New Insight on Immune Tolerance from Transgenic Mouse Models

Myung-Shik Lee

Department of Medicine Division of Endocrinology & Metabolism,
Samsung Medical Center, Seoul, Korea

INTRODUCTION

With the advent of transgenic animal techniques, it became possible to express a specific protein in a specific tissue that would be regarded as a 'self-antigen' by the host. Transgenic mice with specific immunoglobulin molecules or T-cell receptor(TCR) molecules on the majority of lymphocytes also become available allowing pursuit of lymphocytes recognizing specific antigens. Because of these advances, immunological steps leading to self-nonself discrimination such as thymic positive, negative selection and peripheral tolerizing mechanisms were finely dissected and demonstrated *in vivo*, which have been unclear and conceptual until recently.

In this article, the new insight on 'peripheral' T cell immune tolerance acquired through transgenic mouse technology will be discussed, aside from thymic 'central' tolerance or B cell tolerance.

ABERRANT MHC CLASS II EXPRESSION AND MHC TRANSGENIC MOUSE

While class I MHC molecules are expressed on nearly all nucleated cells, class II MHC molecules have been known to be expressed only on antigen-presenting cells(APC) such as dendritic cells, macrophages and Langhans cells specializing in antigen

presentation in normal conditions. However, some investigators have reported ectopic expression of class II MHC molecule in nonlymphoid tissue from the patients with autoimmune diseases such as Graves' disease(Hanafusa et al., 1983) or autoimmune diabetes(Bottazzo et al., 1985), and those results were confirmed by other investigators(Jansson et al., 1984; Lee et al., 1988). The aberrant class II MHC expression was induced by interferon- γ (IFN- γ) secreted from infiltrating T lymphocytes(Piccinini et al., 1987), and involves active protein synthesis(Lee et al., 1991). Based on these observations, immunologists have wondered if aberrant expression of class II MHC antigen in peripheral tissue would trigger autoimmune response to peripheral tissue-specific antigens which could not be recognized by Th lymphocytes in the absence of class II MHC molecules(Bottazzo et al., 1983). However, it was difficult to study *in vivo* immunological impact of aberrant MHC class II MHC expression on epithelial cells, although some reports described antigen-presenting ability of MHC class II-expressing nonlymphoid cells such as fibroblasts using sophisticated *in vitro* techniques(Londei et al., 1984).

Then, with the advent of transgenic technology in early 1980, it became possible to study *in vivo* consequences of ectopic expression of class II MHC molecules in peripheral tissues. Researchers utilized insulin promoter sequences to direct expression of allogeneic or syngeneic MHC class II molecules on pancreatic β -cells. Pancreatic β -cells were chosen because of 1) well-characterized insulin promoter sequences 2) easy monitoring of pancreatic β -cell destruction by measuring blood glucose level and 3)

Address for correspondence: Department of Medicine Division of Endocrinology & Metabolism, Samsung Medical Center, 50 Ilwon-dong Kangnam-ku, Seoul 135-230, Korea.
Tel: 02-3410-3436, Fax: 02-3410-3849.

the clinical and scientific importance of autoimmune diabetes mellitus.

Transgenic mice expressing syngeneic or allogeneic class II MHC molecules on pancreatic β -cells were produced by more than four independent groups (Lo et al., 1988; Sarvetnick et al., 1988; Boehme et al., 1989; Miller et al., 1990). Class II MHC transgenic mice produced by Sarvetnick et al. and Lo et al. developed diabetes mellitus, but surprisingly, there was no insulinitis in histological sections of the pancreata. On the contrary, *in vitro* studies including mixed lymphocyte reaction (MLR) showed decreased proliferative response to the transgene product (MHC) but without deletion of potentially reactive T lymphocytes (Lo et al., 1988). They regarded these findings as evidence for 'peripheral tolerance'—a state of apparent immune tolerance observed when lymphocytes encounter their nominal antigens present only in peripheral tissue (not in the thymus causing clonal deletion).

