified on Lympholyte-M (Cedarlane Laboratories, Hornby, ON, Canada). Lymphocytes were incubated with appropriately diluted anti-class I or anti-class II mAbs with phosphate-buffered saline (PBS) containing 0.1% NaN_3 at 4°C for 30 min. After three washes with PBS, lymphocytes were then incubated with appropriately diluted fluorescein isothiocyanate-conjugated anti-mouse IgG antibodies at 4°C for 30 min. After three washes with PBS, cells were analyzed with the FACScan (Becton Dickinson). The following mAbs were used: 30-5-7 (anti-L^d), 28-13-3S (anti-K^b), 31-3-4S (anti-K^d), 28-16-8 (anti-I-A^b), and 10-2-16 (anti-I-A^{g7}) (30, 34). When IgM mAbs (28-13-3S, 31-3-4S, and 28-16-8) were used, lymphocytes were first incubated with anti-mouse IgM antibodies at 4°C for 10 min, and fluorescein isothiocyanate-conjugated anti-mouse IgM antibodies were used as the second antibody.

Immunohistochemical Analysis. Six-micrometer-thick frozen sections of tissues were fixed by acetone/methanol, 1:1 (vol/vol), at 4°C for 10 min and then incubated with 10% horse serum in PBS at 4°C for 1 hr. After a wash with PBS, the sections were incubated with appropriately diluted mAb 30-5-7 (anti-L^d) at 4°C for 30 min and then with fluorescein isothiocyanate-conjugated anti-mouse IgG at 4°C for 30 min. After several washes with PBS, sections were examined with a fluorescence microscope.

Histological Analysis of the Pancreatic Islets. Partial pancreatectomy was carried out at the age of 20 weeks. Dissected tissues were fixed in 10% formalin containing 75 mM phosphate buffer (pH 7.0). After being embedded in paraffin blocks, 3-μm-thick sections were prepared and stained with hematoxylin/eosin. Sections were examined with a light microscope by two independent examiners without knowledge of the MHC phenotypes of the mice. More than 15 islets of Langerhans were examined for each pancreas. A pancreas with lymphocytic infiltration around or into more than one islet was regarded as displaying insulitis (18).

Chromosome *in Situ* Hybridization. R-banded chromosomes were used for *in situ* hybridization. Lymphocytes were isolated from spleen tissues of male mice and were cultured for 44 hr according to the method described (35). Then, 0.2 μM methotrexate was added to the cultures, and the culture was continued for 14 hr. The cells were washed three times with serum-free RPMI 1640 medium, cultured for an additional 4.5 hr in the presence of bromodeoxyuridine at 25 μg/ml for R-banding, and harvested after the treatment with colcemid at 0.02 μg/ml for 20 min (36). The chromosome slides were stained as described (37).

The 4.5-kilobase genomic DNA containing the *H-2L*^{d} gene was nick-translated by using biotin-16-dUTP (Boehringer Mannheim), and *in situ* hybridization and detection of fluorescence were carried out according to the methods of Lawrence *et al.* (38) and Takahashi *et al.* (39) with some modifications. The chromosomal slides were heated to 65°C for 3 hr, treated with RNase [100 μg/ml in 2× standard saline citrate (SSC)] for 1 hr at 37°C, denatured in 70% formamide in 2× SSC at 70°C for 2 min, and immediately dehydrated in a series of cold ethanol solutions. The biotinylated genomic DNA probes were denatured at 75°C for 10 min in 100% formamide with a 15-fold excess of sonicated mouse genomic DNA, which was used as competitor DNA to remove the effect of interspersed repetitive DNA sequences included in the genomic DNA probe. The hybridization mixture consisted of biotinylated DNA at 12.5 μg/ml, 50% formamide, bovine serum albumin at 2 mg/ml, and 10% dextran sulfate in 2× SSC. After incubation in a humid chamber at 37°C overnight, the slides were washed and stained as described (39). Excitation at wavelengths of 450–490 nm (Nikon filter set B-2A and B-2E), 510–560 nm (G-2A), and near 365 nm (UV-2A) was used for observation of fluorescence hybridization signals (R-band, R-band, and Q-band, respectively).

