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# *Analysis of Pathogenesis of Autoimmune Insulitis in NOD Mice: Adoptive Transfer Experiments of Insulitis in ILI and NOD Nude Mice*

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***Abstract:*** *In an effort to study the pathophysiological events in the development of insulitis in NOD mice, we have developed ILI- and NOD-nu/nu mice. ILI mice are a nondiabetic inbred strain but are derived from the same Jcl:ICR mouse as NOD mice and share the same H-2 allotype with NOD mice. Splenocytes and CD4+ cells from diabetic NOD mice appeared to transfer insulitis to ILI-nu/nu mice, suggesting that ILI mice already express autoantigen(s) responsible for insulitis. But reciprocal thymic grafts from NOD mice into ILI-nu/nu mice and those from ILI mice into NOD-nu/nu mice failed to allow the development of insulitis, implying that ILI mice possess neither precursor T cells nor the thymic environment responsible for the development of insulitis. In addition, splenocytes from ILI mice appeared to contain regulatory cells which suppress the development of diabetes but not that of insulitis in NOD mice. The use of these nude mice should provide more information on the products of insulitis-susceptibility genes of NOD mice.*

***Key words:*** *ILI mice, Insulitis, Insulitis susceptibility gene, NOD mice, nude mice*

## Introduction

The non-obese diabetic (NOD) mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM), and is considered an appropriate model for examining the etiology of human IDDM [11]. The onset of diabetes is preceded by the development of autoimmune insulitis that is an inflammatory infiltrate affecting the islets of Langerhans [5, 8]. Evidence supporting the autoimmune etiology of IDDM in the NOD mouse indicates that the immunosuppressant can prevent the development of IDDM [1] and that IDDM can be adoptively transferred to NOD-*nu/nu* mice by splenocytes from overt diabetic NOD mice [18].

Several recessive genes are known to determine the insulitis [3]. Of the insulitis-susceptibility genes, only in the case of *Idd-1*, located in the murine major histocompatibility complex (MHC) on chromosome 17, is there any evidence for the identity of the gene product. *Idd-1* could be a gene complex with at least two susceptibility

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loci, namely *I-Aβ* and *I-Eα*. Transgenic mouse experiments suggest that the presence of the *I-Aβ*$^{NOD}$ (Ser 57) and the absence of I-Eα molecules in the NOD mouse influence the development of the diseases [12, 13, 15].

Previously we performed restriction fragment polymorphism (RFLP) studies of MHC genes (classes I-III) in the NOD mouse in a comparison with the ILI mouse, which is a nondiabetic inbred strain but is derived from the same closed colony (Jcl:ICR) as the NOD mouse. The data obtained implied that the ILI mouse shares the same class II (*Aβ*, *Aα*, *Eβ*, and *Eα*) and class III genes with the NOD mouse [4]. Furthermore, the *Aβ* second exon nucleotide sequence of the ILI is identical to that of the NOD mouse [10]. NOD and ILI mice bear a deletion spanning the promoter region and signal peptide exon of the *Eα* gene, resulting in the failure of *Eα* gene expression [4]. In addition, NOD and ILI mice share the same MHC class I molecules ($K^d$ and $D^b$) (Y. Koide, unpublished observations), the insufficient expression of which is considered to affect the development of IDDM [2]. These results suggest that not only NOD mice but also ILI mice possess *Idd-1* although the ILI mice do not develop the insulitis.

Our work was undertaken to learn the factor(s) preventing ILI mice from developing insulitis. It appeared that the insulitis can be adoptively transferred to ILI-*nu/nu* mice by splenic mononuclear cells (S-MNC) or CD4$^+$ cells from overt diabetic NOD mice. On the other hand, neither NOD thymus transplanted in ILI-*nu/nu* mice nor ILI thymus in NOD-*nu/nu* mice allowed the development of insulitis. These data imply that ILI mice possess autoantigen(s) responsible for the development of insulitis, although T cells responsible for insulitis should be derived from NOD mice and educated under NOD thymus.

## Materials and Methods

*Mice*: NOD, ILI, ILI-*nu/nu*, and NOD-*nu/nu* mice were bred in our Institute for Experimental Animals under specific pathogen-free conditions. In this NOD colony, the spontaneous incidence of diabetes reaches 80% in females and 45% in males by 30 weeks of age. The ILI mice were kindly provided by Dr. H. Kato (Central Institute for Experimental Animals, Kawasaki, Japan).

