Tolerance in Transgenic Mice Expressing Major Histocompatibility Molecules Extrathymically on Pancreatic Cells

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Transgenic mice with defined expression of major histocompatibility complex (MHC) proteins provide novel systems for understanding the fundamental question of T cell tolerance to nonlymphoid self components. The MHC class II I-E and I-A and class I H-2K molecules expressed specifically on pancreatic islet or acinar cells serve as model self antigens. In these systems, transgenic proteins are not detected in the thymus or other lymphoid tissues. Yet mice are tolerant to the pancreatic MHC products in vivo; this tolerance is not induced by clonal deletion. These studies have been aided by monoclonal antibodies specific for I-E–reactive T cells and indicate that clonal anergy may be an important mechanism of tolerance to peripheral proteins.

THE IMMUNE SYSTEM CONFRONTS A CHALLENGE IN ITS quest to discriminate self from nonself components. A diverse repertoire of antigen-specific lymphocytes capable of responding to an unlimited array of pathogens must simultaneously be unresponsive to all self molecules. Antigen-reactive T cell precursors are both positively selected and tolerized in the thymus (*1*). Thymocyte T cell receptor (TCR) molecules interact with MHC molecules or MHC plus peptide complexes expressed on thymic epithelial cells, selecting ultimately for T cells that can recognize foreign antigen in the context of self-MHC molecules. Most intrathymic tolerance occurs by deletion of autoreactive clones that engage MHC-expressing bone marrow–derived cells. Clonal deletion was elegantly demonstrated with monoclonal antibodies (MAbs) that identify T cells bearing TCRs of known β-chain specificity (*2–5*) and by use of TCR transgenic mice (*6, 7*). In addition, evidence for a nondeletional mechanism of intrathymic tolerance mediated by epithelial cells has been reported (*8, 9*).

Yet how does the immune system establish and maintain tolerance to self components that are expressed extrathymically and are restricted to nonlymphoid tissues? Several hypotheses have been put forth to explain this long unresolved question. Tissue-restricted self molecules may be processed and transported by circulating bone marrow–derived cells back to the thymus for induction of tolerance. Alternatively, peripheral tolerizing mechanisms may operate. Possibilities include (i) clonal deletion, (ii) clonal inactivation or anergy, (iii) suppression, (iv) expression of self antigens in "privileged sites" that are sequestered from the responsive lymphocyte pool, and (v) expression of self molecules on nonlymphoid cells that are incapable of effective antigen presentation. Understanding of this issue has been significantly advanced through the use of transgenic mouse technology (*10*). Transgenic mice with well-defined, peripheral expression of model self antigens have made it possible to address the mechanisms of T cell tolerance directly in vivo.

Transgenic (Tg) mice carry a foreign gene, commonly introduced by direct microinjection of that gene into fertilized mouse embryos. Embryos are transplanted into foster mothers, and progeny with the integrated transgene are detected by DNA hybridization. In most cases, a transgene-positive mouse can be bred to establish a lineage of transgene-carrying individuals, because transmission of the foreign sequence is the same as with endogenous mouse genes. Expression of the transgene is directed by regulatory elements contained within it, and many examples of appropriate, tissue-specific, developmentally regulated, and inducible transgene expression have been reported (*10*). The power of transgenic technology exploited for studies of tolerance is the ability to deliberately target transgene expression to particular cell types. Structural genes fused to well-characterized, tissue-restricted enhancers and promoters have yielded precisely defined expression of desired molecules in vivo. Thus, a gene product chosen as a model self protein can be expressed naturally in a designated site without the considerations of surgical transplantation and the inflammation and trauma that accompany it.

Several groups (*11–17*) have developed Tg mouse strains with limited extrathymic expression of MHC class II and class I molecules in order to study tolerance to these exemplary proteins (Table 1). These studies all provide evidence for nondeletional mechanisms of T cell tolerance.

T Cell Tolerance to MHC Expression in Pancreas

The MHC class II molecules, I-A and I-E, are cell surface molecules each composed of α and β polypeptide chains and are normally expressed on the surface of B cells, macrophages, dendritic cells, and thymus epithelial cells. Class I molecules are also heterodi-

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meric proteins consisting of a heavy chain associated with β_2-microglobulin and are ubiquitously expressed (*18*). Aberrant appearance or increased amounts of MHC molecules have been observed on nonlymphoid cells in diseased states, such as on thyroid cells in autoimmune thyroiditis (*19*) and pancreatic β cells in insulin-dependent diabetes mellitus (IDDM) (*20*). Loss of insulin-producing islet β cells in IDDM and in animal models of diabetes appears to be caused by autoreactive lymphocytes that infiltrate the pancreatic islets (insulitis) and destroy these cells (*21*). Thus, aberrant expression of class II proteins may initiate autoimmune destruction by allowing antigen presentation of self molecules on parenchymal cells that are normally ignored by MHC-restricted T cells (*22, 23*). Alternatively, class II expression may occur secondarily to the inflammatory response, consistent with the inducibility of class II molecules on nonlymphoid cells when exposed to lymphokines (*24*) and with the late timing of class II appearance in the BB diabetic rat model (*25*). With insulin gene regulatory elements, MHC expression can be targeted specifically to islet β cells, allowing a direct test of the role of class II proteins in IDDM pathogenesis. These Tg models also allow an investigation of how tolerance develops to nonlymphoid antigens.

