TOLERANCE IN TRANSGENIC MICE EXPRESSING CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ON PANCREATIC ACINAR CELLS

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Tolerance to self antigens is a critical feature of the immune response. In the T lymphocyte pool, it appears that tolerance induction primarily occurs during lymphocyte development in the thymus. Potentially autoreactive T cells are deleted presumably upon exposure to the appropriate self antigen presented by either bone marrow-derived cells or thymic stromal cells (1-4). While this mechanism is effective for inducing tolerance to antigens expressed on these tissues, it may not be as effective in inducing tolerance to antigens expressed specifically in other nonlymphoid tissues (5). These tissue-specific antigens are of great interest since many of the major failures of tolerance to self (i.e., autoimmune disease) are due to immune responses to these "parenchymal self" (6) antigens.

We have been interested in developing well defined models of self tissue-specific antigens, and in studying the mechanisms used by the immune system to deal with these antigens. In some cases, the study of tolerance to self has been greatly aided by animal models of spontaneous or experimentally induced autoimmunity. We sought to supplement the existing models with a system of well defined model autoantigens using transgenic technology. With this technology, expression of cloned genes can be targeted to specific tissues by use of hybrid gene constructs (7, 8). This approach has been successfully used by a number of laboratories (7-11). The advantages of this system over models of spontaneous autoimmunity include the ability to use antigens well defined in terms of three-dimensional structure and T cell subset responses. Furthermore, expression can be targeted to tissues that are readily accessible for histological analysis, and in some cases, timing of expression can be controlled.

In a previous study, we targeted expression of a class II MHC antigen I-E to the pancreatic islet β cells in I-E$^-$ mice (insulin [INS][1]-I-E transgenic mice; reference 12). The experiment was designed to test whether expression of class II MHC by nonlymphoid cells made them capable of stimulating T cell responses to the class

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[1] *Abbreviations used in this paper:* EL, elastase; GPC, guinea pig complement; INS, insulin; LN, lymph node.

II antigen and initiating an autoimmune response against the β cells. Specific expression was achieved, but the INS-I-E transgenic animals developed a nonimmunological form of insulin-dependent diabetes. Interestingly, the animals were found to be tolerant to the I-E transgene antigen despite the absence of detectable I-E expression in the thymus or spleen. In vitro studies with the isolated I-E$^+$ islet cells (13) suggested that class II antigens on nonlymphoid cells were in fact not stimulatory to T lymphocytes. Instead, they paradoxically induced a specific paralysis; that is, reactive T cells exposed to the islet cells were rendered anergic to subsequent rechallenge by normal lymphoid class II$^+$ stimulators. This T cell paralysis observed in vitro may help explain the tolerance in the original INS-I-E transgenic mice (12–15).

The mechanisms involved in tolerance induction in the transgenic mice are likely to be rather complex. Even if T cell paralysis were the major mechanism for inducing tolerance, it is still necessary to explain how all of the relevant T cells were exposed to the peripheral I-E. There is also the perplexing observation that thymocytes were tolerant to I-E (12). Thus, for a more complete analysis of these phenomena, we extended our system to include transgene expression targeted to another tissue: the acinar cells of the exocrine pancreas (elastase [EL]-I-E transgenic mice). In this situation, there is more tissue available for histological analysis, and the diabetes observed in INS-I-E mice could be avoided. Furthermore, since the new EL-I-E transgenic mice were developed on an inbred C57BL/6J (B6) background, adoptive transfer of lymphocytes could be done without concern for irrelevant histocompatibility antigen differences. In this report, we describe the immune status of EL-I-E transgenic mice with respect to tolerance to the transgene I-E. From our experiments we were able to establish that tolerance in T lymphocytes can be induced and maintained by expression of a class II antigen exclusively in peripheral nonlymphoid cells. Since we were interested in determining what peripheral tolerance-inducing mechanisms may be acting in these mice, we also studied the ability of EL-I-E animals to induce tolerance in mature nontolerant T cells in vivo. Our adoptive transfer studies indicated that T cells from tolerant mice can mediate resistance to organ-specific immune attack, but the resistance may not involve specific Ts cells.

Materials and Methods

Mice. All mice were bred and maintained in our animal facility at the University of Pennsylvania School of Veterinary Medicine. Inbred mouse stocks were obtained from The Jackson Laboratory (Bar Harbor, ME). Eα transgenic line 107-1 (wild-type I-E expression; reference 16), originally generated on inbred C57BL/10 and backcrossed several generations to B6, were bred to be homozygous for the transgene. Eα transgenic line 78-1 (wild-type I-E expression; reference 17), originally generated on (B6 × SJL)F_2, was backcrossed several generations to B6. For generation of EL-I-E transgenic mice, two hybrid genes were constructed, similar to the hybrid constructs described in reference 12. The first gene was constructed by fusing 3.5-kb of the 5′ region of the rat elastase 1 gene (including the elastase enhancer and promoter; reference 18) to the structural gene coding the E^d_α chain of I-E. The second construct used 4.5 kb of the 5′ region of rat elastase 1 fused to the gene coding the E^b_β chain of I-E. EL-I-E transgenic mice (lines 231-3 and 415-1) were generated as previously described (19). Briefly, fertilized eggs, (B6 × SJL)F_2 for line 231-3 and inbred B6 for line 415-1, were microinjected with a few hundred copies of each gene construct, and the eggs were transferred to pseudopregnant foster females. Mice were allowed to develop, and at weaning, tail DNA was tested for integration of the transgene. Transgene-positive founder animals were bred to generate transgenic lines, which are designated by the founder animal number.

Mixed Lymphocyte Responses (MLR). MLR conditions were identical to those described previously (12). Briefly, lymph node (LN) T cells were isolated from LN suspensions by treatment with the anti-B cell antibody J11d plus guinea pig complement (GPC) and added to flat-bottomed 96-well plates at 2×10^5/well. Thymocytes were used untreated at 2×10^6/well. Stimulators were irradiated (1,650 rad) or mitomycin C–treated spleen cells from B6, B10.BR, CBA/CaJ, or 107-1 transgenic mice, and added to cultures at 5×10^5/well. Culture media consisted of RPMI 1640 supplemented with glutamine, 20 mM Hepes, antibiotics, 50 μM 2-ME, and 1% fresh mouse serum. Cultures were pulsed with 1 μCi [^{3}H]TdR after 4 d of culture and harvested on the fifth day. Data are the mean of triplicate cultures and presented as cpm $\times 10^{-3}$.