RESULTS

Classification of Backcross Progeny. Transgenic mice expressing the L^d molecule were first established in C57BL/6 mice (B6-L^d). Eighty-seven [(B6-L^d × NOD)F_1 × NOD)] backcross progeny were produced. These mice were examined for the expression of K^b, I-A^b, and L^d molecules on the surface of PBLs and were classified into four groups based on their H-2 expression (Table 1). Twenty-two (12 females and 10 males) mice expressed L^d but not K^b or I-A^b (NOD-L^{d+}); 24 (14 females and 10 males) mice did not express L^d, K^b, or I-A^b (NOD-L^{d-}); 23 (13 females and 10 males) expressed L^d, K^b, and I-A^b (B6/NOD-L^{d+}); and 18 (6 females and 12 males) mice did not express L^d but did express K^b and I-A^b (B6/NOD-L^{d-}).

Expression of L^d Molecule in Transgenic Mice. As shown in Fig. 1, the level of L^d expression on the surface of PBLs in the NOD-L^{d+} mouse was almost the same as that in control BALB/c mice, which have the endogenous *L^d* gene. Immunohistochemical analysis of a thymus section revealed that L^d molecules were expressed throughout the medulla and the cortex of the thymus (Fig. 2). L^d expression on the islets of Langerhans was observed at a very low but almost the same level as that in BALB/c mice (data not shown). Although it was reported that high-level expression of MHC class I K^b and β_2-microglobulin (40, 41) or class II (I-A) molecules (42, 43) in β cells gave rise to atrophy or destruction of islet cells without lymphocytic infiltration, the low level of L^d expression in our transgenic mice did not cause any pathological changes in the islets (data not shown). This is consistent with the result that the expression of class II molecules on islet cells at levels comparable with those on resting B lymphocytes did not cause diabetes (44).

Expression of endogenous K^d and I-A^{g7} molecules in NOD-L^{d+} mice was the same as that of NOD-L^{d-} mice (Fig. 3), suggesting that the expression of exogenous L^d molecules did not influence the expression of endogenous MHC molecules.

Table 1. Incidence of insulitis in backcross progeny

Transgenic mouse	L^d	H-2	No.	+ insulitis No.	+ insulitis %	− insulitis No.	− insulitis %
NOD		*nod/nod*	11	10	91	1	9
NOD-L^{d+}	+	*nod/nod*	22	4	18	18	82
NOD-L^{d-}	−	*nod/nod*	24	13	54	10	46
B6/NOD-L^{d+}	+	*b/nod*	23	0		23	100
B6/NOD-L^{d-}	−	*b/nod*	18	0		18	100

[(B6-L^d × NOD)F_1 × NOD]BC_1 mice were classified into four groups according to their H-2 types and presence of the *L^d* transgene. Partial pancreatectomy was performed at the age of 20 weeks. More than 15 islets for each pancreas were examined. A pancreas with lymphocytic infiltration into more than 1 islet was regarded as displaying insulitis. No., number of mice.

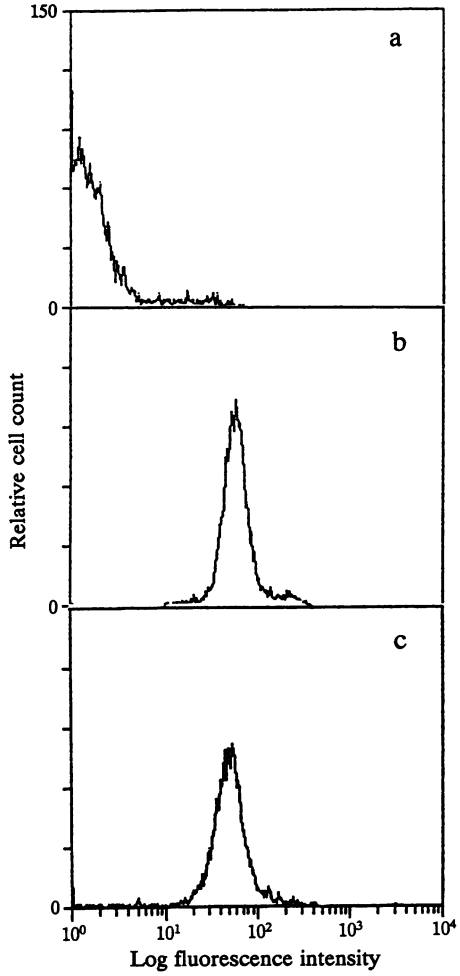

FIG. 1. Immunofluorescence analysis of PBLs for L^d expression. (*a*) NOD-L^{d-} mouse. (*b*) BALB/c mouse. (*c*) NOD-L^{d+} mouse.