Much work has been focused on the class II I-E molecule as a transgene, expressing it in the pancreas of mouse strains that normally lack I-E molecules (*11, 12*). In other studies, class II I-A (*13–16*) or the class I K^b molecule (*26*) has been introduced into mouse strains of the same or different *MHC* haplotype as the transgene. Thus, self antigen is represented by either an allogeneic MHC molecule or pancreas-restricted proteins presented by a syngeneic MHC molecule. In all cases, the MHC molecules were expressed in pancreatic islet or acinar cells by fusing the insulin or elastase enhancer-promoter, respectively, to the coding regions of the class II α and β chains or the class I heavy chain. Insulin regulatory::I-E–coding regions (Ins–I-E) Tg mice (*11*) expressed I-E specifically in pancreatic islet β cells (Fig. 1) and unexpectedly on kidney tubular epithelium. Expression was not detected at the messenger RNA (mRNA) or protein level in any lymphoid organs, including thymus, lymph node, spleen, or on interferon-gamma (IFN-γ)–induced peritoneal macrophages. Severe diabetes developed in 100% of transgenic progeny from three independent lines, indicating aberrant I-E expression in β cells results in diabetes. However, there was no T cell reactivity to I-E, evidenced by the lack of lymphocytic infiltrates in the $I\text{-}E^+$ pancreas. Similarly, Tg mice expressing islet cell–specific I-A (*13–15*) and K^b (*26*) transgenes did not develop lymphocytic infiltrates in the pancreas, yet diabetes also resulted in some of these cases (*13, 15, 26*). It was concluded that diabetes can result from pancreatic β cell *MHC* gene expression. However, in all instances Tg mice appear functionally tolerant to pancreatic transgene products in vivo. This tolerance is complete, since priming of Tg mice to the transgenic alloantigen in vivo also does not induce autoimmunity (*14, 17, 27*).

The absence of insulitis in these Tg systems rules out an autoimmune cause for the defective insulin production. However, the mechanism that causes the diabetic phenotype of these mice is not known. One possibility is inhibition of insulin gene expression at the level of transcription caused by transgene competition for regulatory factors, because expression of A_β (*15*) alone can cause diabetes. In addition, since class II proteins can bind insulin (*28*), inappropriately high-level MHC expression may interfere with insulin secretion (*29*).

Targeting of *MHC* transgenes to pancreatic islets in Tg mice was intended to assess their role in initiating an autoreactive T cell response. Tolerance to the transgenes apparently contradicts the original hypothesis; however, it is possible that tolerance induction is facilitated by the early appearance of *MHC* transgenes, as

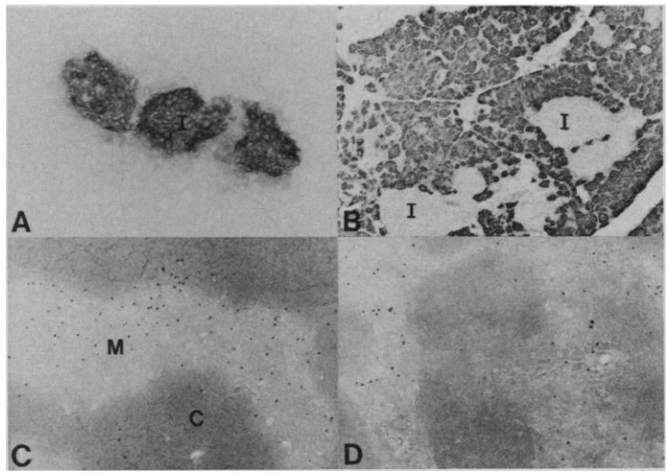

Fig. 1. Expression of I-E transgenic protein in Ins–I-E and El–I-E mice. (**A**) Ins–I-E pancreas shows $I\text{-}E^+$ islet cells (I). (**B**) El–I-E pancreas shows $I\text{-}E^+$ acinar cells. (**C**) Thymus (M, medulla; C, cortex) and (**D**) spleen of El–I-E mice show I-E protein is not detectable in lymphoid tissue. (C) and (D) are also representative of the lack of I-E expression in lymphoid tissue obtained from Ins–I-E mice. Cryostat sections were stained for I-E as described (*11, 12*); (B) to (D) were counterstained with hematoxylin. [Adapted from (*11*) with permission, Cell Press; adapted from (*12*) with permission, Rockefeller University Press]

