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Tolerence toward a novel beta-cell antigen : insights into tissue specific tolerance / Kathleen Marie Smith

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Tolerance toward a Novel Beta-cell Antigen:

Insights into Tissue Specific Tolerance

by

Kathleen Marie Smith

DISSERTATION

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Dedication

I dedicate this work to my husband Hal,
whose unwavering faith in my abilities helped me believe in myself,
and who always reminded me to laugh.

Acknowledgments

I would like to thank my thesis advisor, Doug Hanahan, for allowing me the freedom to pursue my own interests and for providing me the opportunity to develop my talents as a scientist. I would like to thank my thesis committee members, Art Weiss and David Raulet, for providing direction and focus to an immunology project performed in a non-immunology lab. I would like to thank my collaborators, Christine Jolicoeur and Ryo Hirose, whose interest in this work made it possible. I would like to thank the members of the Hanahan lab, past and present, for their companionship and insights. And finally I would like to thank the members of the Hormone Research Institue and the immunology community at UCSF for their support and encouragement throughout my tenure at UCSF. Chapter 2 of this dissertation is a reprint of the article "T-cell Tolerance Toward a Transgenic ß-cell Antigen and Transcription of Endogenous Pancreatic Genes in the Thymus" as it appears in the Proceedings of the National Academy of Sciences, USA.

October 16, 1995

To Whom it May Concern:

I am writing to clarify the contributions of Kathleen M. Smith to a paper included in her thesis in which she is listed as the senior author, rather than as the first author. That paper is entitled, "T-cell tolerance toward a transgenic ß-cell antigen and transcription of endogenous pancreatic genes in thymus", by C. Jolicoeur, D. Hanahan, and K. M. Smith which was published in *PNAS* in 1994. This project was initiated by Christine Jolicoeur when she joined my laboratory as a postdoctoral fellow while I was still at Cold Spring Harbor Laboratory. Christine began to set up the basic assays to characterize the T-cell tolerance we suspected in the Rip Tag transgenic mice. When Kathy Smith joined the lab she began working with Christine on the characterization of the tolerant phenotypes. That work was done jointly. The realization from these results that this rare cell type in the pancreas was conferring systemic tolerance then led Kathy to ask a profound biological question. Namely, might the thymus be expressing the transgene, since how else could one explain the systemic tolerance we observed? Kathy then went on from the foundation that she and Christine had set to perform a difficult and provocative experiment which has revealed that the Rip Tag transgene is indeed expressed in the thymus. Moreover, Kathy then discovered that a series of endogenous pancreatic genes are also expressed at low but detectable levels in the thymus. This is an exciting result which raises new questions about the concept of so-called peripheral tolerance toward rare cell types. Kathy's discovery suggests that many genes expressed by rare cells may be expressed transiently, or at low levels, in the thymus so as to begin the process of educating developing T-cells toward self-antigens. This concept represents by far the most interesting and intriguing part of the paper noted above. The dilemma came in assigning credit. Christine Jolicoeur had begun this project single-handedly. This was to be her only first author paper from my laboratory. We therefore debated submitting Kathy's discovery of the thymic expression of the transgene and the endogenous pancreatic genes as a separate short letter, with her as first author. However, Kathy decided that the two parts were not as strong as the combination and elected for a compromise in which the tolerance assays that she and Christine were performing would be included in the same paper as the discovery of thymic expression, which Kathy was solely responsible for. We agreed that Kathy should be senior author as a measure of the importance of her contribution to this project. It is arguable that she should have been first

author, but in balance our decision was to make Kathy last author. Yet I wish to emphasize that Kathy made seminal contributions to this work and is rightfully proud of her contributions to it. As such, I believe that this manuscript is an appropriate and completely justifiable component of Kathy's thesis, and is presented as such.

Sincerely yours,

Douglas Hanahan

DH/pk

Tolerance toward a Novel ß cell Antigen: Insights into Tissue Specific Tolerance.

by Kathleen M. Smith

The immune system recognizes a vast array of antigens, while many self proteins fail to elicit immune responses, thus motivating the study of tolerance to tissue specific proteins. The rat insulin promoter (RIP) directs expression of the SV40 T antigen (Tag) to the pancreatic ß cells in RIP-Tag mice. RIP-Tag mice develop one of two heritable immunological phenotypes depending on the transgene insertion site into the genone. Mice from embryonic onset families develop tolerance toward T antigen. In other lines, T antigen expression is delayed until adulthood, resulting in non-tolerance and autoimmunity. The tolerance of lymphocyte subsets was characterized in embryonic onset RIP-Tag mice. This study revealed that tolerant $CD4^+$ T cells limit the ability of non-tolerant B cells to produce antibody to T antigen, while $CD8^+$ T cells only mount weak helper-independent cytotoxic responses to T antigen. T antigen RNA was detected in thymus of tolerant, but not non-tolerant RIP-Tag mice, thus correlating with tolerance induction. Surprisingly, thymic T antigen expression recapitulates that of the endogenous insulin genes. Additionally, mRNA's from other endogenous genes, expressed in both endocrine and exocrine cells of the pancreas, were found intrathymically. To assess the contribution of thymic T antigen expression to the development of tolerance, the immune system of athymic mice was reconstituted with transgenic thymus. T antigen expressed by the thymus was sufficient to induce tolerance of $CD4^+$ T cells, but not of $CD8^+$ T cells. Thus, an additional encounter with antigen

in the periphery may be required for tolerance induction of class I MHC restricted $CD8^+$ T cells. The identity of the cells inducing tolerance of $CD4^+$ T cells was investigated. Immunohistochemical and cell fractionation studies suggest that T antigen is expressed by thymic dendritic cells. Moreover, message for several endogenous pancreatic genes is found in the same population of cells. Thus, thymic expression of 'peripheral' tissue specific proteins may play a role in limiting reactivity to proteins expressed in rare cell types, such as the pancreatic ß cell, the target of autoimmune destruction in type I diabetes.

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CHAPTER 1:

Introduction

The immune system has evolved to fight off pathogens: viruses, bacteria, and parasites. T and B lymphocytes, the antigen specific effectors of the immune system, must express a highly diverse set of receptors to recognize these foreign invaders, which they have never seen. To do this, antigen specific receptors are assembled from multiple gene segments and displayed on the surface of the immature lymphocyte. Only those cells bearing receptors useful for the organism are selected for further development. These two classes of lymphocytes, T and B cells, recognize different forms of antigen. The B cells see soluble or cell surface proteins, while T cells interact with peptide fragments, potentially from the same molecules, bound in the antigen binding groove of major histocompatiblity (MHC) molecules.

The majority of T cells develop in the thymus. During thymic maturation, thymocytes with antigen specific receptors which can bind to self-MHC-peptide complexes receive a signal to proceed along their developmental pathway. Those that fail this test do not survive. This process is known as positive selection. In this way, T cells, which can recognize the MHC molecules present throughout the organism, are selected. A consequence of this selection process is that some of the developing T cells will have a high affinity for self peptides presented by those MHC molecules. A second process, know as negative selection, limits this potential hazard. Immature T cells go through a screening process whereby those with high affinity for peptide MHC combinations found in

the thymus receive a signal to die. Thus, one process, positive selection, serves to restrict the T cell repertoire to cells bearing receptors that can bind to self MHC, while a second process, negative selection purges the repertoire of potentially autoreactive T cells (Robey and Fowlkes, 1994). In the end, a mature T cell repertoire is created which is capable of recognizing many foreign antigens, presented as peptides by self MHC antigen presenting molecules.

Negative selection occurs for antigens which are found in the thymus (Nossal, 1994). These include soluble proteins present in the bloodstream and antigens expressed in the thymus. But what about tissue specific proteins expressed by rare cell types? Is tolerance to these ever achieved? The concept of peripheral tolerance was put forward to explain the lack of reactivity to these potential autoantigens.

The Study of Peripheral Tolerance

The bulk of the work on tolerance to tissue specific antigens has not taken into account the interactions of different lymphocytes populations in the organism. Nossal (1994) argues that to understand tolerance it is essential to examine the response of the different lymphocyte types, $CD4^+$ and $CD8^+$ T cells and B cells, since they interact in an immune response and regulate each other's function. For example, B cells present antigens to $CD4^+$ T cells, while help from $CD4^+$ T cells is required for the B cells to produce antibody to protein antigens. Although $CD8^+$ T cells can mount helper-independent cytotoxic responses to many antigens (Doherty, 1993), antigen

specific $CD4^+$ T cells can secrete cytokines which promote cellular immune reactions and allow for the optimal development of a cytotoxic T cell response.

Another consideration is the different ways in which $CD4^+$ and $CD8^+$ T lymphocytes interact with antigen. Although both types of T cells recognize peptide antigens presented by MHC molecules, $CD4^+$ T cells see antigenic peptides in the groove of MHC class II molecules, while $CD8^+$ T cells see a different set of peptides presented by MHC class I molecules. MHC class II molecules are present on specialized antigen presenting cells, such as macrophages and dendritic cells, and display peptides derived from the endocytic pathway. On the other hand, MHC class I molecules are present on most cells in the body, and bind peptide antigens derived from cytoplasmic proteins. The general rule that class I binds endogenous peptides and class II binds exogenous peptides is a function of their biosynthetic pathways (Germain and Margulies, 1993). Of course, exceptions to this rule exists, but can usually be explained by the ability of the antigen in question to gain access to the peptide loading compartment. These differences in distribution and antigen processing requirements for MHC class I and II molecules results in different sets of peptide antigens being presented for recognition by $CD4^+$ and $CD8^+$ T cells.

The advent of transgenic technology has made the study of peripheral tolerance more accessible (Miller and Flavell, 1994). One general approach involves the use of a tissue specific promoter to direct the expression of a novel self antigen to a particular tissue in transgenic mice. In some studies these mice have been directly analyzed for reactivity to the novel self-antigen. More often, they have been bred to a second line of transgenic mice

which express a particular T cell receptor gene pair. In these T cell receptor transgenic mice, the majority of the T cells recognize a peptide derived from the novel tissue specific protein when presented by a specific MHC molecule. In these latter studies, the read-out was made more simple, since the majority of the T cells were of a single specificity and could analyzed directly from the doubly transgenic mice without immunization. A recent study has revealed a limitation of using T cell receptor transgenic mice in the study of peripheral tolerance (Förster et al., 1995). When the majority of the T cells expressed a single T cell receptor, 80-90% as in the previous studies, the tolerization machinery was overwhelmed and the double transgenic mice were non-tolerant of the neo-self antigen. However, when only 10% of the T cells expressed the same receptor, still a very high percentage for a single T cell receptor, the transgenic T lymphocytes were rendered tolerant of the novel tissue specific antigen. Thus, the abundance of T cells capable of interacting with a peripheral protein can influence the experimental outcome. Since normal mice possess a low percentage of T lymphocytes capable of recognizing a given antigen, less than 0.1% of the total T cell pool, the interpretations of previous work employing T cell receptor transgenic mice to study peripheral tolerance needs to be revisited.