Afterwards, several 'costimulatory molecules' such as B7-1 or B7-2 that are essential for effective antigen presentation were cloned, and the peripheral tolerance described above was regarded to be caused by confrontation of peripheral antigens and potentially reactive lymphocytes in the absence of costimulatory signals on nonlymphoid cells. Immune nonresponsiveness was also observed even if lymphocytes were first exposed to the allogeneic class II MHC molecules in postnatal period as was demonstrated in an experiment grafting allogeneic class II MHC-positive islets to nontransgenic littermates (Markmann et al., 1988).

The other class II MHC transgenic mice from Boehme et al. and Miller et al. also did not have insulinitis but were different from the former class II MHC transgenic mice models in that there was no diabetes. 'Apparent immune tolerance' as characterized by the absence of insulinitis was observed as in the former two models; however, actually there was no active immune tolerance to the allogeneic class II MHC molecules in these mice because alloreactivity to the transgenic MHC molecules was preserved in *in vitro* MLR assays (Boehme et al., 1989). The absence of diabetes or active tolerance to the transgene product in these class II MHC transgenic mice may be ascribed to low or more physiological level of protein (allogeneic MHC molecules) expression in the latter two models in which cDNA was used instead of genomic one, which could affect induction of toler-

ance *in vitro* (Sarvetnick, 1991; Quill and Schwartz, 1987). Indeed, the amount of peripheral antigen expressed appears to be an important factor in determining the degree of immune tolerance *in vivo* (Arnold et al., 1993). It may be just ignorance of peripheral antigens, down-regulation of TCR or other accessory molecules or clonal deletion.

The determining factors will include the site and amount of antigen expression, affinity of TCR, expression level of TCR or other accessory molecules, and the developmental stage of T lymphocytes encountering peripheral antigens (Arnold et al., 1993). The site where actual peripheral tolerance occurs is not clearly demonstrated. It may occur in the peripheral lymphoid organs after some 'transport' of allogeneic MHC molecules or directly in the peripheral nonlymphoid organ if sufficient amount of antigens and/or sufficient number of potentially reactive T lymphocytes exist there. Further knowledge of lymphocyte trafficking would reveal the exact site and mechanism of peripheral tolerance induction.

Molecular mechanism of peripheral tolerization process is also not clear. Recently, Fas-Fas ligand interaction has been reported to participate in superantigen-induced peripheral deletion of autoreactive cells (Mogil et al., 1995). This kind of apoptosis-mediated mechanism might work in the deletion of autoreactive cells specific for peripheral antigens.

CYTOKINE TRANSGENIC MOUSE

The above results do not support the Bottazzo hypothesis in that ectopic expression of allogeneic or syngeneic MHC class II molecules do not induce autoimmunity to those molecules or the tissue expressing those molecules. However, aberrant expression of MHC class II molecules induced by cytokines such as IFN- γ in natural disease conditions might have different immunological effects from transgenic expression of the MHC class II molecules alone. The *in vivo* consequences of class II MHC antigen expression induced by cytokine(s) were more closely simulated in transgenic mice expressing IFN- γ in pancreatic β -cells (Sarvetnick, 1991). In these mice, lymphoid infiltration in pancreatic islets and increased expression of MHC molecules in pancreatic parenchymal cells were observed, as well as diabetes. Moreover, lymphocytes from these transgenic mice were sensitized specifically to islet cells since the transgenic mice rejected islet grafts from nontrans-

genic control mice.