suggested by Adams *et al.* (*30*). In this case, Tg lines expressing islet cell–specific SV40 T antigen early in life are tolerant to this protein, whereas Tg lines with delayed onset of T antigen are not tolerant. Therefore, the ability of $I\text{-}E^+$ islet cells to cause autoimmune disease was independently tested by grafting pancreas tissue from Ins–I-E mice into naïve $I\text{-}E^-$ adult recipients (*31*). Interestingly, the Tg tissue persisted, indicating $I\text{-}E^+$ islet cells do not stimulate rejection by mature, nontolerant T cells.

Two groups (*11, 16*) have also generated Tg mice with I-E or I-A molecules targeted to pancreatic acinar cells. This made it possible to determine whether the results obtained in mice with class II–expressing islet cells could be generalized to another nonlymphoid cell type. Also, in this system the coincidence of diabetes could be avoided, and because the Tg mice survive as well as normal mice, more detailed studies of the transgenic immune system are possible. Both *I-E* and *I-A* transgenes were expressed specifically in pancreatic acinar cells with no detectable mRNA or protein in the thymus or other lymphoid or nonlymphoid compartments (Fig. 1). Importantly, no evidence of lymphocytic infiltration was found in the pancreas of any of these lines. Thus the T cells of mice expressing I-E with the elastase promoter (El–I-E mice) or allogeneic I-A are also functionally tolerant to the class II proteins. In our El–I-E mice this was further supported, since priming of El–I-E mice in vivo with subcutaneous injections of $I\text{-}E^+$ spleen cells did not stimulate autoimmune destruction (*11, 27*).

In summary, several independent laboratories have established numerous transgenic lines that have class II or class I molecules specifically in pancreatic islet or acinar cells. In these systems the transgene product is not detected in lymphoid organs, most notably the thymus. Yet T cells of these mice appear to be functionally tolerant in vivo to the transgenic proteins, suggesting extrathymic mechanisms account for the T cell tolerance observed. Closer analysis of transgene expression in these mice using the highly sensitive polymerase chain reaction (PCR) methodology (*32*) may detect transgenic mRNA at very low levels in the thymus. However, it will be difficult to assess the functional significance of this. In the particular case of the El–I-E mice, the tolerant phenotype is

independent of transgenic I-E protein expressed directly in the thymus or in bone marrow–derived cells, as demonstrated by construction of chimeric animals (*12*). Overall, the tolerant phenotype observed in all Tg systems described here is significant, because intrathymic tolerance by clonal elimination is documented (*2–7*), whereas the tolerance in these Tg mice appears to be nondeletional.

T Cell Tolerance Occurs by Nondeletional Mechanisms

The transgenic lines described above and T cells isolated from them were examined by in vivo transfer experiments and by in vitro assays in order to dissect the basis for T lymphocyte tolerance. Ins–I-E Tg mice (*11*) and El–I-E Tg mice (*12*), as well as Tg mice expressing allogeneic I-A molecules on islet (*14, 15*) or acinar cells (*16*), were tested for reactivity to the specific transgenes by assaying the primary mixed lymphocyte reaction (MLR) in vitro with conventional spleen antigen-presenting cells (APC). T cells from either Ins–I-E or El–I-E Tg lines generated very weak proliferative responses to I-E; responses were barely above background in most experiments. In contrast, the I-A–expressing lines (*14–16*) showed strong proliferative responses to the *I-A* transgene (Table 1). The explanation for this apparent difference in reactivity in vitro is unknown. Perhaps the mechanism of tolerance is distinct in different Tg strains because of the precise expression pattern of different constructs or because of the use of I-A versus I-E molecules. Alternatively, it is known that responses to I-A are much stronger than responses to I-E. A partial reduction in functional activity of alloreactive T cells, by whatever mechanism, will have a more profound effect on I-E– than I-A–specific responses. Thus, although all mice are functionally tolerant in vivo (because lymphocytic infiltrates are absent) sufficient I-A reactivity might remain that is detectable in vitro. Like T cells of Ins–I-E and El–I-E mice, T cells of mice expressing class I K^b on islet β cells are functionally tolerant in vivo and in vitro (*33*). In this case T cell function was measured by cytotoxicity to K^b-expressing target cells. However, lysis could be observed by Tg T cells isolated from older mice known to be depleted of transgene K^b-expressing β cells, or by coculture of Tg T cells with interleukin-2 (IL-2). The effect of IL-2 on I-E–specific Tg T cell responses has not yet been tested. However, I-E reactivity did not increase even after Ins–I-E Tg T cells were recovered from thymectomized, irradiated, $I\text{-}E^-$ nontransgenic recipients to which they had been transferred for a 10-week period (*27*).