Another difficulty encountered in the study of peripheral tolerance using transgenic technology has been that many of the transgenic self antigens were also expressed in the thymus, making it difficult to conclude that the tolerant phenotype observed was due to peripheral expression. In one case the thymic expression was attributed to the expression of the keratin gene promoted transgene in thymic epithelium (Husbands et al., 1992). However, in the majority of the cases the reasons for the thymic expression

pattern was not examined. In one study where the peripheral antigen was not expressed in the thymus, the novel self antigen was ignored by the immune system (Ohashi et al., 1991).

These limitations have motivated the design of several experimental systems, using both transgenic and non-trangenic approaches, to address the question of whether extrathymic tolerance actually exists. In these systems, high levels of soluble antigen, either superantigen, the male specific antigen HY, or a virus, were introduced into a mouse with a mature peripheral immune system (Webb et al., 1990; Rocha and von Boehmer, 1991; Moskophidis et al., 1993). In each case the antigen specific T cell population initially expanded, and then the majority of the reactive T cells disappeared. This phenomenon has been called peripheral exhaustion. Although these studies provide evidence for induction of tolerance of mature peripheral T cells to foreign antigens, they do not address the fate of T cells recognizing tissue specific antigens in rare cell types. One study has documented the occurrence of extrathymic tolerance to a transgene product produced by the pancreatic ß cells, a rare cell type, approximately 5×10^5 cell per mouse (Lo et al., 1992). Transgenic mice expressing influenza hemagglutinin under the control of the rat insulin promoter, were thymectomized, lethally irradiated, and reconstituted with non-transgenic bone marrow and a non-transgenic thymus. After reconstitution of the immune system, these mice were unable to mount a cytotoxic T cell response to hemagglutinin. However, this observation has not been followed up. Although peripheral tolerance has been much studied in recent years, an understanding of how the immune system avoids developing spontaneous reactivity toward tissue specific proteins has not been reached.

Initial Studies of RIP-Tag Transgenic Mice

I have chosen to study the immune response to a transgenically expressed ß cell antigen. In these mice the rat insulin promoter (RIP) directs the expression of the SV40 T antigen (Tag) to the pancreatic ß cells. These mice eventually develop ß cell tumors due to the expression of the oncogenic T antigen. RIP-Tag mice have been shown to develop one of two alternative immunological phenotypes depending on the onset of T antigen expression (Adams et al., 1987). Certain families of mice, the prototype being RIP1-Tag2, begin expressing T antigen during embryonic life, concomitant with the endogenous insulin genes (Alpert et al., 1988). These mice were shown to develop tolerance to T antigen, as demonstrated by their inability to produce antibody to T antigen after immunization (Adams et al., 1987). This tolerance is quite profound, since these mice do not develop an immune response to their T antigen expressing tumors. A second embryonic onset lineage RIP3-Tag2 differs from RIP1-Tag2 by using a longer version of the rat insulin promoter.

In other families of mice, T antigen is first detected in adulthood, at approximately 10 weeks of age. The expression of the transgene in these developmental onset lineages is delayed until adulthood, due to a chromosomal position effect at the transgene insertion site (Grant et al., 1991). These mice fail to establish tolerance and instead develop spontaneous autoimmunity. This autoimmune response is characterized by production of autoantibodies to T antigen (Adams et al., 1987; Skowronski et al., 1990) and infiltration of their T antigen expressing islets by $CD4^+$ and $CD8^+$ T cells, B cells, and macrophages (Skowronski et al., 1990). Mice from

the delayed onset lineage RIP1-Tag5 have a similar immunological phenotype to that of RIP1-Tag3 which has been previously described (Skowronski et al., 1990), except that the genetic background of RIP1-Tag5, inbred into C3HeBFe/J, allows for a greater penetrance of the autoimmune phenotype. RIP1-Tag5 mice first express T antigen in scattered pancreatic ß cells, in a few islets, at nine to ten week of age. By the time they are 15 weeks of age, T antigen expressing ß cells can be found in 50 to 70 percent of the islets and in a greater percentage of ß cells within an islet. Circulating autoantibodies to T antigen can be detected in serum, and infiltration of the T antigen expressing islets has begun (C. Jolicoeur, unpublished observations). These delayed onset RIP-Tag mice use the same transgene construct as embryonic onset RIP1-Tag2 mice (Hanahan 1985, Adams et al., 1987).

The availability of this set of transgenic mice, which employ the same transgene construct, but develop different immunological fates, provides the unique opportunity to study the requirements for induction of tolerance and the development of autoimmunity to the same protein expressed in the same rare cell type. In the studies described here, I have focused on the characterization of two of the tolerant lines of mice, RIP1-Tag2 and RIP3-Tag2, and one delayed onset lineage RIP1-Tag5.

CHAPTER 2:

T-cell Tolerance Toward a Transgenic ß-cell Antigen and Transcription of Endogenous Pancreatic Genes in the Thymus

INTRODUCTION

RIP-Tag mice from embryonic onset families were shown to have an impaired ability to produced antibody recognizing T antigen after immunization relative to non-transgenic control mice (Adams et al., 1987). This non-responsiveness to T antigen was attributed to the embryonic onset of T antigen expression in the pancreas, since mice which first express T antigen in their ß cells only in adulthood are competent to produce high titers of antibody recognizing T antigen. The inability of the embryonic onset RIP-Tag mice to produce anti-T antigen antibody may reflect tolerance of the T or of the B lymphocytes. In collaboration with Christine Jolicoeur, I undertook to characterize the tolerance of the T lymphocytes from RIP1-Tag2 and RIP3-Tag2 mice, two embryonic onset lineages. I established a T cell proliferation assay as a measure of $CD4^+$ T cell responsiveness and developed an ELISA assay to test the capacity of RIP3-Tag2 mice to produce T antigen specific antibody. Christine developed a cytotoxic T cell assay to measure the tolerance of the $CD8^+$ T cells. Using these assays we showed that the T lymphocytes in mice from both lineages were tolerant of T antigen. To determine the amount of T antigen which was responsible for inducing this tolerance, Christine quantitated the amount of T antigen protein produced in the pancreas of both RIP1-Tag2 and RIP3-Tag2 mice. In parallel I developed an RNA-PCR assay to examine the expression of T antigen throughout development in pancreas, the main site of T antigen

production, and to investigate whether the thymus, the site of T cell maturation and central tolerance induction, may also express T antigen. Since I detected low levels of T antigen RNA in perinatal thymus from both lines of mice, I used this same assay to show that several endogenous genes, including insulin, which are normally expressed by both endocrine and exocrine cell types of the pancreas, as also expressed in the thymus. Thus, I demonstrated that the expression of T antigen in the thymus is not an artifact of the transgenic system, but in fact is directed by the insulin promoter. Additionally, the detection of RNA for endogenous pancreatic genes in the thymus raises the possiblity that the thymus may play a role in limiting autoreactivity to proteins prodcued by rare cell types. This initial characterization of these two lines of embryonic onset RIP-Tag mice resulted in the publication of the article "T-cell Tolerance Toward a Transgenic ß-cell Antigen and Transcription of Endogenous Pancreatic Genes in the Thymus" which is reprinted in this chapter.

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Immunology

T-cell tolerance toward a transgenic β-cell antigen and transcription of endogenous pancreatic genes in thymus

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ABSTRACT **Transgenic mice expressing T antigen (Tag) in pancreatic β cells establish systemic tolerance toward this self-protein. The self-tolerance in two families of rat insulin promoter (RIP)-Tag mice, expressing different levels of Tag protein, has been characterized. These mice have impaired antibody responses to Tag, show diminished Tag-specific T-cell proliferation, and evidence an inability to generate Tag-specific cytotoxic T cells. The existence of systemic tolerance toward a β-cell-specific protein motivated examination of transgene expression in the thymus. Indeed, low levels of Tag mRNA were detected intrathymically. Remarkably, this expression is a valid property of the insulin gene regulatory region, since insulin RNA was also expressed in the thymus of nontransgenic mice. RNA for other pancreatic genes was also detected in the thymus, thus raising the possibility that many tissue-specific genes could be expressed intrathymically during immunological development and induction of self-tolerance. These results raise important questions for future research into the role of the thymus in tolerance induction toward so-called tissue-specific antigens.**

Much of our knowledge about the cellular mechanisms of self-tolerance comes from studies of the interaction of T cells with major histocompatibility antigens (1–3). It is evident from these studies that the thymus is the primary site for induction of self-tolerance, wherein mechanisms including programed cell death and functional inactivation serve to eliminate self-reactive T lymphocytes. Yet the existence of organ-specific autoimmunity suggests that tolerance toward proteins with restricted patterns of expression may be established by alternative mechanisms (3, 4). Many recent studies have focused on the concept of "peripheral tolerance," whereby nonresponsiveness toward organ-specific antigens is proposed to be elaborated extrathymically, in mature T cells. In one approach, transgenic mice expressing novel antigens in a restricted pattern have been used to study peripheral tolerance (4–6). For example, the insulin gene regulatory region has been utilized to target a variety of antigens to the pancreatic β cell in transgenic mice (7–12). The β cell, a rare cell type localized within the pancreatic islets, is the target cell for immune-mediated destruction in type I diabetes.

The initial demonstration that transgenes utilizing the rat insulin promoter (RIP) could be used to study interactions of the β cell with the immune system came from studies on a viral oncoprotein, simian virus 40 (SV40) T antigen (Tag). Lines of RIP-Tag mice showed two alternative immunological phenotypes, tolerance, or nontolerance and spontaneous autoreactivity (8), which correlated with distinct patterns of transgene expression. The tolerant mice began to express Tag in islet cell progenitors concomitant with activation of the endogenous insulin genes during embryogenesis (13). In contrast, the nontolerant mice activated the transgene in adulthood (8), resulting in failure to establish tolerance and spontaneous autoimmunity (14). With regard to the developmental-onset RIP-Tag mice, previous work documented humoral tolerance toward Tag and a lack of leukocyte infiltration of the pancreatic islets (8, 15). Several other studies that targeted viral antigens to the β cells illustrate the diversity of possible immune responses to this rare cell type (9–12).