Adoptive Transfer Experiments. In most experiments, B6 or 107-1 LN and/or spleen cells were injected intravenously through the tail vein of nonirradiated or irradiated (900 rad) 415-1 EL-I-E transgenic mice. In the experiments described in Tables III and IV, (B6 × SJL)F_1 LN cells were injected into irradiated 231-3 EL-I-E mice. For Fig. 3, (B6 × SJL)F_1 LN cells were injected into irradiated 187-7 INS-I-E mice (12). For some experiments, T cells were isolated from LN and spleen by treatment with J11d plus GPC. Bone marrow was depleted of T cells by treatment with anti-Thy-1 (T24, nonallele specific; references 20 and 21) plus GPC. For in situ priming, 107-1 spleen cells were either mixed with the T cell inoculum for intravenous injection, or injected separately into footpads subcutaneously. 2 wk later (13–15 d after injection), animals were killed, and the pancreata processed for routine histology.

Histology. For routine histology, tissues were fixed in Bouin's, then paraffin embedded, sectioned, and stained with hematoxylin/eosin. For immunohistochemistry, tissues were frozen in Tissue-Tek OCT compound (Miles Scientific, Naperville, IL), sectioned on a cryostat (10 μm), and air dried. Before staining, sections were fixed in acetone, then rehydrated in buffered saline. Sections were then incubated with biotinylated mAb 14.4.4s (anti-I-E, prepared by Berkeley Antibody Co., Berkeley, CA). Antibody binding was revealed by subsequent incubations with avidin/peroxidase (ABC Elite; Vector Laboratories, Inc., Burlingame, CA) and 3'3'-diaminobenzidine. In some cases, the sections were counterstained with hematoxylin (Fig. 1).

For scoring of immune-mediated damage to pancreatic acinar cells in transgenic mice, slides were read by a pathologist, and scored according to the following grading system: 1+, little or no inflammatory infiltrates and <20% loss of acinar cells (Fig. 2 *a*); 2+, significant loss of acinar cells (>20%) with or without infiltrates (Fig. 2 *b*); 3+, >90% loss of acinar cells with or without infiltrates (Fig. 2, *c* and *d*). In the tables, each number represents the score from an individual animal. It should be noted that the acini in pancreas sections from unmanipulated EL-I-E transgenic mice do not look as uniform as those from nontransgenic mice (data not shown). This may reflect some nonimmune-mediated effects of hyperexpression of the class II MHC transgene (10–12, 15).

Results

Expression of Transgene I-E Exclusively in Nonlymphoid Tissues. Our objective was to direct expression of I-E^b (E^d_α/E^b_β) to the acinar cells of the pancreas with hybrid gene constructs produced by fusing the enhancer and promoter of the rat elastase 1 gene separately to the mouse E^d_α and E^b_β structural genes (see Materials and Methods). The elastase sequences used in the hybrid constructs have been previously demonstrated to be effective in specifically directing expression of heterologous structural genes to the acinar cells of exocrine pancreas (18). Two transgenic lines were selected for expansion and further study. The first line, 231-3, was initially generated on a (B6 × SJL)F_2 background and subsequently backcrossed separately to B6 or SJL. The second line, 415-1, was generated directly on an inbred B6 background. Analysis of mRNA and protein expression in both lines showed specific expression limited to pancreas (data not shown). Tissues found to be negative for Eα mRNA included liver, kidney, lung, spleen, and thymus. Stained cryostat sec-

tions demonstrated the presence of I-E only on acinar cells and not on duct cells, pancreatic islets, blood vessels, or connective tissue (Fig. 1 *a*). Furthermore, I-E protein could not be detected in thymus, spleen, LN, or Peyer's patches (Fig. 1 *b* and data not shown).

Tolerance of Transgenic T Lymphocytes In Vitro. Peripheral T lymphocytes and thymocytes from EL-I-E mice were studied in MLR for responses to I-E (Table I). We found that proliferative responses from EL-I-E T cells were much reduced relative to nontolerant controls, although a small detectable proliferative response was often detected. Further studies (described below) led us to believe that these proliferating cells were not strongly reactive to I-E, and might be characterized as producing a "sterile" MLR. That is, although the cells could proliferate in vitro, they could not generate effector function in vivo.

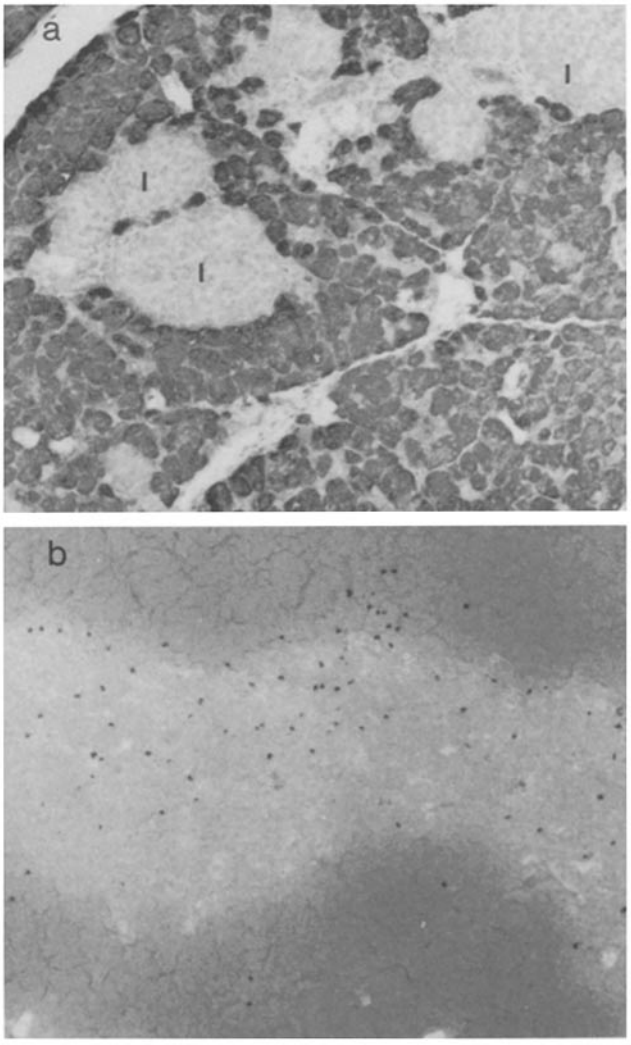

FIGURE 1. Expression of I-E molecules in EL-I-E mice. *a*, Pancreas; *b*, thymus. Cryostat sections of 415-1 EL-I-E mice were stained for I-E as described in Materials and Methods. Note expression of I-E limited to pancreatic acinar cells. No I-E was seen in pancreatic islets (*I*) or ducts, or in thymus. The granules in the thymus reflect endogenous peroxidase activity in granulocytes.