Transgenic T cells from mice with pancreatic *I-A* transgenes showed reactivity to I-A in vitro, suggesting that T cell tolerance apparent in vivo is caused by a nondeletional mechanism. However, the reactivity of T cells specific for pancreatic peptides in the context of I-A is not necessarily reflected by stimulation with $I\text{-}A^+$ spleen cells. Thus, a contribution of T cell deletion to tolerance cannot be entirely ruled out. Which alternative tolerizing mechanisms may also be involved in these systems is not yet clear. However, studies by Murphy *et al.* (*16*), as well as experiments with Ins–I-E and El–I-E mice (*11, 12*), indicate that transfer of nontransgenic, nontolerant T cells into irradiated Tg hosts results in destructive infiltration of the pancreas. Thus, both pancreatic acinar and islet cells are accessible to alloreactive lymphocytes. In the Tg mice with pancreatic K^b, the mechanism of tolerance also appears to be neither deletion nor permanent inactivation, on the basis of in vitro reactivity to K^b under particular circumstances. Furthermore, the mechanism appears to be extrathymic, since thymocytes from these mice are reactive against K^b-expressing target cells in vitro (*33*). However, these experiments do not address deletion of T cells reactive only with pancreas-specific peptides presented by K^b.

Understanding the basis for tolerance induction of this series of mice has been aided by the availability of MAbs to TCRs that are I-E reactive. The ability to identify I-E–reactive cells offers a valuable alternative to functional measurements. Both the KJ23 MAb specific for $V_\beta 17a$ TCR (*34*) and the MR9-4 MAb that recognizes $V_\beta 5^+$ TCR (*35*) were used. $V_\beta 17a^+$ and $V_\beta 5^+$ TCRs are present among the T cells of certain $I\text{-}E^-$ mouse strains, but are deleted in the thymus of $I\text{-}E^+$ mice (*2, 35*). Ins–I-E and El–I-E mice were backcrossed to appropriate $I\text{-}E^-$ strains, SJL/J and C57BL/6, so that they would be homozygous for the $V_\beta 17a$ and $V_\beta 5$ TCR genes, respectively. The frequency of $V_\beta 17a$- and $V_\beta 5$-bearing T cells was then measured by immunofluorescence; no significant differences between Ins–I-E, El–I-E, and $I\text{-}E^-$ nontransgenic control mice were found (*36*) (Fig. 2A). Also, Tg and nontransgenic T cells had similar densities of these TCR molecules. The distribution into $CD4^+$ versus $CD8^+$ cells was appropriate (predominantly $CD4^+$ for $V_\beta 17a^+$ T cells and mostly $CD8^+$ for $V_\beta 5^+$ T cells). Thus, the in vivo tolerance observed in Ins–I-E and El–I-E mice is not caused by clonal deletion of these I-E–reactive populations.

Antigen-Presentation by Pancreatic Islets and Clonal Anergy

A primary alternative for T cell tolerance, if not by deletion, is via clonal inactivation. Paralysis of T cells has been hypothesized to occur by exposure to antigen plus class II molecules in the absence of a second, costimulatory signal normally delivered by APC (*37–39*). T cells paralyzed in this manner are subsequently unresponsive to antigen presented by normal APC. Many examples of T cell anergy have been elucidated in vitro, including inactivation of T cell clones via antigen presented by human T cell clones (*40*), chemically modified spleen cells (*41*), purified MHC class II on planar membranes (*42*), and IFN-γ–induced class II^+ keratinocytes (*43*). We and our collaborators have performed experiments to examine whether clonal anergy is involved in the tolerance observed in Ins–I-E and El–I-E transgenic mice. Two aspects have been studied: (i) antigen presentation by pancreatic islet cells and (ii) the activation properties of Tg T cells.

To assess the ability of $I\text{-}E^+$ islet cells to induce clonal anergy (*31*), T cell lines specific for the herpes glycoprotein D peptide–I-E complex were cultured with Tg $I\text{-}E^+$ islets or nontransgenic control islet cells in the presence or absence of antigen. After 2 days, T cells were recovered and rechallenged with peptide plus $I\text{-}E^+$ spleen cells. T cell reactivity was significantly reduced among T cells precultured with peptide plus Tg islets, but not in those cells precultured with Tg islets alone or control islets with or without antigen. These results show that $I\text{-}E^+$ Tg islet cells can induce T cell unresponsiveness in vitro.