The mechanism of loss of tolerance to self antigens in these mice is not clear. For the development of chronic inflammation such as insulinitis, lymphocytes must transmigrate from vessels and home to inflammatory areas. For lymphocyte homing to the inflammatory foci, a specialized tissue termed high endothelial venules (HEV) is regarded as essential. Thus, the expression of HEV-specific antigens (vascular addressins) which are recognized by monoclonal antibodies MECA-367 or -79 was studied. MECA-367 recognizes a mucosal vascular addressin, and MECA-79 recognizes a peripheral lymph node vascular addressin. Both vascular addressins were induced in the pancreas of Ins-IFN- γ mice (Lee and Sarvetnick, 1994). However, it is still not clear if induction of vascular addressins is responsible for the initial infiltration of inflammatory cells in the pancreas of the Ins-IFN- γ mice.

There is also a possibility that local IFN- γ might play a role in inducing 'costimulatory signals', which leads to overcoming peripheral tolerizing mechanism. Costimulatory molecules can be defined as the molecule that regulate expansion of T lymphocyte after an antigen is recognized by T cell receptor. When the delivery of the signals is blocked, clonal expansion would be blocked and a state of T cell inactivation or anergy would ensue. Expression of B7-1, a prototype of costimulatory molecules, was expressed on APC of Ins-IFN- γ mice but not on nonlymphoid cells (Lee et al., 1995). Although these mice showed evidence of sensitization to islet cells, specific sensitization to islet-specific antigens could not be demonstrated because of the unavailability of islet-specific T cell antigens. This problem was solved using viral antigen transgenic mice as illustrated in the following section.

VIRAL ANTIGEN TRANSGENIC MICE AND PERIPHERAL IGNORANCE

Transgenic mouse technology also allowed investigation of (auto)immune responses against artificial 'self' antigens. A couple of viral antigens were employed as artificial self antigens because antigenic determinant causing such immune responses and humoral and/or cellular immune response against those determinants were well characterized.

Lymphocytic choriomeningitis virus (LCMV) is one of the best characterized viruses in terms of immune response against them, and two groups of investiga-

tors expressed LCMV nucleoprotein (NP) or glycoprotein (GP) antigens in pancreatic β -cells using insulin promoter (Ohashi et al., 1991; Oldstone et al., 1991). No insulinitis was observed, and there was apparent tolerance to the viral antigens transgenically expressed in the pancreas. This appears to be consistent with the classical immunological theory of self-nonself discrimination since the viral antigen would have been expressed well before birth. However, when infected with LCMV, the transgenic mice developed insulinitis and diabetes mellitus. These results indicated that apparent tolerance of lymphocytes rendered nonresponsive to self (transgenic viral) protein could be broken when infected with the immunogenic form of virus later. They also suggest that lymphocytes, potentially reactive to self antigens are not completely deleted or anergized. Instead, they may just 'ignore' the self antigens because of their location on nonlymphoid tissue and attack the antigens when appropriately sensitized. This mechanism might be termed as 'peripheral ignorance', and it is possible that most apparent immune tolerance to peripheral antigens not expressed in the thymus or lymphoid organ might be this type of immune ignorance. These results might also link two separate events of autoimmunity and virus infection although virus infection has long been implicated as a triggering event of autoimmunity without definitive evidence. Namely, if viral genes are present in the genome of animals or human and they are infected with the viruses later in their life, apparent tolerance to those viral (or self) antigens might be broken and (auto)immunity might occur. Instead, infection by a virus resembling host endogenous constituent might cause loss of tolerance to self antigen, which is compatible with the 'molecular mimicry' model (Fujinami and Oldstone, 1985).

The LCMV mouse model was also used to demonstrate islet-specific sensitization in Ins-IFN- γ mice. In Ins-IFN- γ mice, it was difficult to show islet-specific sensitization because of the absence of available T cell antigen. The LCMV model enabled expression of well-characterized LCMV NP or GP antigen in the pancreatic β -cells, and the crossing between LCMV model and Ins-IFN- γ mice allowed demonstration of LCMV-specific T cell immune responses and islet-specific immune sensitization in double transgenic mice (Fig. 1) (Lee et al., 1995).