TABLE I
Primary MLR of EL-I-E LN T Cells and Thymocytes

Responders	Proliferative response to stimulators		
	B6(H-2^b)	B10.BR(H-2^k)	107-1(H-2^b + I-E)
LNT			
(B6 × SJL)F_1 control	0.4	39.8	4.1
EL-I-E (231-3)	0.3	52.4	1.4
Thymus			
(B6 × SJL)F_1 control	0.2	16.2	16.2
EL-I-E (231-3)	0.2	15.4	0.2

Tolerance in thymocytes and peripheral T cells in MLR. LN T cells and thymocytes from control (B6 × SJL)F_1 mice responded well to I-E stimulator 107-1, while cells from EL-I-E (231-3) mice responded only poorly. EL-I-E (231-3) mice were backcrossed to B6(H-2^b). Similar results were obtained from 415-1 mice (see also Table IX).

It was possible that I-E was expressed in the thymus of EL-I-E transgenic mice at levels below our limits of detection in mRNA and protein assays, but at levels sufficient to induce tolerance by clonal deletion. To address this possibility, we took advantage of the fact that one indicator of I-E expression in the thymus and the induction of intrathymic tolerance to I-E is the deletion of T cells using the TCR Vβ17a gene (identified by the mAb KJ23; reference 2). Although the actual specificity of TCRs using the gene is unclear (22), it is well established that expression of I-E in the thymus, especially by bone marrow-derived cells, causes a major reduction in the numbers of KJ23$^+$ T cells leaving the thymus (2, 23). Since the Vβ17a gene is not expressed in B6 mice, the transgenic mice (line 231-3) were backcrossed several generations to SJL/J to make them homozygous for the Vβ17a gene (23).

Peripheral LN T cells from I-E$^-$ control SJL/J, I-E$^+$ control 107-1 Eα transgenics backcrossed to SJL/J, and 231-3 EL-I-E transgenics backcrossed to SJL/J were stained with KJ23. As expected, ~9.8% (SE 2.6, n = 4) of SJL T cells stained with KJ23, while 2.5% (SE 1.7, n = 3) of the I-E$^+$ control 107-1 Eα transgenic T cells stained. The reduction in the number of KJ23$^+$ T cells in 107-1 transgenics was presumably due to clonal deletion in the thymus, mostly by I-E$^+$ bone marrow-derived cells (2, 23). Interestingly, T cells from EL-I-E mice were 8.6% (SE 0.9, n = 5) positive when stained with KJ23, indicating that there was no significant clonal deletion by I-E$^+$ cells in the thymus.

Finally, to confirm that the tolerant phenotype was independent of the presence of the transgene in cells of the immune system, the thymus and bone marrow-derived cells of transgenic 415-1 EL-I-E mice were replaced with nontransgenic tissue. This was achieved by adult thymectomy, grafting of a B6 neonatal thymus under the kidney capsule, and lethal irradiation and reconstitution using T-depleted B6 bone marrow (ATx/Thy/BM). 6–8 wk after reconstitution, T cells from these reconstructed mice were tested for reactivity to I-E in MLR (Table II, Exp. 1 and 2). As with the unmanipulated transgenic mice, T cells responded only poorly, if at all, to I-E$^+$ stimulator spleen cells. Similarly, B6→231-3 irradiation chimeras were also tolerant to I-E (Table II, Exp. 3). These results demonstrate that expression

TABLE II

Primary MLR of "Reconstructed" EL-I-E LN T Cells

Exp.	LNT responders	Proliferative response to stimulators		
		B6(H-2^b)	CBA/Ca(H-2^k)	107-1(H-2^b + I-E)
1	B6 control	0.4	26.4	6.0
	EL-I-E (415-1) ATx/Thy/BM*	0.4	47.9	1.0
2	B6 control	0.3	58.1	14.0
	107-1(H-2^b + I-E)	0.3	71.0	0.3
	EL-I-E (415-1) ATx/Thy/BM*	0.7	27.0	2.3
	EL-I-E (415-1) ATx/Thy/BM*	0.8	18.6	2.3
		B6(H-2^b)	B10.BR(H-2^k)	107-1(H-2^b + I-E)
3	B6 control	1.6	88.0	10.5
	B6 → (B6 × SJL)F_1	2.0	83.2	28.7
	B6 → 231-3	1.9	117.0	2.2

EL-I-E mice whose immune system was replaced with nontransgenic B6 tissue responded poorly to I-E. Although their responses were higher than responses by tolerant 107-1 T cells, there was no histological evidence of autoimmune attack of the EL-I-E pancreas. 6–8 wk later, LN T cells were tested in MLR against stimulators as noted above. In Exp.3, (B6 × SJL)F_1 and 231-3 EL-I-E mice were irradiated and reconstituted with T-depleted B6 bone marrow, and assayed 6–8 wk later.

* 415-1 EL-I-E mice were thymectomized, grafted with a neonatal B6 thymus, then lethally irradiated (900 rad) and reconstituted with 2 × 10^6 T-depleted B6 bone marrow (ATx/Thy/BM).

of I-E in pancreatic parenchymal cells alone can still induce tolerance in all peripheral T cells regardless of the presence of the transgene in lymphoid cells or thymus.