ILI-*nu/nu* (M1) mice were produced by intercrossing (ILI × BALB/c-*nu/nu*) $F_1$ mice and then ILI-*nu/nu* (M2) mice were produced by intercrossing (ILI × M1) $F_1$ mice. Likewise, ILI-*nu/nu* (M3–M10) mice were produced. An identical breeding scheme was followed to produce NOD-*nu/nu* (M11) mice.

*Preparation of S-MNC*: Spleens were removed and teased apart into single cell suspensions. S-MNC were isolated by Ficoll-Paque density-gradient (M-SMF; JIMRO, Takasaki, Japan). The cells were washed and suspended in RPMI-1640 (Nissui Pharmaceutical, Tokyo) containing 10% fetal calf serum (FCS).

*Preparation of CD4$^+$ subset*: CD4$^+$ subset was prepared according to the manufacturer's instructions with the magnetic cell separator, MiniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, S-MNC were incubated with anti-CD4 magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C, washed and CD4$^+$ cells were collected on a magnetic flow-through column. The purity, as assessed by flow cytometry, was >95%.

*Adoptive transfer*: S-MNC or CD4$^+$ cells suspended in 200 μl of RPMI-1640 were injected intraperitoneally. As the control, 200 μl of RPMI-1640 without cells was injected intraperitoneally.

*Thymic graft*: The thymic lobes were obtained from day 16–18 fetus, and were cultured on polycarbonate filters (pore size, 8 μm; Nucleopore Co., Pleasanton, CA) floating on RPMI-1640 supplemented with 10% FCS and 1.5 mM-deoxyguanosine (dGuo) (Sigma Chemical Co.) at 37°C in a humidified atmosphere of 7.5% $CO_2$ [7]. After 5 days of culture, the lobes were washed three times to remove dGuo, and then grafted beneath the renal capsule of mice under nembutal anesthesia.

*Immunofluorescence*: Cells were stained with monoclonal antibodies (mAbs) to CD4 (9861SB; GIBCO BRL, Gaithersburg, MD), CD8a (9868SB; GIBCO BRL), Thy1.1(Meiji, Tokyo, Japan), Thy1.2 (Meiji), and H-2K$^b$D$^b$ (CL9007A; Cedarlane, Ontario, Canada) and analyzed by flow cytometry with the EPICS Profile (Coulter Electronics, Miami, FL).

## Results

*Splenocytes from diabetic NOD mice transfer insulitis to ILI-nu/nu recipients*

Donor S-MNC were harvested from overt diabetic female NOD mice and transferred to female and male ILI-*nu/nu* (M10) mice. As shown in Table 1, two of

**Table 1.** Injection of S-MNC from diabetic NOD mice into ILI-*nu/nu* (M10) mice

| ILI-*nu/nu* No. | F/M | No. of cells injected ($\times 10^6$) | Weeks or post-transfer | Thy1.2$^+$ Cell (%) [a)] | Diabetes | Insulitis |
|---|---|---|---|---|---|---|
| 1 | M | 2.0 | 6 | 3.6 | − | − |
| 2 | F | 10.0 | 5 | 0.2 | − | − |
| 3 | M | 5.0 | 14 | 16.8 | + | + |
| 4 | F | 10.0 | 10 | 10.4 | − | + |

[a)]The surface antigen Thy1.2 of the NOD mouse was analysed directly with the monoclonal antibody by flow cytometry and the population was calculated as ratio to spleen lymphocytes.

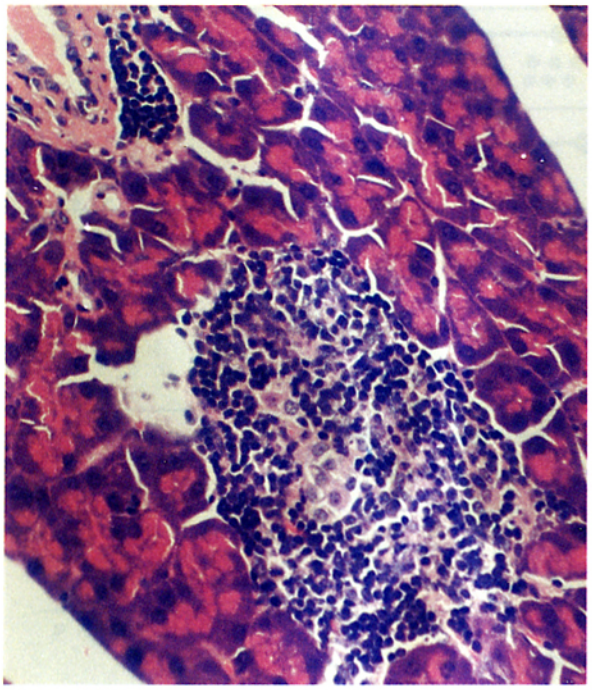

**Fig. 1.** Histological findings of insulitis induced in the ILI-*nu/nu* mouse (No. 3 mouse in Table 1) by transfer of the S-MNC from overt diabetic NOD mice. Marked infiltration of MNC in the pancreas islet was observed.