To determine whether or not Tg T cells were clonally paralyzed in vivo, we attempted to activate them by cross-linking their TCR molecules (*36*). Normally, T cells will proliferate in response to receptor-mediated cross-linking via antibodies to the TCR in the presence of accessory cells or soluble costimulatory signals (*44–47*). Since paralyzed cells are anergic to rechallenge with antigen plus conventional APC, it would not be expected that anergic cells would be stimulated to proliferate by receptor cross-linking. Although control mice proliferated significantly when stimulated with MAbs to $V_\beta 17a$ (anti-$V_\beta 17a$) or $V_\beta 5$ (anti-$V_\beta 5$), Ins–I-E Tg T cells responded weakly, if at all. El–I-E T cells responded strongly with anti-$V_\beta 17a$, but poorly to anti-$V_\beta 5$ (*36*) (Fig. 2B). Thus, these experiments provide direct evidence that some I-E–reactive T cells are apparently rendered anergic in vivo, or at least that the threshold

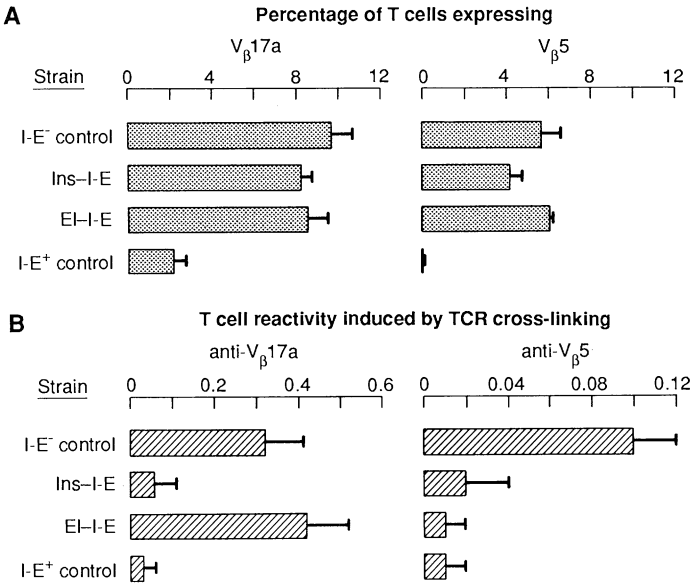

Fig. 2. (**A**) Presence of $V_\beta17a^+$– and $V_\beta5^+$ I-E–reactive T cells in Ins–I-E and El–I-E Tg mice. Percentages represent mean ± SEM (n = 2 to 6 individuals) detected by immunofluorescence. Lymph node T cells were stained for $V_\beta17a^+$ TCR, whereas unseparated lymph node cells were stained for $V_\beta5^+$ TCR. I-E⁻ control strains for $V_\beta17a^+$ and $V_\beta5^+$ T cells were the SJL/J and C57BL/6 strains, respectively. The I-E⁺ control strain was a Tg line expressing I-E on all appropriate lymphoid tissues backcrossed to SJL/J or C57BL/6, respectively. (**B**) T cell proliferation induced by TCR cross-linking with anti-$V_\beta17a$– and anti-$V_\beta5$–specific MAbs. Values indicate mean ± SEM (n = 3 to 7 individuals), and are taken from (*36*). Data are expressed as the change in counts per minute (Δcpm) induced by anti-$V_\beta17a$ or anti-$V_\beta5$ divided by Δcpm induced by an antibody specific for the CD3 molecule of the CD3-TCR complex (*47*). Strong responses were induced by the anti-CD3 MAb for all mouse strains tested, and therefore served as a control for the general responsiveness of the T cell population. Experimental conditions for TCR-mediated activation were as described (*36*). [Adapted from (*36*) with permission, *Nature*]

of activation is different between Tg T cells and T cells that are not tolerant to I-E isolated from a control I-E⁻ strain. The difference in activation of El–I-E T cells with the anti-$V_\beta17a$ versus the anti-$V_\beta5$ MAb suggests a difference between $V_\beta17a^+$ and $V_\beta5^+$ T cells in their fine specificity for I-E. Likewise, the difference between activation of Ins–I-E and El–I-E T cells by anti-$V_\beta17a$ suggests a difference between the I-E expressed by islet and acinar cells. I-E⁺ islet cells and I-E⁺ acinar cells may, for example, present different I-E–self-peptide complexes (*48*).