Immune Destruction of I-E^+ Tissue by Adoptive Transfer of Nontolerant T Lymphocytes In Vivo. Since transgenic animals were tolerant by MLR, they were clearly exposed to I-E in some tolerogenic form. No I-E was detected in the thymus, so it was possible that exposure to I-E in the periphery would result in tolerance. Evidence for tolerance induction to MHC antigens expressed by nonlymphoid cells has been reported previously (24). More recently, studies by a number of workers have suggested that inappropriate presentation of class II MHC antigens on T cells (25), as purified class II on planar membranes (26), as chemically fixed spleen cells (27), or on class II–expressing nonlymphoid cells (13, 28), can induce a state of clonal paralysis in T cells in vitro. Therefore, to see if peripheral tolerance could be induced in nontolerant T cells in vivo by exposure to the nonlymphoid I-E^+ acinar cells, B6 T cells were injected into irradiated EL-1-E (415-1) mice. The irradiation was intended to remove host lymphocytes so that the injected T cells could be recovered later and assayed for reactivity to I-E.

Mature nontolerant T cells were not tolerized by this adoptive transfer. Instead, they caused rapid destruction of the host transgenic I-E^+ exocrine pancreas (Fig. 2 *c*, Table III, and subsequent tables). T lymphocytes recovered from the animals responded well to I-E in MLR (Table IV). Cell depletion experiments established that the destruction of the I-E^+ pancreas depended on I-E-reactive T lymphocytes, since LN cells depleted of T cells did not cause pathology (Table III), and T cells from I-E^+ transgenic mice (tolerant to I-E) also had no effect (Table V).

The irradiation of the transgenic EL-I-E recipients may have contributed to the

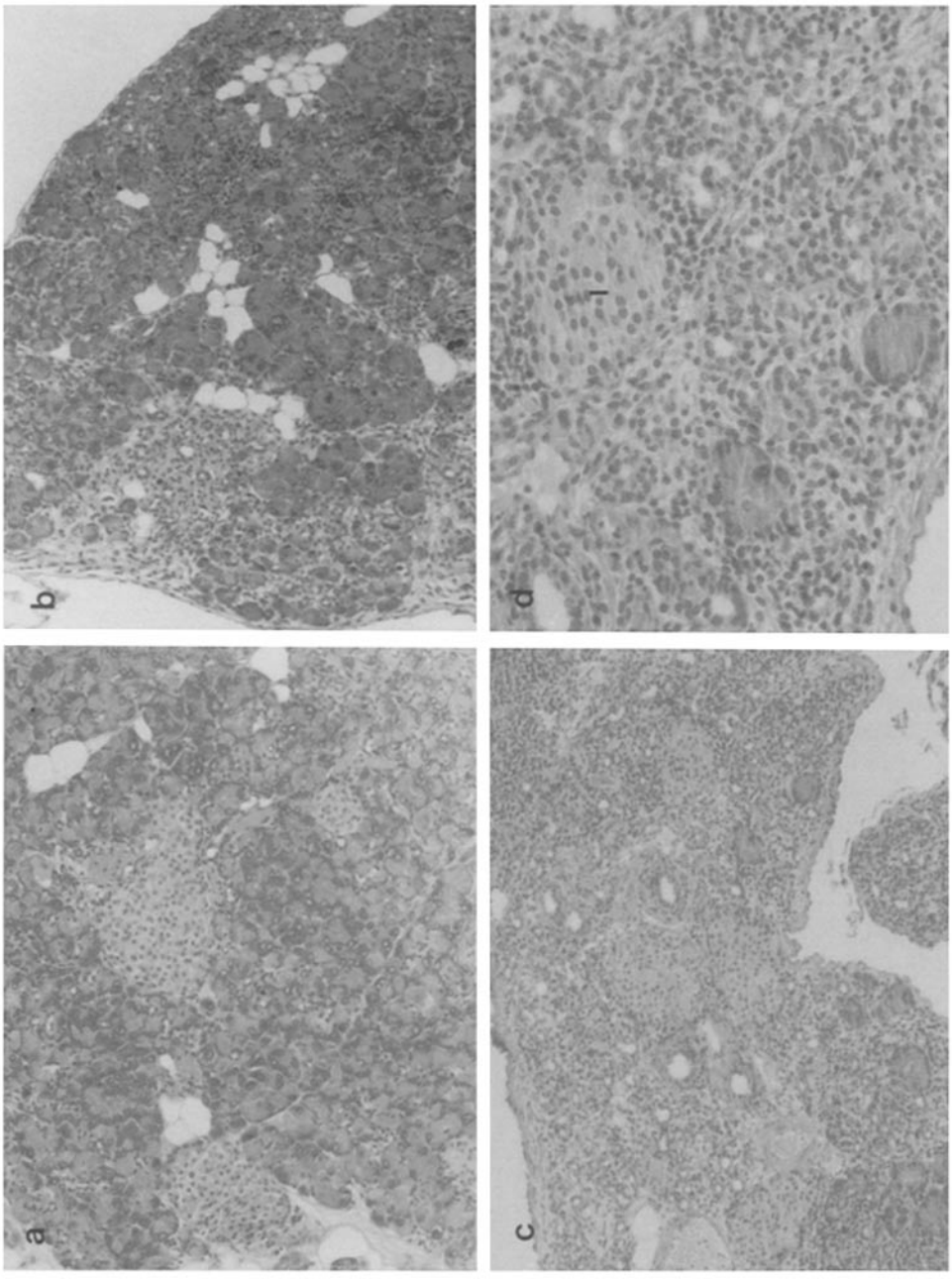

FIGURE 2. Grades used in histological analysis (see Materials and Methods). *a*, 1+; *b*, 2+; *c* and *d*, 3+. Note that although exocrine acinar cells are completely destroyed in *c* and *d*, pancreatic ducts, blood vessels, and endocrine islets are spared.

TABLE III

Transfer of Nontolerant T Cells into Irradiated EL-I-E Mice

Cells injected	Irradiation of recipients	Pathology*
	rad	
None	900§	1+,1+,1+
(B6 × SJL)F_1 LN (10^7)	900§	3+,3+,3+
(B6 × SJL)F_1 LN (10^6)	900§	1+,1+,2+
(B6 × SJL)F_1 anti-Thy-1 LN‡ (10^7)	900§	1+,1+,2+

Nontolerant T cells injected into irradiated EL-I-E mice induce pancreas destruction.