In the present study we have characterized T-cell responsiveness in two developmental-onset lines of RIP-Tag mice that express distinctive amounts of Tag. Remarkably, humoral, proliferative, and cytotoxic responses toward this β-cell antigen are dramatically impaired. The existence of profound systemic T-cell tolerance raises a perplexing question: How is it that T cells throughout the body are rendered nonresponsive toward a pancreatic islet cell antigen? This dichotomy provoked examination of transgene and endogenous insulin gene expression in the developing thymus. We report that the RIP-Tag transgene is expressed at low levels in the thymus. Notably, this expression pattern recapitulates that of the endogenous insulin genes. Moreover, several other pancreatic genes are also transcribed intrathymically, which implicates the thymus in establishment and maintenance of tolerance to these "peripheral proteins."

MATERIALS AND METHODS

Mice. RIP1-Tag2 mice have been described (7). To generate the RIP3-Tag construct, a 9.5-kb *Eco*RI fragment 5′ to the *Eco*RI site at −451 bp was used to replace the sequences between −695 bp and −451 bp in the original RIP1-Tag construct. This construct was injected into B6D2F2 embryos. The RIP1-Tag2 mice studied were backcrossed between 9 and 12 generations to C57BL/6 and the RIP3-Tag2 mice between 6 and 8 generations. C57BL/6, B6D2F1, and BALB/c mice were purchased from either The Jackson Laboratory or Bantin & Kingman (Fremont, CA). Male and female mice were studied and were immunized at 6 weeks of age.

T Antigen Protein. Tag was purified from Sf9 or High Five (Invitrogen) insect cells infected with a baculovirus expression vector carrying the SV40 early region (16) by affinity chromatography (17).

Antibody Response. Mice were primed i.p. with 10 μg of Tag and 5 μg of β-galactosidase (β-gal) in complete Freund's adjuvant (CFA), given booster immunizations 14 days later with the same amount of protein in incomplete Freund's adjuvant, and bled on day 20. Sera were tested in a standard ELISA. Briefly, plates were coated with 10 μg of Tag or β-gal

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Abbreviations: RIP, rat insulin promoter; SV40, simian virus 40; Tag, SV40 T antigen; β-gal, β-galactosidase; CTL, cytotoxic T lymphocyte; RT, reverse transcriptase; LCMV, lymphocytic choriomeningitis virus; GP, glycoprotein; GAD65 and GAD67, two isoforms of glutamic acid decarboxylase; β_2m, β_2-microglobulin.

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per ml and blocked with 1% bovine serum albumin. Serial dilution of sera and positive control antibodies, 419 (7) and Z378 (Promega), were tested. Plates were developed with biotinylated goat anti-mouse IgG (Fisher), streptavidin-horseradish peroxidase (Vector Laboratories), and *o*-phenyldiamine (Zymed).

Proliferation Assay. This assay has been described (18). Briefly, female mice were immunized s.c. at the base of the tail with 25 or 50 μg of Tag protein in CFA. Ten days later lymph node cells were cultured in 96-well flat-bottomed plates in HL-1 medium (Ventrex Laboratories, Portland, ME) in triplicate. Wells containing 4×10^5 cells and serial dilutions of Tag protein, nonviable desiccated *Mycobacterium tuberculosis* H37RA (Difco), or medium alone were incubated at 37°C for 72 hr and pulsed with 1 μCi of [^{3}H]thymidine the last 6 hr (1 Ci = 37 GBq). Plates were harvested and counted on a Betaplate Reader (Pharmacia LKB).

Cytotoxic T Lymphocyte (CTL) Assay. This assay has been described (19). Briefly, mice were immunized i.p. with 2×10^7 C57SV, SV40-transformed fibroblasts from C57BL/6 mice. Seven to 10 days later, 2×10^7 spleen cells were cultured with 2.5×10^5 irradiated (3000 rads; 1 rad = 0.01 Gy) C57SV or 5×10^7 irradiated (3000 rads) BALB/c spleen cells. CTLs were tested using either a ^{51}Cr release assay (19) or a DNA fragmentation assay (20) on C57SV or control allogeneic P815 (American Type Culture Collection) H-2^d targets.

RNA Preparation and cDNA Synthesis. Tissues were removed from mice at various ages. To prevent potential cross-contamination of samples, dissecting instruments were sequentially rinsed in 2 M NaOH, autoclaved water, and ethanol between samples. RNA was prepared using the RNAzol (Tel-Test, Friendswood, TX) method. Aliquots (3 μg) of RNA were treated with DNase and split in half. cDNA was synthesized under standard conditions using random primers. One tube received buffer (−RT) and the other buffer plus Moloney murine leukemia virus reverse transcriptase (BRL) (+RT). For the dilution series, pancreatic RNA from a newborn RIP1-Tag2 mouse was diluted into thymus RNA from an adult C57BL/6 mouse.

Detection of RNA in Pancreas and Thymus. RNA-specific fragments were amplified from cDNA using *Taq* DNA polymerase in reaction mixtures spiked with 5 μCi of [α-^{32}P]-dATP. Amplification was carried out for 30 cycles of 1 min at 95°C, 30 s at 60°C, and 2 min at 72°C. The primers for Tag (21), β_2-microglobulin (β_2m) (22), and pancreatic genes (23) have been reported, except for trypsin (GATTCTGCCAAGATCATCCG and GTATACACCAGGAGCATCTG), elastase (TCCACGGTGAAGACGACCATG and GGCAATGACATTGTTCATCCA), and two isoforms of glutamic acid decarboxylase, GAD65 (CCTGGTGAGTGCCACAGCTG and CTGGCGCCACCTTTGAGAGG) and GAD67 (TGCAACCTCCTCGAACGCGG and CCAGGATCTGCTCCAGAGAC). For all primer sets, the two primers are in different exons. Eight percent polyacrylamide gels were run, fixed, dried, and used to expose x-ray film.

Western Blotting. Pancreata from 1-week-old mice were disrupted in 50 mM TRIS, pH 8.0/0.3% SDS/1.0% 2-mercaptoethanol/2 μM leupeptin/2 μM pepstatin/0.5 mM *p*-nitrophenyl *p'*-guanidinobenzoate. Extracts were heated for 10 min at 95°C and clarified by centrifugation. The protein level was determined and samples were run on 10% SDS/PAGE. The gel was blotted onto nitrocellulose, blocked with 5% nonfat milk, probed with monoclonal antibody 416 (7), detected with anti-mouse IgG-horseradish peroxidase (Vector Laboratories), and developed using the ECL system (Amersham).

RESULTS

RIP1-Tag2 and RIP3-Tag2 mice use different versions of the rat insulin II promoter to direct the expression of Tag to the pancreatic β cell. RIP1-Tag comprises 695 bp of 5′ flanking DNA, whereas RIP3-Tag extends ≈10,000 bp 5′ from the promoter. Both lines of mice begin expressing Tag in the pancreatic bud at embryonic day 9 (ref. 13; unpublished data). Since Tag is a nuclear oncoprotein, its expression inevitably leads to the formation of β-cell tumors (ref. 7; unpublished data). Tumors are first detected between 10 and 12 weeks of age. Therefore, mice studied in the immunological assays were immunized at 6 weeks of age and analyzed over the next several weeks, prior to overt tumor formation.

RIP-Tag Mice Develop Systemic Tolerance. To characterize the tolerance toward the β-cell antigen Tag, RIP1-Tag2 and RIP3-Tag2 mice were tested in three T-cell-dependent assays. In the first assay, mice were immunized with a combination of Tag and, as a control antigen, β-gal, and their ability to make Tag-specific antibody was assessed (Fig. 1*A*). Transgenic mice had an impaired Tag-specific antibody response relative to controls. Humoral tolerance has been described for RIP1-Tag2 mice (15) but not for RIP3-Tag2 mice. The mean anti-Tag response of transgenic mice was 350-fold lower than that of the control group.

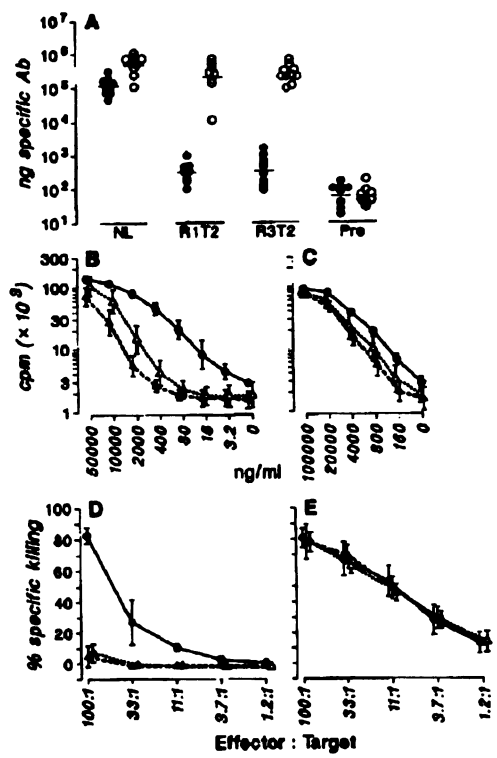

FIG. 1. RIP-Tag mice develop systemic tolerance toward Tag. (*A*) Secondary antibody (Ab) response to Tag and β-gal. The response to Tag (●) and β-gal (○) was calculated for individual mice. The mean of all animals in the group is represented by a bar. Nontransgenic mice, RIP1-Tag2, and RIP3-Tag2 are abbreviated as NL, R1T2, and R3T2, respectively. Pre indicates the values obtained from the preimmune serum of the nontransgenic littermates. (*B* and *C*) *In vitro* proliferation of RIP-Tag lymphocytes. Lymph node cells were cultured with dilutions of Tag (*B*) or *M. tuberculosis* (*C*) and pulsed with [^{3}H]thymidine. (*D* and *E*) CTL activity generated by RIP-Tag splenocytes cultured with either Tag-expressing fibroblasts or BALB/c stimulators. Cultured splenocytes were tested for their ability to kill ^{51}Cr-loaded C57SV (*D*) or allogeneic P815 (*E*) targets. In *B–D*, the mean for all animals in each group, nontransgenic littermates (●), RIP1-Tag2 (▲), and RIP3-Tag2 (△), is shown.

Next we evaluated the ability of T cells from immunized mice to proliferate in response to Tag (Fig. 1 *B* and *C*). Cultured lymph node cells from transgenic mice had diminished Tag-dependent proliferation, relative to controls. By comparing the amount of Tag protein that elicits 20% of the maximal proliferation in the control group, it can be determined that 35 and 90 times more Tag is required for RIP3-Tag2 and RIP1-Tag2 T-cells, respectively, to proliferate to an equivalent extent. Although the Tag protein was affinity purified, we cannot exclude a contribution to the observed response by minor contaminants in the preparation. In independent experiments the proliferative response of transgenic mice to other foreign antigens, hemocyanin, ovalbumin, and β-gal, was indistinguishable from that of controls (data not shown).