Given the interest in measuring activation properties of class II I-E–reactive cells and the tight association of class II recognition with $CD4^+$ cells, it was surprising to find that unfractionated Tg T cells were not responsive to anti-V_β stimulation, even though 35% of $V_\beta17a^+$ cells are $CD8^+$ and the majority of $V_\beta5^+$ cells are $CD8^+$. Both $CD4^+$ and $CD8^+$ T cells normally respond to receptor cross-linking (*46, 49*). It is possible that use of particular V_β genes confers sufficient I-E reactivity even among $CD8^+$ cells. To address this question, novel $CD8^+$ T cell hybridomas expressing $V_\beta17a^+$ were generated (*50*) and examined for reactivity to I-E. CD8-dependent I-E reactivity was not observed among a large panel of hybrids. However, I-E reactivity of $CD8^+$ T cells has also been investigated in an independent system based solely on measuring I-E alloreactivity in vivo (*51*). T cells bearing $V_\beta11^+$ TCR are also specifically deleted in I-E–expressing mouse strains, but even $CD4^+$ $V_\beta11^+$ T cell hybrids do not display reactivity to I-E in vitro. Transfer of T cells from I-E⁻ mice into I-E⁺ hosts showed sequestering of $V_\beta11^+$ cells in the host early after transfer. Interestingly, blast cells recovered several days after transfer contained a significant enrichment of $V_\beta11^+$ cells, which included $CD4^+$ as well as $CD8^+$ blasts. Thus, capability for I-E reactivity in vivo may not be fairly reflected by in vitro measurements. Using $P \rightarrow F_1$ radiation bone marrow chimeras to examine tolerance imposed by thymic epithelial cells, Ramsdell *et al.* (*9, 52*) also provide evidence for tolerance by clonal anergy among $CD4^+$ and $CD8^+$ T cells. In this case, both I-E–reactive $V_\beta17a$ and Mls^a-reactive $V_\beta6$-bearing T cells were studied.

The unresponsiveness of Tg T cells to anti-$V_\beta17a$ and anti-$V_\beta5$ stimulation provides evidence that clonal anergy of T cells can occur in vivo, possibly by exposure to I-E⁺ pancreatic cells in vivo. However, we cannot exclude that some clonal deletion among T cells outside of the $V_\beta17a^+$ and $V_\beta5^+$ subsets is necessary for the development of the tolerant phenotype. Also, it is perplexing that thymocytes as well as peripheral T cells from these mice also fail to respond to I-E by primary MLR in vitro (*11, 12*). Expression of the I-E transgene was never detected in thymus sections from Ins–I-E or El–I-E Tg mice. In addition, to show that tolerance was independent of transgene expression in the thymus or bone marrow–derived cells (*12*), adult El–I-E mice were thymectomized, engrafted with the thymus from a genetically I-E⁻ C57BL/6 strain, then lethally irradiated and reconstituted with C57BL/6 bone marrow. Lymph node T cells isolated 6 to 8 weeks after reconstitution still responded only weakly to I-E by primary MLR. Collectively these data are consistent with the possibility that I-E was transported from the pancreas or kidney and presented in the thymus to induce tolerance

Table 1. Mouse strains with pancreas-specific *MHC* transgene expression. Transgene expression was targeted to the pancreatic islet or acinar cells with enhancer-promoter sequences of the insulin or elastase gene, respectively. T cell reactivity in vivo was measured by the occurrence (+) of lymphocytic infiltrates in pancreatic tissue; minus indicates the absence of insulitis in all cases. T cell reactivity in vitro is measured by primary MLR to MHC class II *I-E* or *I-A* transgenes, or by the ability to generate CTL recognizing the MHC class T K^b transgene. In vitro reactivity was determined for cases in which the haplotype of the transgene was different from that of the host strain. NA, not applicable. For the K^b transgenic mice, reactivity can be detected under certain circumstances in vitro [(*33*) and text].

MHC transgene	Site of transgene expression	MHC of host	T-cell reactivity to transgene		Reference
			In vivo	In vitro	
$I\text{-}E^b$	Islet β cells, kidney	$I\text{-}E^-$ or $I\text{-}E^b$	–	–	(*11*)
$I\text{-}E^b$	Acinar cells	$I\text{-}E^-$	–	–	(*12*)
$I\text{-}A^d$	Islet β cells	$I\text{-}A^d$	–	NA	(*13*)
$I\text{-}A^k$	Islet β cells	$I\text{-}A^b$ or $I\text{-}A^k$	–	+	(*14*)
$I\text{-}A^d$	Islet β cells	$I\text{-}A^b$	–	+	(*15*)
$I\text{-}A^d$	Acinar cells	$I\text{-}A^b$	–	+	(*16*)
K^b	Islet β cells	$I\text{-}A^k$, $I\text{-}A^{kb}$, $I\text{-}A^{bb}$, $I\text{-}A^{bs}$, $I\text{-}A^{ss}$	–	–	(*17*)

there, although at present we cannot exclude a very low level of I-E expression undetected by our assays directly in the thymus of Ins–I-E mice. An alternative explanation is that responding cells taken from the thymus are not representative of newly generated T cells and may instead consist of cells that have recirculated from the periphery (*53*). Nonetheless, given the presence of $V_\beta 17a^+$ and $V_\beta 5^+$ T cell populations, the conclusion that the tolerant state involves anergy appears to be still valid. It is possible that tolerance occurs in the thymus by paralysis and not deletion (*9*), depending on the quantity or site of presented antigen.