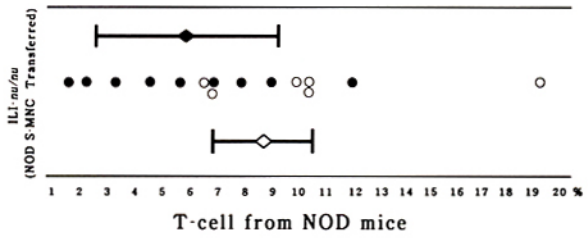


**Fig. 2.** The percentage of donor T cells (Thy1.2$^+$) to splenic lymphocytes in ILI-*nu/nu* recipients injected with S-MNC from overt diabetic NOD mice. S-MNC ($1–2 \times 10^6$) from overt diabetic NOD mice were transferred to ILI-*nu/nu* mice (M5–7). The splenic lymphocytes were analyzed 9–16 weeks after transfer on EPICS Profile flow cytometry to determine the percentage of Thy1.2$^+$ cells. The rhombuses indicate the mean values. The vertical bars represent the standard deviations. Open circles and rhombuses indicate the mice with developing insulitis and closed circles indicate the mice without insulitis.

four recipients of diabetic NOD S-MNC developed insulitis. The histological findings of insulitis observed in No. 3 mouse are shown in Fig. 1. Furthermore, No. 3 male mouse became diabetic, but neither No. 1 nor No. 2 recipient showed any sign of insulitis at 6 and 5 weeks of post-transfer, respectively. We therefore examined the number of surviving NOD T cells in the recipients, since NOD T cells are able to be distinguished from ILI T cells by Thy1 allotype (NOD, Thy1.2; ILI, Thy1.1). The recipients with no insulitis appeared to have fewer Thy 1.2$^+$ cells in the spleen as than those with insulitis (Table 1). Transfer studies with ILI-*nu/nu* (M5–7) indicate that adoptive transfer of insulitis requires at least 6% Thy1.2$^+$ T cells in the splenic lymphocytes of recipients (Fig. 2). Furthermore, CD4$^+$ T cells from diabetic NOD mice were capable of transferring insulitis in two of four ILI-*nu/nu* (M10) recipients (Table 2).

In contrast, S-MNC from diabetic NOD mice failed do transfer insulitis in any of four BALB/c-*nu/nu* recipients (data not shown).

*Thymic grafts from NOD mice failed to allow development of insulitis in ILI-nu/nu mice*

To determine whether the thymic environment of NOD mice is responsible for the development of insulitis, NOD thymus was transplanted under the cap-

**Table 2.** Injection of the CD4 positive lymphocyte from diabetic NOD mice into ILI-*nu/nu* (M10) mice

| F/M | No. of cells injected (× $10^6$) | Weeks of post-transfer | Thy1.2$^+$ Cell (%)[a)] | Diabetes | Insulitis |
|---|---|---|---|---|---|
| M | 2.2 | 8 | 8.9 | − | + |
| F | 1.0 | 13 | 13.3 | − | − |
| M | 1.0 | 13 | 15.1 | − | + |
| M | 2.6 | 10 | 6.5 | − | − |

[a)]The surface antigen Thy1.2 of the NOD mouse was analysed directry with the monoclonal antibody by flow cytometry and the population was calculated as ratio to spleen lymphocytes.