* Pathology graded as described in Materials and Methods.

‡ LN cells were depleted of T cells by treatment with anti-Thy-1 plus GPC.

§ Plus 3 × 10^6 ATBM.

pancreas destruction by setting up a nonspecific inflammatory process in the pancreas, leading to the nonspecific accumulation and activation of lymphocytes. Any paralysis-inducing capacity of the pancreas might be obscured by this possible source of costimulatory activity (29). Previous experiments suggested, however, that nonspecific inflammation could not provide effective costimulatory activity, since the inflammation associated with pancreas grafting did not induce rejection of INS-I-E islets (13). Furthermore, fixed spleen cells were still capable of inducing specific nonresponsiveness in vivo despite the possible presence of costimulatory activity (27). Nonetheless, to address this possibility, a group of EL-I-E mice was injected with nontolerant T cells 10 d after irradiation. By this time after irradiation, the nonspecific inflammation is gone (data not shown). In this situation, the pancreas was still destroyed (3/3 mice scoring 3+), suggesting that the nonspecific inflammation was not required for the destruction of the pancreas.

To remove host lymphocytes without using irradiation, a group of EL-I-E 415-1 mice were thymectomized, then treated with a combination of anti-Thy-1 antibody T24 with or without cortisone acetate (ATx/anti-Thy-1/cortisone, ATx/anti-Thy-1). This treatment has been shown to be effective at depleting T lymphocytes in vivo (20, 21). The mice were then injected with lymphocytes from either nontolerant B6 donors or I-E-tolerant 107-1 donors. Interestingly, here too the pancreas was specifically destroyed within 2 wk by B6 LN cells but not by 107-1 LN cells (Table V). To confirm that the destruction of the pancreas in this situation was due to I-E-reactive T lym-

TABLE IV

MLR of T Cells Recovered from Adoptive Transfer

	Proliferative response to stimulators		
LNT responders	B6	B10.BR	107-1
(B6 × SJL)F_1 control	0.3	57.4	7.0
Recip-1	0.5	42.9	11.4
Recip-2	0.6	35.0	4.4

Nontolerant T cells injected into irradiated EL-I-E mice are not tolerized to I-E. Recip-1 and Recip-2 were 231-3 EL-I-E mice given 900 rad then 4 × 10^7 F_1 plus BM. 4 wk later, LN cells were recovered and used as responders in MLR. In all EL-I-E recipients, the pancreas was destroyed (graded 3+).

TABLE V
Transfer of Nontolerant T Cells into EL-I-E Mice Depleted of T Cells without Irradiation

Exp.	Cells injected	Treatment of recipients	Pathology
1	None	ATx/anti-Thy-1/cortisone	1+,1+
	107-1 LN/spleen (10^8)	ATx/anti-Thy-1/cortisone	1+,1+
	B6 LN/spleen (10^8)	ATx/anti-Thy-1/cortisone	1+,3+,3+,3+
2	107-1 LN (6×10^7)	ATx/anti-Thy-1	1+,1+,2+
	B6 LN (6×10^7)	ATx/anti-Thy-1	1+,3+,3+

Nontolerant T cells injected into T-depleted EL-I-E mice destroy the pancreas, as seen in irradiated EL-I-E recipients. EL-I-E (415-1) mice were thymectomized, then treated with anti-Thy-1 with or without cortisone acetate (ATx/anti-Thy-1/cortisone or ATx/anti-Thy-1). 2 wk later, they were injected with nontolerant B6 or tolerant 107-1 cells as indicated, and pathology was assessed 2 wk later (see also Table IX).

phocytes, T cells were recovered from the ATx/anti-Thy-1/cortisone animals 2 wk after cell injection to assay for reactivity in MLR. As expected, T cells recovered from recipients of B6 cells responded very strongly against I-E$^+$ stimulators, while T cells recovered from recipients of 107-1 cells responded poorly (Table VI).

To determine if the presence of host T cells could inhibit the response of transferred nontolerant T cells, B6 T cells were injected into unmanipulated (no irradiation, no thymectomy, etc.) EL-I-E transgenic mice to see if these animals would also develop pancreatic infiltrates (Table VII). In this case the nonirradiated recipient animals did not develop infiltrates, and the pancreas was not destroyed within 2 wk. 4 wk after injection, the pancreas was still intact (Table VII, Exp. 1), indicating that there was no chronic immune response in these animals. Thus, it appeared that the presence of host T lymphocytes provided resistance to pancreas destruction.

TABLE VI
MLR of LN T Cells Recovered from ATx/Anti-Thy-1/Cortisone 415-1 Recipients

	Proliferative response to stimulators		
LNT responders	B6	B10.BR	107-1
B6	0.4	30.9	41.8
Recip Tol-1	1.1	41.0	1.2
Recip Tol-2	1.0	12.5	0.9
Recip Nontol-1*	0.2	4.2	0.2
Recip Nontol-2	9.1	14.9	23.7
Recip Nontol-3	3.1	15.7	42.9

LN T cells were recovered from the EL-I-E adoptive transfer recipients and assayed in MLR. Recip Tol-1 and Tol-2 were 415-1 EL-I-E recipients of tolerant 107-1 T cells (from Table V, Exp. 1, group 2). Recip Nontol-1-3 were from the same experiment group 3, recipients of nontolerant B6 T cells.

* The pancreas of Nontol-1 remained intact in contrast to the other three animals in that group, and appeared to be a failure of injected cells to survive (note poor response to B10.BR).

TABLE VII

Transfer of Nontolerant T Cells into Unmanipulated EL-I-E Mice

Exp.	Cells injected	Irradiation of recipients	Pathology
1	78-1 T cells (4×10^7)	None	1+,1+,1+ (2 wk)
	B6 T cells (4×10^7)	None	1+,1+,1+ (2 wk)
			1+,1+,1+ (4 wk)
	B6 T cells (10^7)	None	1+,1+,1+ (2 wk)
			1+,1+,1+ (4 wk)
2	B6 thymus (4×10^7)	None + 2×10^6 B6 ATBM	1+,1+,1+
	B6 thymus (10^7)	None + 2×10^6 B6 ATBM	1+,1+,1+
	B6 thymus (4×10^7)	900 rad + 2×10^6 B6 ATBM	3+,3+,3+

Nontolerant T cells injected into nonirradiated EL-I-E mice do not induce pancreas destruction.