In the third assay, we asked whether RIP1-Tag2 and RIP3-Tag2 mice could generate Tag-specific CTLs. Spleen cells from immunized mice were tested for their ability to kill Tag-expressing fibroblasts or allogeneic targets in a ^{51}Cr release assay (Fig. 1 *D* and *E*) and a more sensitive DNA fragmentation assay (data not shown). Although control mice generated CTLs capable of killing Tag-expressing cells, no anti-Tag CTL activity was detected in cultures from transgenic mice in either assay.

In two of the assays used to characterize the tolerant phenotype of RIP1-Tag2 and RIP3-Tag2 mice, immune responses toward Tag could be detected, albeit at significantly reduced levels. Therefore we asked if these reactive T cells were potentially autoreactive toward Tag protein in β cells. Pancreata from mice that had been immunized and given booster immunizations with Tag s.c. were examined for islet infiltration and destruction by immunohistochemistry. No evidence of lymphocytic infiltration or immunopathology was observed (data not shown). Thus, RIP1-Tag2 and RIP3-Tag2 T-cells respond normally to foreign antigens and weakly to exogenous Tag but are systematically tolerant toward the endogenous antigen Tag in islet β cells.

Quantitation of Tag Protein in the Pancreas. The level of Tag in the pancreas was quantitated to provide an estimate of the amount of this protein being produced by these systemically tolerant mice. First we developed a semiquantitative PCR-based assay (Fig. 2*C*) to examine Tag mRNA expression. The pattern of Tag expression in the developing pancreas of the two lines of mice differed (Fig. 2*A*) but stabilized by 1 week of age. Therefore, we chose to quantitate Tag protein from pancreatic extracts of 1-week-old mice by Western blotting (Fig. 3). Approximately 1 ng of Tag was detected in 30 μg of pancreatic extract from RIP3-Tag2 mice. Since 1.8 mg of extract was obtained from a single pancreas on average, we estimate that 60 ng of Tag is present in a 1-week-old RIP3-Tag2 pancreas. RIP1-Tag2 pancreata contained ≈10 ng of Tag protein.

Tag Expression in the Thymus of RIP-Tag Mice. While the primary site of Tag expression is the pancreatic β cell, documentation of systemic tolerance toward this self-protein led us to investigate whether the transgene might be expressed in the thymus. Thymic cDNAs were tested for the presence of Tag-specific message by PCR (Fig. 2*B*). As expected, a Tag-specific signal was not amplified from nontransgenic thymus cDNA or from samples to which no RT had been added (−RT). Surprisingly, faint Tag bands appeared at distinct times during development in the two lineages. In RIP3-Tag2 mice Tag message was detected at 1 week, whereas in RIP1-Tag2 it was detected in newborn mice. To confirm the specificity of this expression, we examined other tissues from transgenic mice. A Tag-specific signal could be amplified from cDNA made from brain, testis, and small intestine of adult RIP1-Tag2 mice and from brain and skeletal muscle of adult RIP3-Tag2 mice, but not from adult kidney, heart, liver, large intestine, skin, lung, salivary gland, adrenal, thyroid, lymph node, or bone marrow, nor

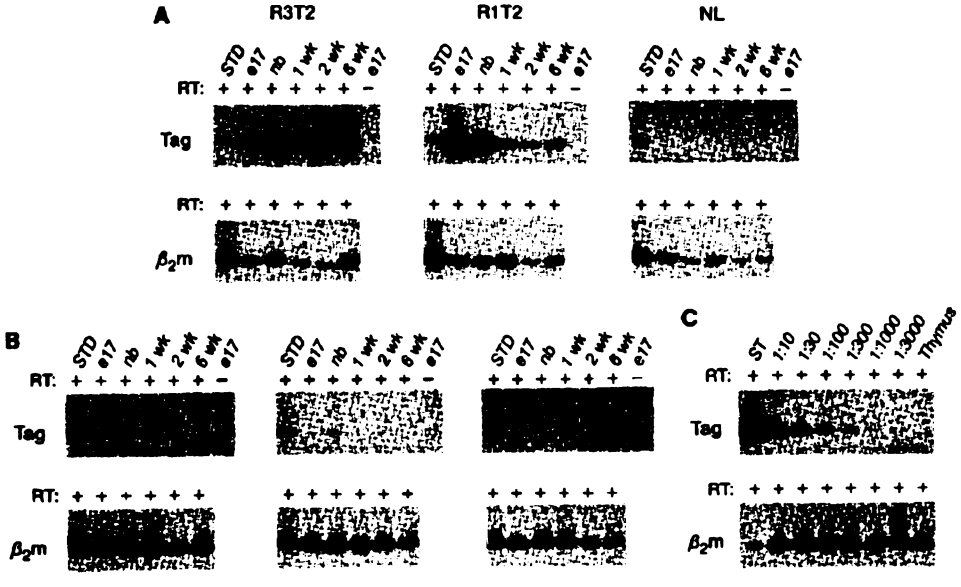

FIG. 2. Expression of Tag during development in pancreas and thymus. Pancreatic and thymic cDNAs were tested by PCR for the presence of Tag- and β_2m-specific sequences. The large T antigen band (156 bp) is shown. Ages examined are embryonic day 17 (e17), newborn (nb), 1, 2, and 6 weeks. + and − indicate whether RT had been added to the sample. (*A*) Tag expression in the pancreas of transgenic mice. (*B*) Tag expression in the thymus of transgenic mice. (*C*) Sensitivity of RNA-PCR assay. Pancreatic RNA from a newborn RIP1-Tag2 was diluted into thymus RNA of a C57BL/6 adult at the ratios indicated above the lanes prior to cDNA synthesis. The first sample (ST) is undiluted pancreatic RNA. To compare the reaction products from different experiments, the 1:100 sample from the dilution series was included as a standard (STD) in each experiment.

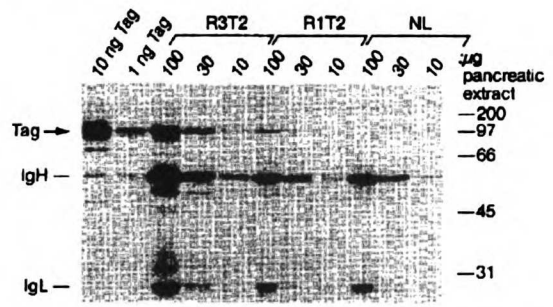

FIG. 3. Quantitation of Tag protein in RIP-Tag pancreata. Pancreatic extracts from 1-week-old RIP3-Tag2 (R3T2), RIP1-Tag2 (R1T2), and nontransgenic littermate (NL) mice were run on SDS/PAGE and immunoblotted. The Tag-specific band is marked by an arrow. Mouse IgG was present in the extracts and the heavy (IgH) and light (IgL) chains are detected by the secondary reagent.

newborn liver (data not shown). Although Tag mRNA in brain and testis of RIP1-Tag2 was easily detectable, both are considered immunologically privileged sites. The levels of Tag message in intestine of RIP1-Tag2 as well as skeletal muscle and brain of RIP3-Tag2 mice are quite low. A faint Tag-specific signal was detected in spleen from an adult RIP3-Tag2, but not a RIP1-Tag2 mouse. The inability to detect Tag RNA in most tissues supports the specificity of the thymic Tag expression. When cDNAs prepared from five times as much RNA were tested in the PCR assay, Tag-specific message was detected in newborn and 1- and 2-week-old transgenic thymus from both lineages, but not in nontransgenic littermates, thus indicating that this expression is not transient but persists until at least 2 weeks of age.

To determine whether Tag protein could be detected in the thymus, we examined thymic sections from newborn RIP1-Tag2 and 1-week-old RIP3-Tag2 mice for Tag immunoreactivity (data not shown). No Tag-expressing cells could be identified, supporting the notion that the level of Tag expressed by thymic residents is low.

Thymic Tag Expression Recapitulates Endogenous Insulin Gene Expression. The above experiments reveal low-level Tag expression in thymus of RIP-Tag mice. This expression could be an artifact of the chimeric transgene and its random integration or, rather, reflect an intrinsic property of the insulin gene regulatory region. To distinguish between these two possibilities, cDNAs from thymus, liver, and pancreas of C57BL/6 mice of various ages were assayed for the presence of insulin transcripts by RNA-PCR (Fig. 4). Insulin RNA was detected in thymus and pancreas, but not liver, of all animals tested. Insulin message was also undetectable in spleen and lymph node (data not shown). Thymic insulin expression was

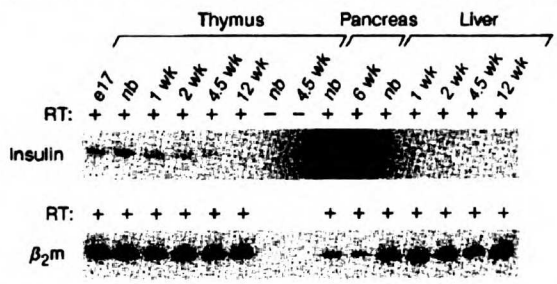

FIG. 4. Insulin expression in the thymus. cDNAs from thymus, pancreas, and liver of C57BL/6 mice were tested for the presence of insulin or β_2m-specific transcripts in independent reactions. The figure is labeled as in Fig. 2.

Table 1. Expression of pancreas-specific genes in the thymus

Gene	Tissue			
	Pancreas	Thymus	Liver	Brain
Tag	+++++	+	−	
β_2m	++++	+++++	+++++	+++++
Glucagon	++++	++	−	
Insulin	+++++	+	−	
Pancreatic polypeptide	++++	++	−	
Somatostatin	++++	++	−	
Trypsin	++++	+	−	
Amylase	+++++	−	+++	
Carboxypeptidase A	++++	−	−	
Elastase	+++++	+	+	
GAD65	−	−	−	+++++
GAD67	+	+	−	+++++

cDNAs from newborn RIP1-Tag2 and nontransgenic mice were tested for transcripts of genes normally expressed in the pancreas. All samples are from newborn mice except the brain sample, which is from a 6-week-old mouse. Data from four independent experiments are summarized. The intensity of each signal was compared to the Tag bands in the dilution series in Fig. 2*C*. Bands with intensities greater than or equal to the undiluted sample are represented as +++++. Bands greater in intensity than the 1:10, 1:30, 1:100, and 1:300 samples are designated by ++++, +++, ++, and +, respectively. Inability to detect specific mRNA is indicated by −. Although GAD65 protein has been detected at very low levels in mouse islets (24), as well as in cell lines derived from RIP1-Tag2 mice (25), we have been unable to detect GAD65 transcripts in RNA extracted from newborn or adult pancreas, from isolated islets, or from the thymus, despite its clear expression in brain.

strongest in perinatal mice although it persisted until 12 weeks of age. Thus, thymic Tag expression is not an artifact of the transgenic system but, rather, reflects a property of the insulin genes.