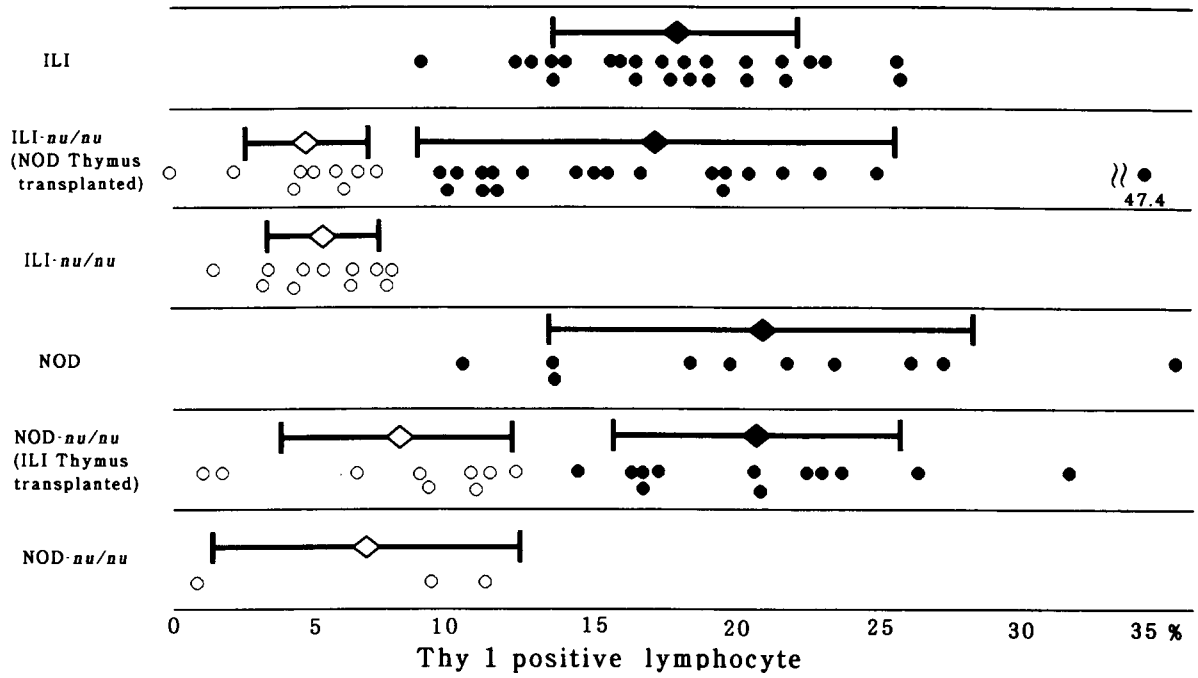


**Fig. 3.** The percentage of mature T cells to splenic lymphocytes in ILI, NOD, ILI-*nu/nu*, NOD-*nu/nu* and thymus-grafted nude mice. Fetal NOD thymus and ILI thymus were grafted beneath the kidney capsule of ILI-*nu/nu* and NOD-*nu/nu*, respectively, after eliminating thymocytes with dGuo. The splenic lymphocytes were analyzed by EPICS Profile flow cytometry to determine the percentage of host type T cells (ILI, Thy1.1; NOD, Thy1.2). The rhombus indicates the mean value and the vertical bar indicates the standard deviation. Open circles in thymus-grafted mice represent less than 8.9% Thy1.1 cells and less than 11.1% Thy1.2 cells in ILI-*nu/nu* and NOD-*nu/nu* recipients, respectively. Closed circles in thymus-grafted nude mice indicate the percentage of Thy1$^+$ cells comparable to those in euthymic mice.

sule of the ILI-*nu/nu* kidney after eliminating thymocytes with dGuo [7], but no insulitis was observed in 29 transplanted ILI-*nu/nu* mice (16 females and 13 males) at 21 weeks after transplantation. It seems most likely that the grafted thymuses actually functioned in at least 20 ILI-*nu/nu* recipients since they expressed more mature T cells of the ILI type (Thy1.1) than nontransplanted ILI-*nu/nu* mice expressing less than 8.9% Thy1.1 cells of splenic lymphocytes (Fig. 3).

*Thymic grafts from ILI mice failed to allow the development of insulitis in NOD-nu/nu mice*

ILI thymuses transplanted into 21 NOD-*nu/nu* mice (10 females and 11 males) appeared not to allow the development of insulitis. At least 12 of 21 transplanted

**Table 3.** Injection of S-MNC from ILI mice into neonatal NOD mice

| NOD F/M | No. of cells injected ($\times 10^7$)[a] | Diabetes | Insulitis |
|---|---|---|---|
| F | 0 | 16/17 | 17/17 |
| | 3 | 2/16 | 15/16 |
| M | 0 | 5/ 8 | 8/ 8 |
| | 3 | 0/12 | 12/12 |

[a]S-MNC from ILI mice or control medium were injected intraperitoneally into NOD mice aged 4–5 days. Diabetes and Insulitis were examined 28–32 weeks after injection.