The experimental data indicate that clonal anergy of Tg I-E–tolerant T cells occurs during normal T cell development in vivo. Evidence has been previously reported for clonal paralysis of T cells in vivo in systems that parallel those described for in vitro T cell paralysis. For example, Jenkins *et al.* (*41*) injected mice intravenously with APC chemically coupled in the presence or absence of antigen cytochrome c, then immunized by injecting the foot pad with cytochrome c emulsified in adjuvant. T cells of mice exposed to antigen chemically coupled to APC responded poorly on challenge with cytochrome c in vitro, whereas T cells exposed to APC not chemically coupled to antigen responded vigorously. Consistent with this finding, mice that have received intravenous infusions of MHC-expressing L cell fibroblast transfectants are less responsive to subsequently transplanted allografts bearing the appropriate alloantigen (*54*). Also, T cells of mice administered Mls-different spleen cells intravenously lose their capacity to proliferate to that Mls difference in vitro (*55*). In this system, the Mls-reactive T cells can be identified with a $V_\beta 6$-specific MAb, and the continued presence of $V_\beta 6^+$ T cells in tolerant mice implicates clonal anergy. However, these systems show induction of tolerance in mature T cells to exogenously administered antigen. In this context, a study with $I\text{-}E^+$ islet cells (*31*) was performed to determine their ability to induce tolerance in mature T cells. We have already mentioned that $I\text{-}E^+$ islet tissue grafted to $I\text{-}E^-$ naïve recipients persists, showing it is incapable of stimulating its own rejection. When these mice were primed 40 days after grafting with $I\text{-}E^+$ spleen cells, the grafts were rejected within 2 weeks. Moreover, the MLR reactivity to I-E was detectable in host cells as much as 120 days after grafting. Thus, the $I\text{-}E^+$ islet cells were not tolerogenic to all mature I-E–specific T cells in this circumstance. Several critical questions arise from these data. (i) At what stage are T cells "susceptible" to tolerance induction? (ii) Can T cells at different maturational states be tolerized by distinct pathways? Continued investigation of our Tg models should contribute to resolving how tolerance by anergy occurs under natural circumstances in vivo.

Conclusions

Evidence has been obtained through the use of Tg mice and gene targeting that self-reactive T lymphocytes become tolerant by nondeletional mechanisms. Future experiments should more finely delineate how T cell tolerance is established and maintained in these mice, and whether or not it is truly extrathymic. These studies also addressed other parameters influencing tolerance, including target organ accessibility and antigen-presenting capability. The data suggest that although the pancreas is accessible to circulating autoreactive T cells, pancreatic cells are ineffective APC. These characteristics of pancreatic cells types may or may not be generalizable to other target organs. New transgenic systems can be designed now to address these issues.