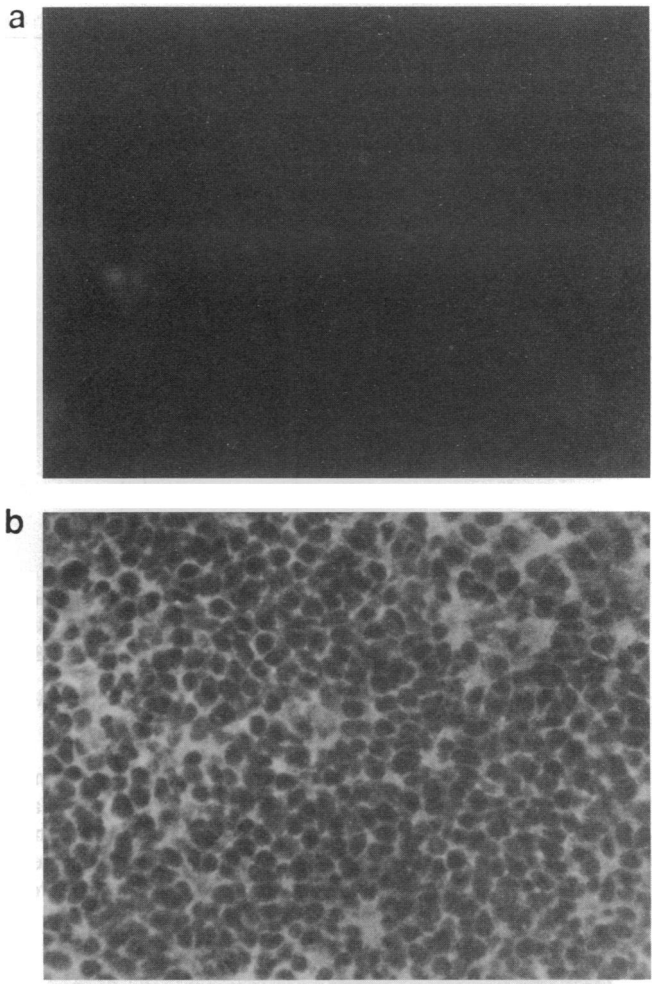

FIG. 2. Immunohistochemical analysis of thymic tissue for L^d expression. (*a*) NOD-L^{d-} mouse. (*b*) NOD-L^{d+} mouse. (×180.)

Prevention of Insulitis in NOD-L^{d+} Mice. To examine the effects of the expression of the introduced L^d gene on the development of insulitis, partial pancreatectomy was carried out at 20 weeks of age, and the presence of insulitis was analyzed histologically (Table 1). As the incidence of insulitis at 20 weeks of age was >80% in both male and female NOD mice of our colony, the results of both males and females were combined and then analyzed statistically. Thirteen out of 24 NOD-L^{d-} mice (54%) developed insulitis. This value is consistent with the notion that the transgene is not located on the same chromosome as the *Idd* loci, as was demonstrated by chromosome *in situ* hybridization analysis (see below).

In NOD-L^{d+} mice, the incidence of insulitis was significantly reduced as compared with that in NOD-L^{d-} mice. Four out of 22 mice (18%) developed insulitis. The difference in the incidence of insulitis between NOD-L^{d+} mice and NOD-L^{d-} mice was statistically significant by χ^2 analysis ($0.01 < P < 0.05$). The levels of L^d expression were not different between insulitis-positive and insulitis-negative NOD-L^{d+} mice. None of the B6/NOD-L^{d+} or B6/NOD-L^{d-} mice, which are heterozygous at the *H-2* locus, developed insulitis.