Other viral antigen transgenic mice used in the analysis of immune tolerance were those expressing influenza haemagglutinin in their pancreatic β -cells (RIP-

Fig. 1. Secondary CTL response to LCMV antigens in double transgenic mice and single transgenic littermates used as controls. Lymphocytes from NP⁺/IFN- γ ⁺ mice killed both LCMV-infected(A) and vaccinia-NP-infected targets(B) to a moderate degree, whereas lymphocytes from NP⁺/IFN- γ ⁺ mice exerted only negligible killing of these targets(A and B). These results suggest that lymphocytes from the double transgenic mice are spontaneously and specifically sensitized to LCMV-NP antigen. Lymphocytes from GP⁺/IFN- γ ⁺ mice killed a small portion of LCMV-infected targets(C). Lymphocytes from LCMV-infected mice used as positive controls showed strong CTL response to LCMV antigens(A-C).(From Lee et al., 1995 with permission)

HA mice). However, neither insulinitis nor diabetes in the RIP-HA mice was observed even after infection with vaccinia-HA recombinant virus(Lo et al., 1992). This seems to be in apparent contradiction with the results obtained using LCMV transgenic mice(Ohashi et al., 1991; Oldstone et al., 1991), and it was speculated that the inability of vaccinia, not a mouse pathogen, to infect host cells and subsequent limitation of T cell stimulatory activity might be responsible for the deficient T cell response after virus challenge.

TCR TRANSGENIC MICE

TCR transgenic mice were initially produced to enlarge the lymphocyte population with a specific TCR by allelic exclusion and to allow the pursuit of lymphocytes specific for non-MHC molecules, which has been impossible because of the small number of those lymphocytes. TCR transgenic mice were also bred with other transgenic mice expressing the authentic antigen in the peripheral tissue.

In the case of LCMV transgenic mice which showed the evidence of peripheral ignorance, the addition of TCR transgene encoding TCR specific for LCMV antigen on over 70% of peripheral lymphocytes further demonstrated that there was no clonal deletion, abortion or a decrease in the TCR density of the lymphocytes in the double transgenic offsprings(Ohashi et al., 1991). They proposed that unprimed lymphocytes harboring TCR were not 'anergized' on the basis that cytotoxic T lymphocyte(CTL) response were similar between LCMV-transgenic and -non-transgenic mice, and only could not recognize the antigen due to the inability of pancreatic β -cells to stimulate T lymphocytes. These results further support their concept of immunological ignorance.

On the other hand, in SV40 T transgenic mice, additional expression of the transgenic SV40 T-specific TCR led to (auto)immune response to transgenic SV40 T protein and pancreatic destruction in double transgenic mice expressing the antigen on pancreatic acinar cells and TCR on the majority of lymphocytes(Geiger et al., 1992). Consistent with this result, expression of HA-specific TCR transgene from Th2 clone on RIP-HA transgenic mice showed that autoimmune response to HA antigen and autoimmune diabetes occurred, according to the strain of breeding mice(Scott et al., 1994). Additionally, TCR transgene from HA-specific Th1 cells also induced insulinitis and

autoimmune diabetes in RIP-HA transgenic mice, indicating no anergy to peripheral antigen on pancreatic islet cells (Sherman L, personal communication).

Based on these findings from TCR transgenic mice, it can be inferred that transgenic TCR expression on the majority of lymphocytes is usually not associated with their deletion or anergy to peripheral antigens. They just ignore the peripheral antigens on account of their location in non-APC in some cases or recognize peripheral antigens leading to (auto)immune destruction in another cases, probably according to the affinity of TCR and/or expression level of peripheral antigens. Also, lymphocytes expressing TCR transgene specific for peripheral antigens do not appear to need specific sensitization signal or events for the (auto)immune responses. However, it is not clear if these results can be extrapolated to natural situation because of the possibility that overwhelming number of potentially reactive lymphocytes might override the peripheral tolerizing capacity of peripheral antigens that might be sufficient for the tolerance induction in a very small number of self-reactive lymphocytes. The limited capacity for the tolerization of lymphocytes specific for peripheral antigens was demonstrated in double transgenic mice expressing both SV40 T antigen in pancreatic β -cells and TCR specific for the SV40 T antigen. A subline expressing that TCR in 10% of peripheral T lymphocytes showed partial deletion of specific T lymphocytes and nonresponsiveness of the remaining lymphocytes, while another line expressing that TCR in 90% of peripheral T lymphocytes was nontolerant to that antigen both *in vivo* and *in vitro* (Foester et al., 1995).