NOD-*nu/nu* mice were found to possess more mature T cells of the NOD type (Thy1.2) than the control nude mice expressing less than 11.1% Thy1.2$^+$ cells of splenic lymphocytes (Fig. 3). The percentage of Thy1.2$^+$ cells in the NOD mice and the 12 thymus-grafted nude mice was not significantly different, indicating that the grafted thymuses functioned in the 12 nude mice.

*S-MNC from ILI mice prevented the onset of diabetes but not the development of insulitis*

We investigated whether S-MNC of ILI mice contain regulatory cells preventing the development of the disease. A total of $3 \times 10^7$ S-MNC from ILI mice were injected into neonatal NOD mice. As demonstrated by the data in Table 3, the injection failed to prevent the development of insulitis although the treatment was able to decrease the incidence of diabetes.

## Discussion

Evidence has accumulated for the recessive genes which determine insulitis in NOD mice. It has been suggested that at least nine genes (*Idd-1–9*) are the diabetes susceptibility genes in NOD mice [16]. Of the recessive genes, however, only in the case of *Idd-1* is there any evidence for the identity of the gene product [12, 13, 15]. Previously, we have demonstrated that the ILI mouse, which is a non-diabetic inbred strain but derived from the same Jcl:ICR mouse as the NOD mouse, also possessed *Idd-1* linked to MHC [4, 10]. Furthermore, [ILI × NOD] × NOD backcross experiments suggest that one recessive gene, which is not linked to MHC, is responsible for the insulitis [9].

In an effort to study the gene product, we have developed adoptive transfer models employing NOD and ILI nude mice. In the experiments, we demonstrated that S-MNC from diabetic NOD mice can transfer insulitis to ILI-*nu/nu* recipients, suggesting that ILI mice already possess autoantigen(s) responsible for the development of insulitis, but two of 4 recipients failed to develop insulitis. In a separate set of experiments, the adoptive transfers of insulitis were suggested to require at least 6% Thy1.2$^+$ (NOD type) T cells surviving in the splenic lymphocytes of recipients. This is consistent with our observation that two recipients developing no insulitis had only 3.6 and 0.2% Thy1.2$^+$ cells in splenic lymphocytes. It seems most likely that NOD type MHC (H-2$^{g7}$) molecules expressed in ILI mice play a pivotal role in the development of insulitis in the recipients since T cells from NOD mice possibly recognize the insulitis-related antigen(s) in the context of NOD type MHC molecules. We have observed that NOD and ILI mice share the same MHC class I and II molecules and that no mixed lymphocyte reaction was demonstrated between NOD and ILI splenocytes (Y. Koide, unpublished observation). In fact, none of the BALB/c-*nu/nu* (H-2$^d$) mice receiving S-MNC from diabetic NOD mice exhibited insulitis although a sufficient number of NOD (Thy1.2$^+$) T cells were detected in the spleen.

Adoptive transfer of insulitis was also achieved in ILI-*nu/nu* mice with purified CD4$^+$ cells from diabetic NOD mice, but none of these recipients developed diabetes. This is consistent with the observation that adoptive transfer of diabetes requires both CD4$^+$ and CD8$^+$ cells [17].

Given that ILI mice express autoantigen(s) responsible for the development of insulitis, we explored the pathophysiological events that prevent differentiation of T cells responsible for insulitis in ILI mice. NOD-thymus transplanted in ILI-*nu/nu* mice appeared not to allow the development of insulitis although mature T cells derived from ILI bone marrow were detected in the spleen of recipients. Similarly, functioning ILI thymus grafts prevented the development of insulitis in NOD-*nu/nu* mice. These data imply that ILI mice may possess neither precursor T cells nor the thymic environment required for the development of effector T cells in insulitis.

One can envision that ILI mice possess regulatory cells which suppress the development of insulitis [6],

but this seems unlikely since the injection of S-MNC from ILI mice into neonatal NOD mice failed to prevent insulitis. It is, however, noteworthy that the injection substantially decreased the incidence of diabetes. This observation is somewhat consistent with a previous report stating that lymphocytes from prediabetic NOD mice prevented the rapid destruction of $\beta$-cells after inflammatory insulitis [14]. It has therefore been proposed that the development of diabetes depends on an imbalance between diabetogenic and regulatory cells. In this context, ILI mice seem to possess the regulatory cells but not diabetogenic cells.

Taken together, our data obtained with NOD and ILI nude mice suggest that ILI mice express autoantigen(s) responsible for insulitis although they possess neither precursor T cells nor the thymic environment required for the development of T cells recognizing the autoantigens. The findings cannot be attributed to one recessive gene described above. The issue is currently under further investigation.

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