REFERENCES AND NOTES

1. J. Sprent, D. Lo, E.-K. Gao, Y. Ron, *Immunol. Rev.* **101**, 173 (1988).
2. J. Kappler, N. Roehm, P. Marrack, *Cell* **49**, 273 (1987).
3. J. Kappler, U. Staerz, J. White, P. Marrack, *Nature* **332**, 35 (1988).
4. H. R. MacDonald *et al.*, *ibid.*, p. 40.
5. A. M. Pullen, P. Marrack, J. W. Kappler, *ibid.* **335**, 796 (1988).
6. P. Kisielow, H. Blüthmann, U. D. Staerz, M. Steinmetz, H. Von Boehmer, *ibid.* **333**, 742 (1988).
7. W. C. Sha *et al.*, *ibid.* **335**, 271 (1988).
8. P. Marrack *et al.*, *Cell* **53**, 627 (1988); G. Widera *et al.*, *ibid.* **51**, 175 (1987).
9. F. Ramsdell, T. Lantz, B. J. Fowlkes, *Science* **246**, 1038 (1989).
10. R. D. Palmiter and R. L. Brinster, *Annu. Rev. Genet.* **20**, 465 (1986).
11. D. Lo *et al.*, *Cell* **53**, 159 (1988).
12. D. Lo, L. C. Burkly, R. A. Flavell, R. D. Palmiter, R. L. Brinster, *J. Exp. Med.* **170**, 87 (1989).
13. N. Sarvetnick, D. Liggitt, S. L. Pitts, S. E. Hansen, T. A. Stewart, *Cell* **52**, 773 (1988).
14. J. Böhme *et al.*, *Science* **244**, 1179 (1989).
15. J. Miller, L. Daitch, S. Rath, E. Selsing, *J. Immunol.* **144**, 334 (1990).
16. K. M. Murphy, C. T. Weaver, M. Elish, P. M. Allen, D. Y. Loh, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10034 (1989).
17. J. F. A. P. Miller, G. Morahan, J. Allison, P. S. Bhathal, K. O. Cox, *Immunol. Rev.* **107**, 109 (1989).
18. R. A. Flavell *et al.*, *Science* **233**, 437 (1986); J. E. Coligan, T. J. Kindt, H. Uehara, J. Martinko, S. J. Nathenson, *Nature* **291**, 35 (1981).
19. G. F. Bottazzo, R. Pujol-Borrell, T. Hanafusa, M. Feldmann, *Lancet* **ii**, 1115 (1983).
20. G. F. Bottazzo *et al.*, *N. Engl. J. Med.* **313**, 353 (1985).
21. A. A. Rossini, J. P. Mordes, A. A. Like, *Annu. Rev. Immunol.* **3**, 289 (1985).
22. G. F. Bottazzo, I. Todd, R. Mirakian, A. Belfiore, R. Pujol-Borrell, *Immunol. Rev.* **94**, 137 (1986).
23. C. Cowing, *Immunol. Today* **6**, 72 (1985).
24. R. Pujol-Borrell *et al.*, *Nature* **326**, 304 (1987).
25. B. M. Dean, R. Walker, A. J. Bone, J. D. Baird, A. Cooke, *Diabetologia* **28**, 464 (1985).
26. J. Allison *et al.*, *Nature* **333**, 529 (1988).
27. D. Lo, unpublished observations.
28. M. L. Phillips, C. C. Yip, E. M. Shevach, T. L. Delovitch, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5634 (1986).
29. P. Parham, *Nature* **333**, 500 (1988).
30. T. E. Adams, S. Alpert, D. Hanahan, *ibid.* **325**, 223 (1987).
31. J. Markmann *et al.*, *ibid.* **336**, 476 (1988).
32. R. K. Saiki *et al.*, *Science* **239**, 487 (1988).
33. G. Morahan, J. Allison, J. F. A. P. Miller, *Nature* **339**, 622 (1989).
34. J. Kappler *et al.*, *Cell* **49**, 263 (1987).
35. J. Bill, E. Palmer, O. Kanagawa, in preparation; J. Bill, V. Appel, E. Palmer, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9184 (1988).
36. L. C. Burkly, D. Lo, O. Kanagawa, R. L. Brinster, R. A. Flavell, *Nature* **342**, 564 (1989).
37. P. Bretscher and M. Cohn, *Science* **169**, 1042 (1970).
38. D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *Annu. Rev. Immunol.* **7**, 445 (1989).
39. R. H. Schwartz, *Science* **248**, 1349 (1990).
40. J. R. Lamb, B. J. Skidmore, N. Green, J. M. Chiller, M. Feldmann, *J. Exp. Med.* **157**, 1434 (1983).
41. M. K. Jenkins and R. H. Schwartz, *ibid.* **165**, 302 (1987).
42. H. Quill and R. H. Schwartz, *J. Immunol.* **138**, 3704 (1987).
43. A. A. Gaspari, M. K, Jenkins, S. I. Katz, *ibid.* **141**, 2216 (1988).
44. J. Frelinger, A. Sing, A. Infante, C. G. Fathman, *Immunol. Rev.* **81**, 21 (1984).
45. B. Manger, A. Weiss, C. Weyland, J. Goronzy, J. D. Stobo, *J. Immunol.* **135**, 3669 (1985).
46. I. N. Crispe, M. J. Bevan, U. Staerz, *Nature* **317**, 627 (1985).
47. O. Leo, M. Foo, D. H. Sachs, L. E. Samelson, J. A. Bluestone, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1374 (1987).
48. P. Matzinger and M. J. Bevan, *J. Cell. Immun.* **29**, 1 (1987); P. Marrack and J. Kappler, *Nature* **332**, 840 (1988).
49. O. Kanagawa, unpublished observations.
50. H.-G. Burgert, J. White, H.-U. Weltzien, P. Marrack, J. Kappler, *J. Exp. Med.* **170**, 1887 (1989).
51. E.-K. Gao, O. Kanagawa, J. Sprent, *ibid.*, p. 1947.
52. F. Ramsdell and B. J. Fowlkes, *Science* **248**, 1342 (1990).
53. P. J. Fink, M. J. Bevan, I. L. Weissman, *J. Exp. Med.* **159**, 436 (1984); S. A. Michie, E. A. Kirkpatrick, R. V. Rouse, *ibid.* **168**, 1929 (1988); Y. Naparstek *et al.*, *Nature* **300**, 262 (1982).
54. J. C. Madsen, R. A. Superina, K. J. Wood, P. J. Morris, *Nature* **332**, 161 (1988).
55. H.-G. Rammensee, R. Kroschewski, B. Frangoulis, *ibid.* **339**, 541 (1989).
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