Chromosomal Localization of the L^d Transgene. The above results suggest that the transgene is not located on the same chromosome as the *Idd* loci. To confirm this, we performed chromosome *in situ* hybridization analysis. The hybridization signals were detected in the E area of one of chromosomes 6 and also on the C area of both chromosomes 17, where the MHC locus is located (Fig. 4). The latter signal is considered to be due to cross-hybridization of the L^d gene probe with endogenous class I genes on chromosome 17. Backcross progeny were classified into four groups including NOD-L^{d+}. The presence of this group rules out the possibility that the transgene was integrated into the C area on chromosome 17 adjacent to the MHC locus. Thus, the difference of the incidence of insulitis between NOD-L^{d+} mice and NOD-L^{d-} mice is considered to solely reflect the effects of L^d expression on the development of insulitis.

DISCUSSION

In this report, we demonstrate that the expression of exogenous MHC class I L^d molecule could decrease the incidence of insulitis in NOD mice. In these experiments, the L^d transgene was introduced into NOD mice through crosses to B6-L^d mice. Therefore, it was crucial that the L^d transgene was not linked to the MHC or the other *Idd* loci. The presence of four groups, including NOD-L^{d+} mice, ruled out the possibility that the transgene was integrated into chromosome 17, where the MHC resides. It was also unlikely that the transgene was located on the same chromosome as the non-MHC *Idd* locus. If this was the case, NOD-L^{d-} mice should have shown a higher incidence of insulitis. Consistent with this, chromosome *in situ* hybridization analysis showed that the transgene was located on the E area of one of chromosomes 6.

It is possible that expression of L^d molecules might nonspecifically suppress the immunocompetence of the transgenic mice, leading to the prevention of insulitis. However,

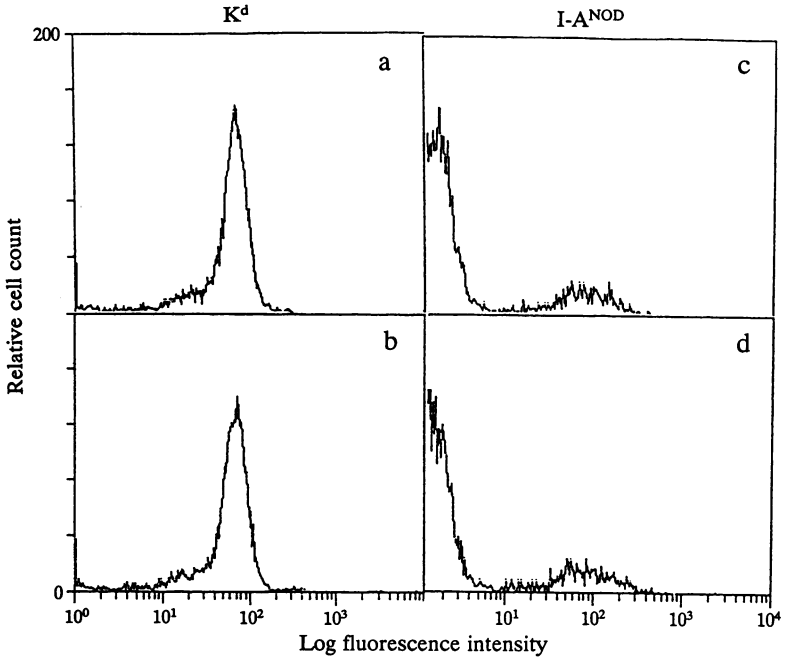

FIG. 3. Immunofluorescence analysis of PBLs for K^d and I-A^{g7} expression. PBLs were stained with mAb 31-3-4S (anti-K^d) (*a* and *b*) or with mAb 10-2-16 (anti-I-A^{g7}) (*c* and *d*). (*a* and *c*) NOD-L^{d-} mouse. (*b* and *d*) NOD-L^{d+} mouse.

we regard this unlikely because no alteration was examined in the level of expression of endogenous MHC class I or class II molecules, and normal immune responses were retained against several antigens so far tested. Taken together, these results suggest that the prevention of insulitis was the direct effect of L^d expression. But, unlike I-E transgenic NOD mice (19), the prevention of insulitis by L^d expression was not complete. This situation is very similar to that in I-A^k transgenic NOD mice (20). Whatever the mechanisms of suppression of insulitis (see below), it should be noted that endogenous counterparts of the transgenes are normally expressed in both I-A^k and L^d transgenic mice.