Destruction of the pancreas in irradiated and T-depleted EL-I-E animals may have been mediated by donor T cells previously activated in the donor animals by an environmental antigen in the context of self I-A (self I-A plus X complex) that was crossreactive with I-E. Thymocytes were injected in another experiment (Table VII, Exp. 2), based on the possibility that thymocytes are less likely to have been activated by environmental antigens. The thymocytes had no effect on the pancreas of nonirradiated EL-I-E mice, but they still destroyed the pancreas of irradiated recipients.

It could be argued that the destruction of the I-E$^+$ acinar cells by injected nontolerant T cells might be due to some peculiar property of these exocrine cells. Therefore, similar experiments were also done using irradiated INS-I-E mice as recipients of nontolerant lymphocytes. INS-I-E transgenic mice specifically express I-E on endocrine islet β cells. In this situation, infiltrates were found specifically in pancreatic islets in a manner resembling the insulitis described in models of spontaneous autoimmune diabetes (Fig. 3).

Assays for Suppression In Vitro and In Vivo. Since the evidence suggested that host T cells in EL-I-E mice were responsible for resistance to pancreas destruction, it was possible that they may mediate the resistance through specific suppression. Cell mixing was tried to see if MLR responses to I-E would be suppressed by tolerant cells. T cells from EL-I-E mice mixed with nontolerant T cells did not suppress the response (Table VIII). To test for the presence of I-E-specific suppressor cells in vivo, mixtures of tolerant transgenic T cells and nontolerant T cells were injected into irradiated EL-I-E mice. Using 1:1 mixes, there was no evidence for suppression, as all groups injected with nontolerant T cells showed complete destruction of the I-E$^+$ pancreas (Table IX, Exp. 1).

It was possible that the suppressor cells needed to be "primed" by an injection of a large number of nontolerant T cells (30, 31). We attempted to prime suppressors in 415-1 EL-I-E mice by injecting 10^8 LN and spleen cells from nontolerant B6 mice. 2 wk later, spleen cells from the primed EL-I-E mice were used as a source of suppressors. Mixtures of spleen cells and nontolerant B6 LN cells were injected at 2:1 and 4:1 ratios into irradiated EL-I-E (415-1) recipients. Again, all injected groups showed complete destruction of the pancreas within 2 wk (Table IX, Exp. 2). This suggests that suppressor cells were not present in EL-I-E mice even after priming in vivo.

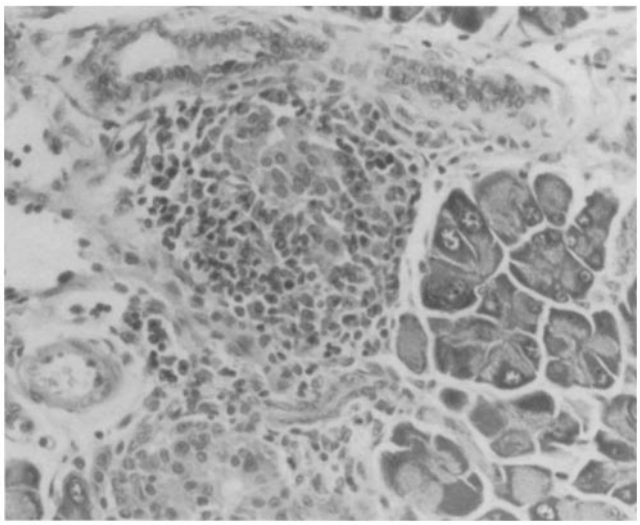

FIGURE 3. Islet infiltrates in INS-I-E mice after irradiation and injection of nontolerant LN cells. Note that infiltrates are specific for the I-E$^+$ islets rather than exocrine tissue.

Nontolerant T Cells Can Be Primed In Situ in EL-I-E Mice. Since cell mixing studies failed to provide direct evidence for specific suppression, we sought to identify other potential mechanisms to explain the failure of nontolerant T cells to destroy the pancreas of nonirradiated EL-I-E mice. It was formally possible that the injected cells died soon after injection. We do not believe this to be the case, however, since studies have demonstrated survival of a significant proportion of injected T cells, even across minor histocompatibility antigen differences (32, 33). In the studies described here using the 415-1 EL-I-E line, completely syngeneic B6 combinations (except for the transgene) were used. Considering this, we believed it more likely that the transferred nontolerant cells persisted in EL-I-E recipients, but were diluted out by the greater numbers of tolerant host T cells. Activation of some nontolerant T cells in the pancreas might be damped as the host cells would absorb the released lymphokines. Yet if the nontolerant cells remained in the animal, it was possible that a stronger stimulus would be sufficient to induce an effective immune response against the I-E$^+$ acinar cells.

We attempted to prime the injected nontolerant T cells in situ by two methods

TABLE VIII

Cell Mixing in MLR to Assay Suppression

	Proliferative response to stimulators		
LNT responders	B6	B10.BR	107-1
B6	0.9	24.7	34.1
415-1	0.7	39.9	3.0
B6 (2×10^5) + 415-1 (2×10^5)	4.7	-	38.8
B6 (2×10^5) + 415-1 (4×10^5)	22.7	-	52.8

Cell mixing in vitro fails to reveal suppression.

TABLE IX

Cell Mixing in Adoptive Transfer to Assay for Suppression

Exp.	Cells injected	Irradiation of recipients	Pathology
		rad	
1	B6 LN (5×10^6)	900*	3+,3+,3+
	415-1 LN (5×10^6)	900*	1+,1+,1+
	107-1 LN (5×10^6)	900*	1+,1+,1+
	B6 LN (5×10^6) + 415-1 LN (5×10^6)	900*	3+,3+,3+
	B6 LN (5×10^6) + 107-1 LN (5×10^6)	900*	3+,3+,3+
2	B6 LN (2×10^7)	900*	3+,3+,3+
	B6 LN (2×10^7) + primed EL-I-E spleen (4×10^7)	900*	3+,3+,3+
	B6 LN (2×10^7) + primed EL-I-E spleen (8×10^7)	900*	3+,3+,3+

Cell mixing in vivo fails to reveal suppression. In Exp. 2, suppressor donors (415-1 EL-I-E mice) were primed with 10^8 B6 LN/spleen intravenously. 2 wk later, spleen cells from these mice were mixed with B6 LN cells before injection into irradiated 415-1 EL-I-E mice.