Expression of Pancreas-Specific Genes in the Thymus. The detection of insulin gene transcription in the thymus raised the larger issue of whether other pancreas-specific genes are expressed there. To address this question, cDNAs from thymus, liver, and pancreas were analyzed for gene transcripts selectively expressed by distinct pancreatic cell types (Table 1). Primers that amplify RNA-specific products were designed for endocrine- (glucagon, insulin, pancreatic polypeptide, and somatostatin) and exocrine- (trypsin, amylase, carboxypeptidase A, and elastase) specific genes and for GAD67 and the β-cell autoantigen, GAD65. The results from newborn RIP1-Tag2 and nontransgenic mice are summarized in Table 1. Similar results were obtained for adult C57BL/6 mice. As expected, mRNAs for all endocrine and exocrine genes were detected in the pancreas at high levels. We failed to detect GAD65 RNA in the pancreas but were able to detect low levels of GAD67 message in newborn pancreas. Weak signals for glucagon, pancreatic polypeptide, somatostatin, trypsin, and GAD67 were detected in the thymus but not in liver cDNA. RNA for amylase was detected in liver, as expected, but not thymus, while carboxypeptidase A and GAD65-specific RNA were found in neither. Elastase transcripts were detected in low abundance in thymus and liver. Thymic expression of somatostatin has been previously reported (26). Thus, the thymus expresses low levels of several pancreatic genes.

DISCUSSION

We have shown that RIP3-Tag2 and RIP1-Tag2 mice develop systemic T-cell tolerance toward the self-antigen Tag. The pancreatic β cells comprise the main site of Tag protein synthesis, containing 9 and 60 ng of Tag in 1-week-old RIP1-Tag2 and RIP3-Tag2 mice, respectively. Should the entire contents of the β cells be released into the circulation,

Tag would be present at 0.5 and 3 nM in the intravascular fluid volume of RIP1-Tag2 and RIP3-Tag2. These levels fall into the range of rare serum proteins that may be ignored by the immune system (27). There is no evidence that Tag protein is quantitatively released by the β cells. Thus the amounts released, for example, by cell death, should be several orders of magnitude lower than these maximal calculated values and therefore below the threshold for tolerization as a serum protein.

How is it then that systemic tolerance toward Tag is established? It is unlikely that all recent thymic emigrants circulate past the β cells and become tolerized in the pancreas (28). Another possibility is that Tag, released by the β cells, is carried back to the lymph nodes or thymus. As discussed above, this is also an inadequate explanation for the systemic tolerance observed. In addition to the Tag produced by the β cells, we now report expression of Tag RNA in the thymus itself. This expression is directed by the insulin gene regulatory region and is 3000–10,000 times lower than that in the pancreatic β cells. Two other groups using insulin-promoted transgenes have provided evidence that peripheral (12) and thymic (29) expression of the transgene can contribute to systemic tolerance.

The surprising observation that the endogenous insulin genes are expressed in the thymus motivated similar evaluation of other tissue-specific genes. Remarkably, thymic expression of several pancreatic genes from endocrine and exocrine cell types was detected. Whether other genes, normally thought of as being "peripheral," are expressed intrathymically awaits further study. If expression of "tissue-specific" genes is a general property of the thymus, it would support a hypothesis in which the thymus plays a role in limiting autoreactivity to proteins thought of as being peripheral. A set of observations lends credence to this notion. Oldstone *et al.* (11) described mice that express the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) in the pancreatic β cells. GP is normally ignored by the immune system. When mice are infected with LCMV, a slow onset of diabetes ensues. This is in contrast to RIP-GP mice developed by Ohashi *et al.* (10), which develop rapid onset of disease after LCMV infection. Recently, Oldstone's group has discovered GP expression in the thymus only in those mice with slow-onset disease (M. B. Oldstone and M. Vonnerrath, personal communication). These results argue that thymic expression of a peripheral protein contributes to nonresponsiveness but is inadequate to tolerize all potentially autoreactive T cells. Arnold *et al.* (4) have proposed a model in which lymphocytes undergo multiple antigen encounters that drive them further and further into a state of nonresponsiveness. The initial encounter may occur in the thymus. If thymic expression of tissue-specific genes plays a role in self-tolerance toward rare cell types, then it will be of interest to assess the possibility that defects in thymic expression are a component to organ-specific autoimmunity.

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CHAPTER 3:

Expression of T antigen in the Thymus Correlates with the Development of the Tolerant Phenotype

INTRODUCTION

RIP-Tag mice with embryonic onset of T antigen expression develop systemic tolerance toward T antigen as shown in Chapter 2. The tissue specific expression of T antigen has been well documented in RIP-Tag2 mice. In the initial report describing these mice, an immunopercipitation study demonstrated that T antigen protein is produced in pancreas and not in other tissues (Hanahan 1985). In subsequent studies, T antigen was shown to be transiently expressed in the central nervous system of embryos by immunohistochemistry (Alpert et al, 1988). Very low levels of T antigen have been inferred to be synthesized in the intestine, since a small percentage of RIP1-Tag2 mice develop metastatic intestinal tumors, which may relate to the transgene insertion site (Grant et al. 1991). This type of extensive study has not be undertaken for RIP3-Tag2 mice. In addition to the pancreatic expression of T antigen, I have discovered that low levels of T antigen RNA can be detected in the thymus of perinatal transgenic mice from both lineages, estimated to be 3000-10,000 times lower than present in the pancreatic ß cells of newborn RIP1-Tag2 mice. Thymic T antigen expression in mice from both the tolerant lineages suggests that it may contribute to the development of systemic tolerance.

Mice from the delayed onset lineage, RIP1-Tag5, fail to establish tolerance to T antigen, and instead develop spontaneous autoantibodies to T antigen and a lymphocytic infiltrate of their T antigen expressing islets. This

spontaneous anti-self reactivity has been attributed to the failure of delayed onset mice to express T antigen during perinatal life, when tolerance is first established. If the intrathymic expression of T antigen detected in mice of the embryonic onset lineages, RIP1-Tag2 and RIP3-Tag2, contributes to the tolerant phenotype, one would predict that RIP1-Tag 5 mice would fail to express T antigen intrathymically.

In this chapter I examine the tissue specificity of T antigen expression in RIP1-Tag2 and RIP3-Tag2 mice by RNA-PCR to determine if T antigen is synthesized in any other tissues at a level which may impact tolerance induction. I also explore whether T antigen RNA persists until immunolgical maturity in the thymus of mice from both embryonic onset lineages and whether T antigen transcripts can be detected in thymus of delayed onset RIP1-Tag5 mice. Finally, I test whether peripheral lymphocytes from RIP1-Tag5 mice exhibit any signs of tolerance induction or activation toward T antigen.

MATERIALS AND METHODS

RNA-PCR. RNA-PCR was performed as described in chapter 2, except that for some of the RNA samples, more RNA, 3.75 μg or 7.5 μg, was present in the reverse transcription reaction, instead of the typical 1.5 μg. If not otherwise noted, the reverse transcription reaction contained 1.5 μg of RNA.

In Vitro Proliferation Assay. The in vitro proliferation assay was performed as described in chapter 2.

RESULTS

Tissue Specificity of T antigen Expression

The initial characterization of RIP1-Tag2 mice failed to detect T antigen protein in the thymus. However, by using a sensitive PCR based assay, I have recently shown that T antigen message can be detected in perinatal thymus of both RIP1-Tag2 and RIP3 Tag2 mice, albeit at levels 300 to 1000 fold lower than in total pancreas (Chapter 2). Since the thymus is the site of T cell maturation and central tolerance induction, this finding indicates that the thymus may play a role in establishing tolerance to T antigen in these mice.

To discover if any other tissues synthesized T antigen at a level that could also contribute to tolerance induction, I used RNA-PCR to examine the expression of T antigen in 18 different tissues of adult RIP1-Tag2 and RIP3-Tag2 mice (Table 2). In RIP1-Tag2 mice T antigen was detected in pancreas, as expected, as well as in brain, testes, and small intestine, but not in any other tissues. The expression in small intestine and brain could be predicted from what is know about the expression pattern of RIP1-Tag2 mice (Alpert et al., 1988; Grant et al., 1991). Recently, neuroendocrine cells, expressing T antigen have been detected in the small intestine of RIP1-Tag2 mice, but these are rare (D. Olson, personal communication). In testes T antigen RNA was detected at a higher level than in total pancreas. In the initial immunopercipitation study, no T antigen protein was detected in the testes. It may be that this T antigen message, although spliced, is not translated, or that the expression pattern has changed upon maintaining the

Table 2: *Tissue specificity of T antigen expression.* cDNA from various tissues of six week old transgenic mice were assayed for T antigen expression by PCR. The intensity of the signal is indicated in the table. The grading of the signal intensity is the same as utilized in Table 1.

	RIP1-Tag2	*RIP3-Tag2*
Brain	++	+
Salivary Gland	-	-
Heart	-	-
Thymus	-	-
Lung	-	-
Liver	-	-
Pancreas	++	++++
Spleen	-	+/-
Kidney	-	-
Adrenal	-	-
Testes	+++	++*
Mesenteric LN	-	-
Small Intestine	+	-
Large Intestine	-	-
Skeletal Muscle	-	+
Bone Marrow	-	-
Skin	-	-
Thyroid	-	-

**** Abundant unspliced band, not DNA***

Table 2

lineage for ten years. In any case, the testes, as well as the brain, are immunologically privileged sites (Streilein, 1993) and should not contribute greatly to the tolerant phenotype of RIP1-Tag2 mice.