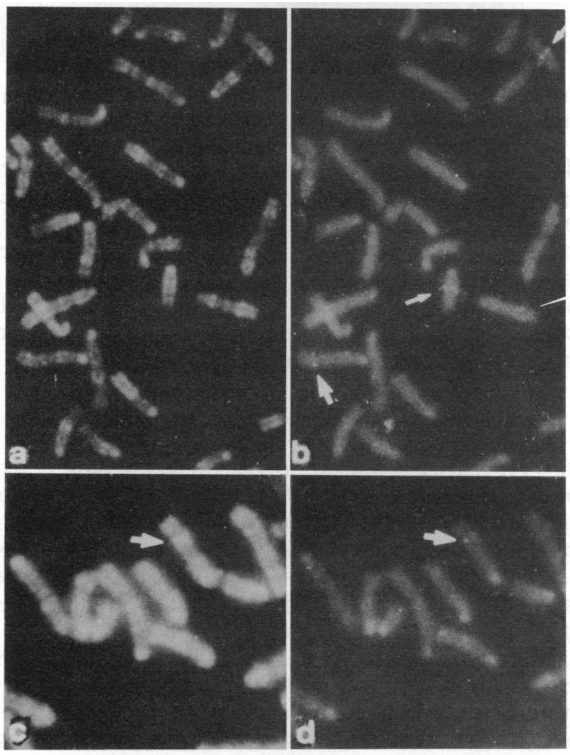

FIG. 4. Chromosomal localization of the integrated *H-2L^d* DNA sequence by using *in situ* hybridization with a biotinylated DNA probe. The metaphase spreads were photographed with G-2A (*a*) (R-band), B-2E (*b* and *d*), and B-2A (*c*) (R-band) filters. The hybridization signals of the transgene on chromosome 6 and the endogenous class I gene sequences on chromosome 17 are indicated by large and small arrows, respectively.

There are three major possible mechanisms for the prevention of insulitis by L^d expression. First, L^d expression in the thymus or in peripheral tissues may alter the T-cell repertoire either by clonal deletion or clonal anergy. Thus, autoreactive T cells against islet β cells might be deleted or anergized. Such an explanation has often been proposed for the mechanism of prevention of insulitis in I-E transgenic NOD mice (45, 46). Second, suppressor T cells may be induced to suppress the immune reaction to β cells. Although several reports have demonstrated the presence of I-E-restricted suppressor T cells (47, 48), class I-restricted suppressor T cells have not been demonstrated yet. A third mechanism is that transgenic L^d molecules may induce tolerance to nominal autoantigens. It is well established that MHC class I molecules tightly bind to peptides that are endogenously processed by antigen-presenting cells and that the binding of peptides to MHC molecules is highly specific (49–51). At least three kinds of proteins have been identified as the β-cell autoantigens (52–54). It is possible that these antigens may bind to NOD-type class I molecules followed by the presentation of these antigens to cytotoxic T cells. In accordance with this, increased expression of class I molecules is observed in both endocrine and exocrine tissues in the vicinity of intraislet infiltration (22, 55). But, in NOD-L^{d+} transgenic mice, these antigens may not be recognized by these T cells although they may bind to L^d molecules, possibly because they mimic self-peptides to which tolerance may have been induced.

In recent years, accumulating data, especially from transgenic experiments (17–22), have demonstrated the contribution of the unique MHC class II molecule to the development of insulitis in NOD mice. On the other hand, the involvement of class I molecules has been a controversial matter, partly because the NOD mouse does not have unique class I

molecules (10). Our data strongly support the idea that the NOD-type MHC class I molecules as well as the unique MHC class II molecule are involved in the susceptibility to insulitis in NOD mice.

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