* Plus 2×10^6 B6 ATBM.

(Table X). With the first method, I-E$^+$ spleen cells were coinjected intravenously with the nontolerant T cells. With the second method, I-E$^+$ spleen cells were injected subcutaneously into the rear footpads of mice injected the same day intravenously with nontolerant T cells. Control EL-I-E mice were injected with equivalent numbers of nontolerant T cells only. As expected from previous experiments (Table VII), the pancreata of the control animals remained intact at the end of 2 wk, without evidence of lymphocytic infiltrates. However, after priming by either protocol, the recipient EL-I-E pancreas was destroyed within 2 wk.

The results suggest that injected nontolerant T cells survive in nonirradiated EL-I-E recipients and remain receptive to priming to I-E antigens by I-E$^+$ spleen cells in situ. Destruction of the pancreas was probably due to activation of the injected

TABLE X

Priming of Adoptively Transferred Nontolerant T Cells In Situ

Exp.	Cells injected	Treatment of recipients	Pathology
1	B6 LN/spleen intravenously (10^8)	None	1+,1+,1+
	B6 LN/spleen intravenously (10^8)	107-1 spleen intravenously (2×10^7)	1+,3+,3+
	B6 LN/spleen intravenously (10^8)	107-1 spleen subcutaneously (2×10^7)	2+,3+,3+
2	B6 T cells (3×10^7)	None	1+,1+,1+,1+
	B6 T cells (3×10^7)	107-1 spleen intravenously (2×10^7)	1+,1+,1+,1+
	B6 T cells (3×10^7)	107-1 spleen subcutaneously (2×10^7)	1+,1+,2+,3+
3	B6 T cells (5.5×10^7)	None	1+,1+
	B6 T cells (5.5×10^7)	107-1 spleen subcutaneously (4×10^7)	3+,3+

Nontolerant T cells do not destroy EL-I-E pancreas when injected alone, but they can be induced in situ to attack the pancreas by coinjections of I-E$^+$ spleen cells. In all three experiments, B6 cells were injected intravenously. In some groups, I-E$^+$ 107-1 spleen cells were coinjected intravenously, while in other groups, 107-1 spleen cells were injected subcutaneously into the foo pads.

nontolerant T cells, since infiltrates were not found in EL-I-E mice primed with I-E$^+$ spleen but not injected with nontolerant T cells (data not shown). The activation of reactive T cells appeared to be more effective when the stimulator I-E$^+$ spleen cells were injected subcutaneously in the footpads rather than intravenously. This may reflect a requirement for a concentration of stimulator cells in a single LN. Interestingly, LN cells from donor animals primed to I-E by the same protocol did not destroy the pancreas when transferred to nonirradiated EL-I-E mice, even though primed cells generated a much greater MLR response to I-E than unprimed cells (data not shown). The number of nontolerant T cells injected appears to be important, since the priming induced pancreas destruction most effectively when 10^8 LN plus spleen or 5.5×10^7 LN T cells were injected as a source of nontolerant cells (Table X, Exp. 1 and 3) as compared with when only 3.0×10^7 LN T cells were injected (Table X, Exp. 2).

Discussion

From the studies presented here, we draw two major conclusions. First, mice expressing a transgene class II antigen exclusively in nonlymphoid tissues do not develop spontaneous autoimmune disease, and both thymocytes and peripheral T lymphocytes are tolerant assayed both by MLR and adoptive transfer studies. These results complement our previous observations on INS-I-E transgenic mice (12–14), suggesting that our conclusions may also be generalized in some manner to nontransgenic mice. That is, self antigens may be capable of inducing tolerance in T cells by associating with class II MHC antigens on nonlymphoid cells. The mechanism for inducing this tolerance is still unclear, but our in vitro studies with isolated I-E$^+$ islet cells from INS-I-E mice suggested that clonal paralysis of T cells may be an important factor (13, 14). Our second conclusion is that I-E-expressing parenchymal cells are susceptible to immune destruction by nontolerant T cells, but host T lymphocytes can provide some resistance to immune damage. This resistance is probably distinct from the tolerance induction described above.

It is important to note that the transgenic mice used in the present study were developed to address mechanisms of tolerance only to nonlymphoid organ-specific antigens, not to all self antigens nor to injected exogenous soluble antigens. Our results do not conflict with studies on tolerance to lymphoid self antigens, which most likely involves clonal deletion of reactive cells in the thymus (2–4). In addition, the adoptive transfer studies presented here were intended to specifically study whether peripheral tolerance-inducing mechanisms were present in the transgenic mice that might induce tolerance in transferred nontolerant T cells. Those studies do not necessarily bear on the question of why the unmanipulated transgenic mice were themselves already tolerant to the transgene I-E.

The transgenic T cells were actually tolerant to I-E by two criteria. First, they showed reduced reactivity in MLR. Second, they were functionally unable to generate an immune response to I-E$^+$ acinar cells in vivo, even after in vivo priming by I-E$^+$ spleen cells. Although the absence of dominant nonresponsiveness is consistent with clonal deletion in the thymus, T cells expressing Vβ genes associated with reactivity to I-E were not deleted from the T cell pool, suggesting that clonal deletion did not occur.

Clonal paralysis may be an explanation for the persistence of potentially autoreactive T cells, but it is still unclear how it occurs in vivo. It is possible that newly generated cells from the thymus are more susceptible to paralysis, but it seems unlikely that all of these T cells would encounter the transgene I-E expressed only in the pancreas. Pancreas destruction induced by T cells injected into irradiated or T-depleted EL-I-E mice clearly show that nontolerant mature T cells are not universally susceptible to paralysis in vivo. This is consistent with our previous observations that I-E$^+$ islet grafts did not induce tolerance in naive graft recipients in vivo, nor could they prevent immune destruction by I-E-primed T cells (13).