For RIP3-Tag2 mice, T antigen was detected at the highest level in the pancreas. T antigen mRNA was also present at low levels in brain and skeletal muscle. Abundant unspliced message was detected in testes, and a faint signal in the spleen of two different mice. A similar pattern of expression is also detected for RIP3-Tag3 mice, a separate lineage which utilizes the same transgene as RIP3-Tag2 mice, but has a different integration site (data not shown). Although brain and testes again have low levels of message for T antigen, these tissues are not readily accessible to the immune circulation. A faint T antigen signal could be found in the spleen, but not the thymus of adult RIP3-Tag2 mice. This splenic expression was only seen in RIP3-Tag2 mice, not in RIP3-Tag3 (data not shown) or RIP1-Tag2 mice, which have a lower level of pancreatic T antigen expression. Since the spleen sits on top of and is connected to the pancreas, which expresses high levels of T antigen, it is difficult to rule out that the spleen was not contaminated with a small amount of pancreatic tissue. Alternatively, the same cell type expressing T antigen in the thymus may also be found in the spleen. The expression of T antigen in the skeletal muscle is a little harder to discount as a possible source of tolerogen. No tumors have been found in muscle of RIP3-Tag2 mice. Since this expression is found only in RIP3-Tag2 mice, and not in RIP1-Tag2 mice, and is present at a low level, it is unlikely to contribute significantly to the development of tolerance in embryonic onset RIP-Tag mice. Thus, the main source of T antigen in both RIP1-Tag2 and RIP3-Tag2 mice is the pancreas. Although T antigen RNA is detected in

brain and testes in mice of both lineages, these are not likely depots of T antigen which contribute to tolerance induction.

T antigen Is Detected in the Thymus of Mice from Tolerant Families Throughout Development.

Initially, T antigen transcripts were detected in perinatal thymus at discrete time points in RIP1-Tag2 and RIP3-Tag2 mice, newborn and one week, respectively. The level was very low, and appeared to be at the limit of detection of the PCR based assay. Since the thymus is a critical site for tolerance induction, even this vanishingly small amount of T antigen could have an important effect on the induction of tolerance. However, it would be difficult to envisage a role for thymically expressed T antigen in the establishment of tolerance, if its expression were truly transient.

To investigate whether the expression of T antigen in the thymus was transient or constitutive, I developed a more sensitive version of the same RNA-PCR assay. I had noticed that using more RNA in the reverse transcription reaction allowed for increased sensitivity in the PCR assay. Therefore, five times as much RNA was used in the reverse transcription reaction as in the standard assay. The cDNAs were analyzed as before. Figure 5 shows a typical assay. Since I had observed sporadic faint signals for T antigen in thymic cDNAs from one week old RIP1-Tag2 and two week old RIP3-Tag2, I resynthesized the cDNA using 7.5 μg of RNA, instead of the standard 1.5 μg, for a group of newborn and one week RIP1-Tag2 and one and two week old RIP3-Tag2 samples. As can be seen in Figure 5, all samples

Figure 5: *T antigen expression in thymus is not transient.* cDNA from thymus was reverse transcribed using 7.5 μg of RNA. Samples were tested by PCR for the presence of spliced T antigen and $ß_2$-microglobulin transcripts. The amount of RNA used in the reverse transcription reaction, the age of the mice, and their lineage is indicated over the lanes. The 156 bp band for the spliced large T antigen is shown. RNA samples to which no reverse transcriptase were added (-RT controls) were assayed by PCR in parallel and did not show T antigen specific bands (data not shown).

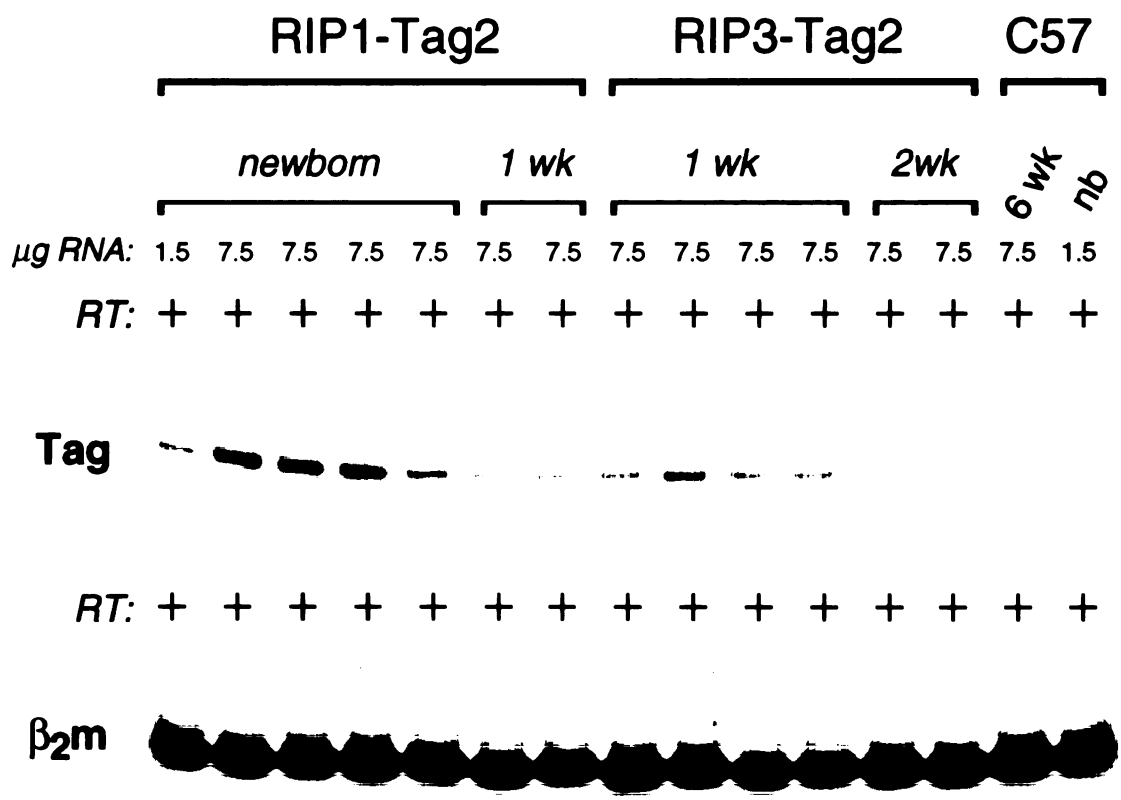

RIP1-Tag2
RIP3-Tag2
C57
newborn
1 wk
1 wk
2wk
6 wk
nb
µg RNA: 1.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 7.5 1.5
RT: + + + + + + + + + + + + + + +
Tag
RT: + + + + + + + + + + + + + + +
β2m

Figure 5

from transgenic mice, but not the C57BL/6 controls, now reveal spliced message for T antigen.

Using this improved assay, I examined thymic RNA for the rest of the ages examined in Figure 2: neonate, one, two, and six weeks. Samples from embryonic day 17 were not included since there was not enough RNA from individual mice to perform the analysis. The data is summarized in Table 3. T antigen message could be detected in thymus from transgenic mice, but not non-transgenic mice, at all ages examined, except for six week old RIP3-Tag2. The data show that T antigen is expressed in the thymus at birth and persists until at least two weeks of age in both lineages and up to six weeks of age in RIP1-Tag2. Although the level of expression is very low, close to the limit of detection of a sensitive PCR based assay, T antigen may be expressed in a rare cell type involved in tolerance induction. Thus, it appears that T antigen expression, like that of several endogenous pancreatic genes including insulin, as shown in Figure 4 and Table 1 persists in the thymus.

Lack of T antigen Expression in the Thymus of RIP1-Tag5 Mice Correlates with the Non-tolerant Phenotype.

Although delayed onset RIP1-Tag5 mice use the same insulin promoted transgene construct as embryonic onset RIP1-Tag2 mice, they fail to establish tolerance to T antigen. If thymic expression of T antigen plays a role in the development of the tolerant phenotype, one would predict that T antigen should not be expressed in the thymus of RIP1-Tag5 mice. To test

Table 3: *Summary of thymic T antigen expression.* Data from several experiments examining the expression of T antigen in the thymus of transgenic and non-transgenic mice at various ages is summarized. cDNAs were prepared from 7.5 μg total RNA. The number of animals in which T antigen RNA could be detected intrathymically is indicated over the total number tested.

Age	***Non-Transgenic***	***RIP1-Tag2***	***RIP3-Tag2***
neonate	0/4	7/7	1/3
1 week	0/2	2/2	4/4
2 weeks	0/3	2/2	2/2
6 weeks	0/1	1/2	0/2

Table 3

this hypothesis, RNA was extracted from thymus and pancreas of RIP1-Tag5 mice and cDNA tested by PCR for the expression of T antigen and $ß_2$-microglobulin (Figure 6). Newborn and one week old mice were examined since T antigen message is detected at these ages in RIP1-Tag2 and RIP3-Tag2 mice. Since T antigen is initially detected in the pancreas at nine weeks, thymic RNA from nine and 11 week old animals was also studied. In the pancreas, T antigen message was first detected at 15 weeks, a time when a large proportion of the ß cells are producing it. As mentioned above, T antigen protein is observed in a few scattered pancreatic ß cells at nine weeks, and thus its RNA is unlikely to be detectable at early times using this technique.

Remarkably, no T antigen message could be detected in the thymus at any of the ages examined, even though the very sensitive assay described above was used. The possibility remains that T antigen could be expressed below the detection limit of this assay. It can be estimated, from the dilution series in Figure 2C, that the level of thymic T antigen RNA in RIP1-Tag5 would have to be at least three to ten times lower than in the thymus of mice from the embryonic onset lineages. It should be noted that 2.5 times more RNA was used to synthesize the cDNA for the RIP1-Tag5 samples than was used for the dilution series, making it even more unlikely that T antigen message is present in RIP1-Tag5 thymus.

RIP1-Tag5 mice exhibit the hallmarks of an autoimmune response directed at their T antigen expressing islets. However, their T cells have never been directly tested in an in vitro proliferation assay for responsiveness to T antigen. It is possible that T lymphocytes in RIP1-Tag5 have been either activated or rendered unresponsive to T antigen after T

Figure 6: *T antigen RNA is not detected in thymus of RIP1-Tag5 mice.* 3.75 μg RNA, prepared from thymus and pancreas of RIP1-Tag5 mice of various ages, was assayed for the presence of T antigen and $ß_2$-microglobulin messages by RNA-PCR. + and - indicate whether reverse transcriptase (RT) was added to the sample. The age of each mouse is indicated over the lane, newborn (nb), 1, 9, 11, 15, 23, and 26 weeks. The standard (STD) is the same as in Figure 2.