Besides the recognition of transgenic antigens, active recognition of natural self antigens by lymphocytes expressing TCR from self-reactive T cell clones was also observed. Transgenic mice expressing rearranged V β 8.2- and V α 2.3-containing TCR genes cloned from myelin basic protein-specific hybridoma were generated (Goverman et al., 1993). Mice in a sterile environment did not show evidence of experimental allergic encephalomyelitis (EAE); however, transgenic mice in a non-sterile facility developed spontaneous EAE. This result suggests that lymphocytes expressing self-reactive TCR could actively recognize self antigens leading to autoimmune diseases (EAE) without any immunological intervention, at least in non-sterile conditions. Some unknown environmental factor(s) might play a role in triggering the

disease.

Similar results were obtained in nonobese diabetic (NOD) backcross mice expressing TCR from an islet-specific T cell clone. Those mice showed accelerated onset of diabetes, suggesting active recognition of self antigens by self-reactive TCR without specific immune intervention (Katz et al., 1993). These results indicate the complexity of the mechanism determining immune tolerance vs. autoimmunity, and shows that active autoimmune diseases could occur if a sufficient number of self-reactive lymphocytes exist, probably with the help of environment. However, it is far from clear if this conclusion could be applied to natural *in vivo* circumstances because thymic or peripheral tolerizing mechanism might be overridden by the enormous number of self-reactive lymphocytes in those TCR transgenic mice which could not occur in natural situation.

The power of TCR transgenic mouse technology in elucidation of immune tolerance was recently shown in a paper describing the putative immunological mechanism of a well-known phenomenon—oral tolerance. Oral administration of antigen has long been used to induce antigen-specific immune tolerance; however, the mechanism was not clearly demonstrated. By using transgenic mice expressing ovalbumin-specific TCR, it was demonstrated that a high dose of oral antigen (ovalbumin) led to deletion of antigen-reactive T cells in Peyer's patches while low-dose administration induced T cell clones secreting immunoinhibitory cytokines such as TGF- β , IL-4 and IL-10. TCR transgenic technology also provided some clues on the immunological mechanism of acceptance of paternal alloantigens during pregnancy. Transgenic mice expressing K^b-reactive TCR transgene and bearing K^b-positive conceptus had reduced number of alloreactive T cells and accepted K^b-bearing tumor grafts only during pregnancy. The number of clonotypic TCR⁺ cells with CD4⁻ CD8⁻ double negative features were also significantly higher in those pregnant mice suggesting some features of clonal abortion (Tafari et al., 1995). These results clearly demonstrate the resolving power of transgenic mouse technology in analyzing specific antigen-TCR interaction and the mechanism of immune tolerance.

CONCLUSION

Transgenic mouse technology contributed much to the understanding of immune tolerance and autoim-

munity by providing direct *in vivo* evidence which was unavailable in conventional approaches. However, it should be remembered that unphysiologic or artifactual effects sometimes occur confounding proper interpretation. It is also true that current transgenic technology is not advanced enough to handle multiple genes and to solve the mystery of autoimmunity—the opposite of immune tolerance. Despite these caveats, further transgenic studies combined with other sophisticated *in vitro* techniques or more advanced methods of gene targeting are expected to provide answers to many unsolved questions regarding immune tolerance.

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