As noted previously, it is especially perplexing that thymocytes also appeared to be tolerant despite the absence of detectable I-E in the thymus. Our studies appear to have ruled out thymus expression of I-E as a cause of tolerance in EL-I-E T cells, since EL-I-E mice whose thymus and bone marrow had been replaced by nontransgenic tissue were still tolerant to I-E in MLR. One possible explanation is that I-E from the pancreas was transported back to the thymus (assuming that processed I-E is tolerogenic). Yet if processed I-E is at all similar to native I-E, we might have expected deletion of Vβ17a$^+$ T cells.

Alternatively, it may be possible that the thymocyte MLR was produced only by mature T cells that had returned to the thymus from the periphery. Thus, transgenic mature T cells may have encountered I-E in the pancreas, became paralyzed there, and returned to the thymic medulla. Although kinetic studies suggest that recirculation of T cells back to the thymus cannot account for the majority of mature phenotype T cells there (34), it has not been determined which cells respond in thymocyte MLRs. A number of studies have suggested that T cells, especially activated cells, are capable of returning to the thymic medulla (20, 33, 35), and such activated cells are probably very effective responders in MLR. Paralyzed cells may resemble activated T cells and may be equally capable of returning to the thymus, since it has been shown that encounter with tolerogenic class II can still induce cell volume increase and some lymphokine production (26).

When nontolerant T cells were transferred to irradiated or T-depleted EL-I-E or INS-I-E mice, the I-E$^+$ parenchymal tissue was destroyed. Destruction was probably mediated by T cells that were previously activated in the nontolerant donor animal by an environmental antigen in the context of self I-A (I-A^b plus X complex) that was crossreactive with I-E. Yet nontolerant T cells in recipients of INS-I-E islet grafts generally ignored the islets (13), and nontolerant cells did not destroy the pancreas in nonirradiated EL-I-E mice. T cell depletion experiments (Table V) indicated that host T cells were responsible for the resistance to pancreas destruction in EL-I-E mice. Ts cells might be invoked to explain this phenomenon, but numerous experiments based on conventional in vitro and in vivo assays suggested that resistance in EL-I-E mice is not maintained by specific Ts cells. First, dominant nonresponsiveness could not be demonstrated in either in vitro or in vivo cell mixing experiments. Second, "priming" for suppressors did not reveal dominant nonresponsiveness. Finally, nontolerant T cells injected into EL-I-E mice can still be activated by I-E$^+$ spleen cells in situ to destroy the pancreas, suggesting that host resistance can not interfere with the specific activation of nontolerant T cells to self antigens.

We propose an alternative explanation for the apparent T cell–mediated host resistance to pancreas destruction by injected nontolerant T cells. The injected non-

tolerant T cells were probably diluted by an excess of tolerant host T cells. Acting as an effective "lymphokine sink," these cells could absorb lymphokines produced by the small number of reactive cells and modulate the immune response. Since immune responses probably require the expansion and differentiation of T cells through the action of lymphokines, the absorption of lymphokines by tolerant cells may prevent the immune response from developing. In any immune response the vast majority of T cells are not specific for the antigen, so this effect might nonspecifically dampen any immune response. However, it could be overcome through at least two mechanisms. First, the precursor frequency of reactive cells may be high enough such that the response cannot be damped. Second, the stimulus may be enhanced by concentrating the antigen in a LN or by increasing the effectiveness of the stimulator cells (as with the injected I-E$^+$ spleen cells; Table X).

The absence of lymphokine absorption capacity could then explain why tissue destruction occurs in T-depleted or irradiated hosts. Lymphopenia probably allows the development of immune responses when the precursor frequency to a particular antigen is very low and antigen presentation is suboptimal. In the absence of a large number of T cells to absorb excess lymphokines, a small number of reactive cells may develop into effector cells through autocrine stimulation.

Observations on a number of rodent models of autoimmunity to organ-specific antigens appear to fit well in this context. For example, spontaneous autoimmune diabetes in the BB rat is associated with lymphopenia (36). Lymphopenia is also a major feature of a whole complex of organ-specific autoimmunity induced by neonatal thymectomy. Day 3 thymectomized mice can develop a whole spectrum of spontaneous autoimmune diseases involving the thyroid, gonads, gastric mucosa, and prostate (37–39). The demonstrated ability to transfer resistance to disease in these models might be interpreted in part as an augmentation of the immune system's capacity to absorb lymphokines.

Given the above hypothesis, a number of testable predictions can be made. For example, the ability of cells to confer resistance to autoimmune destruction may best correlate with their capacity to bind lymphokines. Paralyzed T cells with the phenotype described by Schwartz and others (25–27, 29) may be one type of cell ideal for this function, since these cells can recognize antigen, and may respond by increasing lymphokine receptors. If specific immune responses are damped by absorption of lymphokines by T cells of irrelevant specificity (or paralyzed specific cells), then the absorbed lymphokines might induce a higher state of activation in the entire immune system. Such a phenomenon has already been described in the case of neonatal tolerance to lymphoid antigens (40). Thus, injection of nontolerant T cells into nonirradiated EL-I-E mice may induce a greater proportion of blast T cells in LN draining the pancreas. We are currently addressing these possibilities.

Summary

To study the nature of tolerance to antigens not expressed by cells of the lymphoid system, expression of class II MHC I-E was targeted to the acinar cells of the exocrine pancreas in transgenic mice (elastase [EL]-I-E). Despite the absence of detectable I-E in the thymus of EL-I-E transgenic mice, both thymocytes and peripheral T lymphocytes were tolerant to I-E, and the pancreas was free of autoimmune infiltrates. Nontolerant T cells adoptively transferred into irradiated or T-depleted

transgenic mice rapidly destroy the I-E$^+$ components of the pancreas; however, adoptive transfer of nontolerant T lymphocytes into nonirradiated transgenic mice do not. These results suggest that tolerance in transgenic mice is maintained by some peripheral tolerance mechanism. However, further studies indicate that tolerance in transgenic mice is not maintained by specific Ts cells. For example, cell mixing experiments both in vitro and in vivo fail to reveal dominant unresponsiveness. Furthermore, nontolerant T cells injected into otherwise unmanipulated EL-I-E mice can be primed in situ (by injections of I-E$^+$ spleen cells) to destroy the I-E$^+$ acinar cells.

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