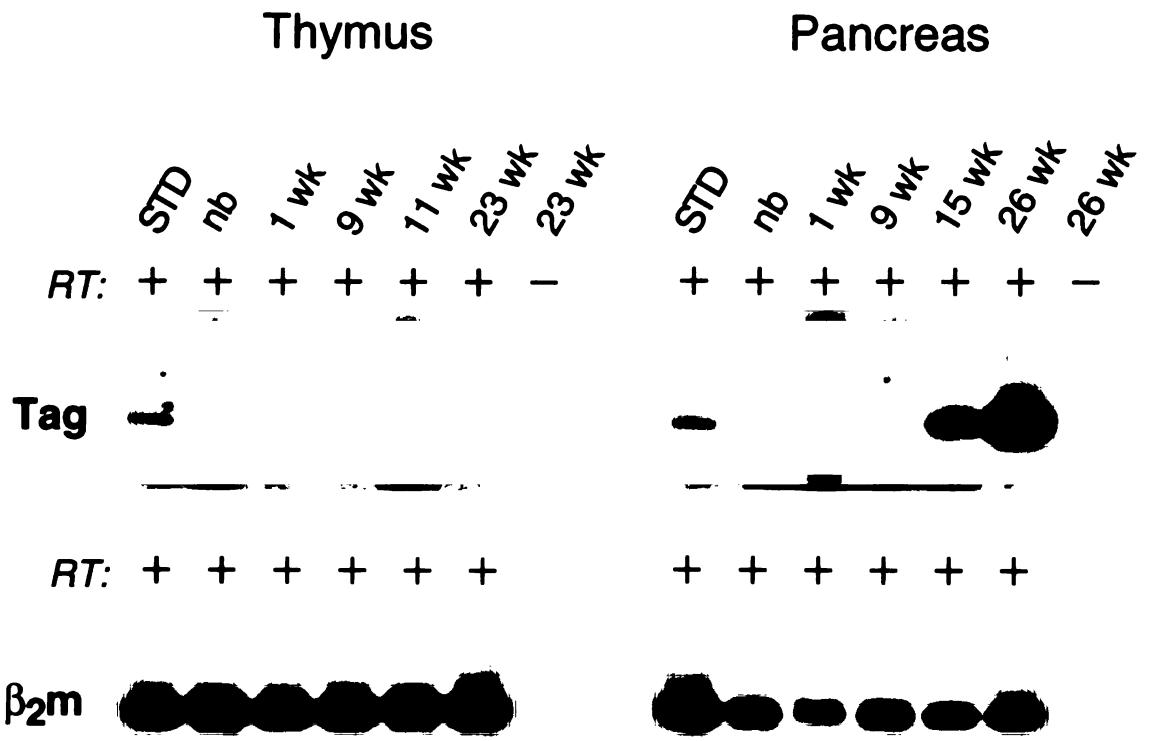

Thymus
Pancreas
STD
nb
1 wk
9 wk
11 wk
23 wk
23 wk
STD
nb
1 wk
9 wk
15 wk
26 wk
26 wk
RT: + + + + + + −
RT: + + + + + + + + + + + + + −
Tag
RT: + + + + + +
RT: + + + + + +
β2m

Figure 6

antigen expression in the pancreatic ß cells has begun. Since T antigen could not be detected in the thymus at any age, any decreased responsiveness would likely be attributed to peripheral expression of T antigen. To ask if RIP1-Tag5 mice exhibit any signs of increased or decreased responsiveness to T antigen as compared to non-trangenic controls, lymphocytes form immunized RIP1-Tag5 mice were tested in an in vitro proliferation assay (Figure 7). Eighteen week old mice were selected for study since all mice at this age express high levels of T antigen and exhibit all the signs of an autoimmune response directed at their T antigen expressing ß cells. As controls, six week old RIP1-Tag5, which had not yet begun expressing T antigen, and non-transgenic mice of both ages were included. Lymphocytes from all mice responded strongly to both T antigen and the control antigen, *M. tuberculosis*. In fact the dose response curves for the three groups are superimposable. No evidence of either increased or decreased responsiveness to T antigen could be observed in either 6 or 18 week old RIP1-Tag5 mice.

SUMMARY AND CONCLUSIONS

In this chapter mice carrying a hybrid insulin T antigen transgene, which exhibit one of two heritable immunological phenotypes, tolerance or non-tolerance, were studied. In adult mice from the embryonic onset lineages RIP1-Tag2 and RIP3-Tag2, which develop tolerance to T antigen, the pancreatic ß cells were shown by RNA-PCR to be the main source of T antigen, which is accessible to the immune system. In initial studies (see

Figure 7: *RIP1-Tag5 T cells are not tolerant of T antigen.* Lymph node cells from six (◇, solid line) and eighteen (O, solid line) week old immunized RIP1-Tag5 mice and non-transgenic controls (■, dashed line) were tested for their ability to proliferate in response to T antigen *(A)* and *M. tuberculosis (B).* Non-transgenic controls of both ages are included. The mean response and standard deviation of all animals in each group is shown: non-transgenic, n=4; RIP1-Tag5, 6 weeks, n=2; and RIP1-Tag5, 18 weeks, n=5. The response to T antigen of the RIP1-Tag5 mice of both ages is similar to that of controls, unlike the greatly reduced responsiveness of tolerant RIP1-Tag2 and RIP3-Tag2 mice as shown in Figure 1.

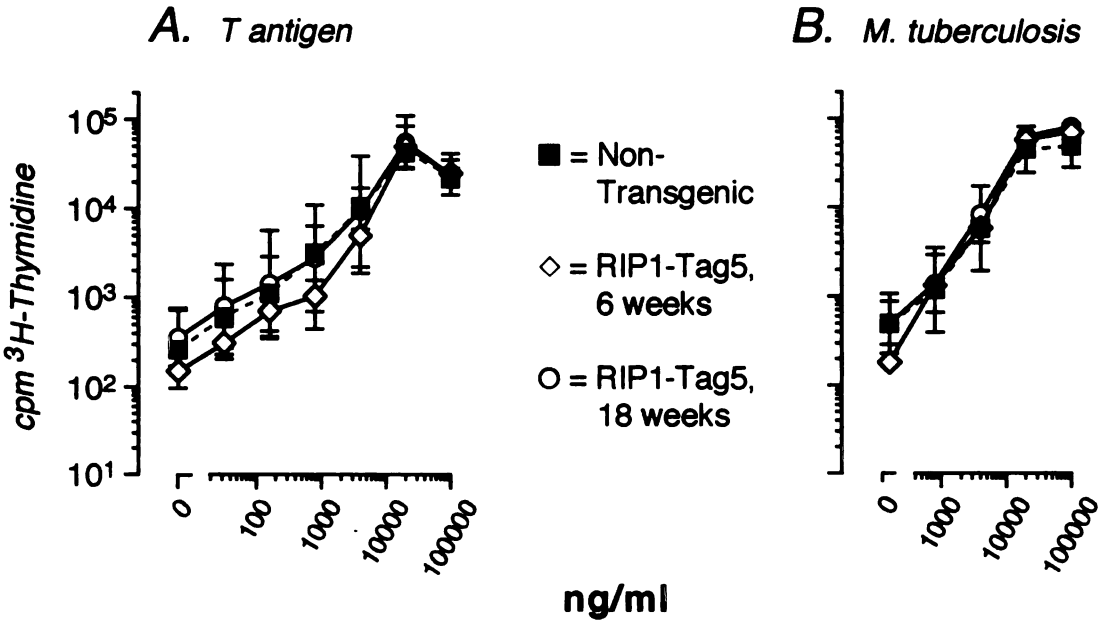

A. T antigen
B. M. tuberculosis
cpm 3H-Thymidine
10^5
10^4
10^3
10^2
10^1
0
100
1000
10000
100000
■ = Non-Transgenic
◇ = RIP1-Tag5, 6 weeks
○ = RIP1-Tag5, 18 weeks
ng/ml
0
1000
10000
100000

Figure 7

Chapter 2) low levels of T antigen mRNA are also found in perinatal thymus, the major site of T cell development. By increasing the sensitivity of the RNA-PCR assay, this expression was shown to persist until at least two weeks of age in RIP3-Tag2 mice and six weeks of age in RIP1-Tag2 mice. In contrast, in mice from the delayed onset lineage RIP1-Tag5, which fail to establish tolerance to T antigen, no T antigen message was detected in the thymus, either during perinatal life or in adulthood when T antigen is produced by the pancreatic ß cells. Furthermore, an in vitro proliferation assay has now been employed to demonstrate that peripheral T cells from young, prior to the onset of T antigen expression, and older, after the initiation of the autoimmune response, RIP1-Tag5 mice showed no indication of tolerization or activation toward T antigen.

In RIP1-Tag5 mice, T antigen protein is first detected in the pancreatic ß cells in adulthood, around 10 weeks of age, after a mature immune system has developed. Although T antigen transcripts were undetectable in the thymus at any time point examined, it is possible that T antigen may be expressed in the thymus at a level below the detection limit of the sensitive PCR based assay employed. However, young six week old RIP1-Tag5 mice showed no signs of tolerance induction toward T antigen in an in vitro proliferation assay, nor did 18 week old RIP1-Tag5 mice, which express T antigen in a large percentage of their ß cells, show any signs of reduced responsiveness toward T antigen. Thus, two lines of evidence, an in vitro proliferation assay and an expression study of T antigen in the thymus, indicate that RIP1-Tag5 mice do not express T antigen in the thymus at a level that impacts tolerance induction of T cells.

T antigen mRNA has been detected in the thymus of mice from both embryonic onset lineages, RIP1-Tag2 and RIP3-Tag2, throughout the early stages of development. The persistence of this thymic expression supports the hypothesis that the thymus plays a role in the development of tolerance to T antigen. This hypothesis is further strengthened by the inability to detect T antigen specific message in the thymus of non-tolerant RIP1-Tag5 mice. Thus, the development of the alternative phenotypes, tolerance and non-tolerance, correlates with the expression and lack of expression of T antigen intrathymically, respectively.

CHAPTER 4:

The Role of $CD8^+$ T Cells in Tolerance and Autoimmunity

INTRODUCTION

The systemic tolerance observed in RIP1-Tag 2 and RIP3-Tag2 mice implies that the T cells in these mice have encountered T antigen in a tolerogenic form. Since tolerance appears as a decreased ability to make antibody recognizing T antigen and a reduced in vitro proliferative response to T antigen protein, it can be inferred that the $CD4^+$ T cells are tolerant. However, the most profound manifestation of tolerance in these mice is their inability to generate T antigen specific cytotoxic T cells. Although the lack of help from $CD4^+$ T cell may also contribute to this non-responsiveness, it is reasonable to infer that the $CD8^+$ T cells have received a tolerogenic signal. Alternatively, tolerant $CD8^+$ T cells may exert a regulatory effect on T antigen specific $CD4^+$ T cells, thus limiting their reactivity toward T antigen.

In contrast to the tolerance observed in embryonic onset RIP-Tag mice, delayed onset RIP1-Tag5 mice develop an autoimmune response directed at their T antigen expressing islets. This autoimmune response is characterized by the spontaneous production of anti-T antigen autoantibodies and infiltration of the islets by $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and macrophages. The relative roles that $CD4^+$ and $CD8^+$ T cells play in the initiation and development of autoimmunity in RIP1-Tag5 mice is unknown. In another model of autoimmunity directed at the pancreatic ß cells, the NOD mouse, it has been postulated that the $CD4^+$ T cells are first

attracted to the islets, but that islet cell destruction is initiated by the cytotoxic $CD8^+$ T lymphocytes (Bendelac, et al., 1987; Miller et al., 1988; Hayakawa, et al., 1991; Yagi, et al., 1992).

To induce tolerance of or activate the $CD8^+$ T lymphocytes, T antigen peptides must be presented by MHC class I molecules. This is relatively easy to envisage, since T antigen, a nuclear protein, is translated in the cytoplasm, where some of it will be degraded into peptide fragments and transported into the endoplasmic reticulum for loading onto newly synthesized MHC class I molecules. Mature MHC class I molecules are produced as a trimolecular complexes, composed of a polymorphic heavy chain, and invariant light chain $ß_2$-microglobulin, and a cellular peptide. Only after trimer formation do stable class I molecules efficiently reach the cell surface. MHC class I molecules are expressed on the majority of cells in the body, including the pancreatic ß cells. Moreover, T antigen is a potent stimulator of $CD8^+$ cytotoxic T cells (Gooding, 1977; Knowles et al., 1979; Tevithia, 1990). Therefore, T antigen peptides should be well presented by class I on the pancreatic ß cells.

Here, I examine the role of MHC class I and $CD8^+$ T cells in the development of the tolerant phenotype of RIP1-Tag2 mice, the autoimmune phenotype of RIP1-Tag5 mice, as well as immune surveillance of tumors by the immune system in both lineages, by crossing mice from these two families to mice genetically deficient for the expression of the class I MHC light chain, $ß_2$-microglobulin. In addition, the breeding of the $ß_2$-microglobulin deficient mice to RIP1-Tag2 mice has revealed that the location of the RIP1-Tag2 transgene insertion site.

MATERIALS AND METHODS

Generation of RIP-Tag mice deficient for the expression of MHC class I. Mice heterozygous for the disrupted ß$_2$-microglobulin gene were obtained from R. Jaenish (Zijlistra et al., 1990). These mice were generated from strain 129 embryonic stem cells. Founders were crossed to C57BL/6 mice. The mice obtained by our laboratory had been intercrossed for an unknown number of generations and carried contributions from both strains. Both the C57BL/6 and 129 strains express the H-2^b haplotype.

RIP1-Tag2 mice, which had been backcrossed to C57BL/6 for a minimum of six generations, were mated to mice heterozygous for the targeted disruption of the ß$_2$-microglobulin gene (ß$_2$mKO/+). Double transgenic mice, carrying one copy of the RIP1-Tag2 transgene and one copy of the disrupted allele of ß$_2$-microglobulin, ß$_2$mKO/+; RIP-Tag2, were crossed to ß$_2$mKO/+ littermates, to generate ß$_2$mKO/KO; RIP1-Tag2 progeny. For the tolerance studies, ß$_2$mKO/KO; RIP1-Tag2 males were crossed to ß$_2$mKO/+ females to generate littermates with all four possible genotypes. For the tumorgenicity and longevity studies, mice were generated from all possible mating combinations. For the mapping studies, crosses are as indicated in Table 5. RIP1-Tag3; ß$_2$mKO mice were generated in the same manner as RIP1-Tag2; ß$_2$m KO mice. The genotypes of the mating combinations are indicated in Table 6.

Mice heterozygous for the disrupted ß$_2$-microglobulin gene were backcrossed to C3HeBFe/J for 11 generations and then bred to RIP1-Tag5 mice, which are inbred in same strain (Förster et al., 1995). Double transgenic ß$_2$mKO/+; RIP1-Tag5 mice were crossed to ß$_2$mKO/+ mice which were from

the twelfth backcross generation into C3HeBFe/J. Progeny of these crosses were studied as well as progeny from crosses of ß$_2$mKO/KO; RIP1-Tag5 to ß$_2$mKO/+ and ß$_2$mKO/+; RIP1-Tag5 to ß$_2$mKO/KO.

Immunological Assays. Both the antibody and proliferation assays were performed as reported in chapter 2, except that the mice tested in the antibody response were given an additional boost of both antigens, T antigen and ß-galactosidase, and the tertiary antibody response is shown. For the autoantibody response, sera were collected from unprimed mice.

Tumor Incidence and Longevity. Tumor incidence for RIP1-Tag2; ß$_2$mKO mice was determined by gross inspection of the pancreas of mice which were obviously suffering from hypoglycemia due to overproduction of insulin by their insulinomas. Life span was determined in a separate group of mice.

Tumor incidence of RIP1-Tag5; ß$_2$mKO mice was determined by collagenase release of tumors (Christofori et al., 1994). Briefly, pancreases were perfused via the common bile duct with collagenase, and further digested at 37° C. Tumors were identified by their size and color, typical red, and counted.

Incidence of Infiltration. RIP1-Tag5 which had been intercrossed to ß$_2$-microglobulin deficient mice, N12 into C3HeBFe/J, were sacrificed at 17 and 20 weeks of age. Their pancreases were frozen in OCT embedding compound and sectioned on a cryostat. Sections were taken from three different regions of the pancreas and adjacent sections immunostained for T antigen, CD4 (GK1.5), CD8 (53.6.7), macrophages (F4/80), MHC class II (M5/114), and MHC

class I (M1/42) as previously described (Skowronski et al., 1990). The total number of islets, the number of T antigen expressing islets, and the number of infiltrated islets, islets exhibiting either peri- or intra-islet accumulation of $CD4^+$ T and B lymphocytes, were counted.

Genetic Mapping. RIP1-Tag2 and RIP1-Tag3 mice were generated in our laboratory and are in the C57BL/6 genetic background. $ß_2$-microglobulin knockout mice, were generated from embryonic stem cells, which were derived from 129 strain mice and carry a dominant allele at the agouti locus, A^w, white-bellied agouti. These mice were backcrossed to C57BL/6 mice in our laboratory. C57BL/6 mice carry a recessive allele, a, at the agouti locus. In the initial backcrosses, the $ß_2$mKO allele segregated with the agouti coat color. These genes are known to be linked on mouse chromosome 2 (see Figure 13). Agouti mice carrying a single copy of the targeted disruption, $ß_2$mKO/+, were crossed to RIP-Tag mice. For testing the linkage of the transgene insertion sites to the $ß_2$-microglobulin locus, double transgenic mice were then crossed to $ß_2$mKO/KO mice, to generate progeny of all four possible genotypes, $ß_2$mKO/+, $ß_2$mKO/KO, $ß_2$mKO/+; RIP-Tag, and $ß_2$mKO/KO; RIP-Tag. Presence of RIP-Tag and the $ß_2$-microglobulin alleles were analyzed by PCR. To test the linkage of the RIP1-Tag2 transgene insertion site and agouti, agouti RIP1-Tag2 mice, A^w/a; RIP1-Tag2, which had received the dominant agouti allele from one parent, A^w, and the recessive agouti allele, a, and RIP1-Tag2 from the other parent, were bred to non-transgenic C57BL/6J mice, a/a. Progeny were analyzed for coat color and presence of the transgene.

RESULTS

Tolerant Phenotype of RIP1-Tag2 Mice Deficient for Expression of Class I MHC

To examine the role of presentation of T antigen by class I MHC molecules and the role of $CD8^+$ T cells in establishing tolerance toward T antigen, RIP1-Tag2 mice were crossed to mice deficient in MHC class I expression due to the targeted disruption of the $ß_2$-microglobulin gene (Zijlistra et al., 1990). These mice do not develop cytotoxic $CD8^+$ T cells, and only possesses low levels of functional MHC class I on the cell surface (Bix and Raulet, 1992). In addition, $ß_2$-microglobulin deficient mice are devoid of natural killer cell function, since natural killer cells distinguish between the presence and absence of class I MHC on the cell surface as part of their recognition mechanism (Liao et al., 1991; Höglund et al., 1991). A prediction can made from the hypothesis that $CD8^+$ T cells and MHC class I presentation of T antigen are essential for the induction of tolerance. If $CD8^+$ T cells are essential for the development of tolerance, then RIP1-Tag2 mice deficient for $ß_2$-microglobulin would be less tolerant than their RIP1-Tag2 littermates which express class I and have $CD8^+$ T cells. If $CD8^+$ T cells do not influence the development of $CD4^+$ T cell tolerance, then RIP1-Tag2 deficient and sufficient mice would exhibit the same tolerant phenotype.

To test these alternatives, littermates of all four possible intercross progeny were tested for tolerance to T antigen in two immunological assays. First, mice were immunized with T antigen and, as a control antigen, ß-galactosidase, and their specific antibody responses to both antigens

determined (Figure 8). RIP1-Tag2 mice, whether or not they were heterozygous or homozygous for the disruption of the ß$_2$-microglobulin gene, had similarly impaired antibody responses to T antigen relative to control mice. Moreover, neither the lack of CD8$^+$ T cell nor of class I MHC impaired the ability of any of the mice to produce high titer antibodies recognizing ß-galactosidase. Thus, the lack of CD8$^+$ T cells and of MHC class I had no effect on humoral responsiveness or antigen-specific non-responsiveness.

Next, littermates of the four possible genotypes were tested in an in vitro proliferation assay. Draining lymph nodes from immunized mice were cultured with dilutions of T antigen or as a control antigen, *M. tuberculosis*, that was present in the complete Freund's adjuvant (Figure 9). Once again, the presence or absence of the functional MHC class I and CD8$^+$ T cells did not influence the induction of tolerance in RIP1-Tag2 mice. Although the response of RIP1-Tag2 mice heterozygous for the ß$_2$-microglobulin deficiency is slightly less than that of the RIP1-Tag2 mice homozygous for the targeted disruption, their response to the control antigen is also slightly decreased. This experiment has been repeated and the result confirmed. Thus, it appears that CD8$^+$ T cells do not play a role in establishing tolerance of CD4$^+$ T cells.

Figure 8: *Antibody response of β_2-microglobulin deficient RIP1-Tag2 mice.* Tertiary antibody response to T antigen (●) and ß-galactosidase (O) of individual immunized mice is shown. The mean of all animals in each group is shown by the bar.

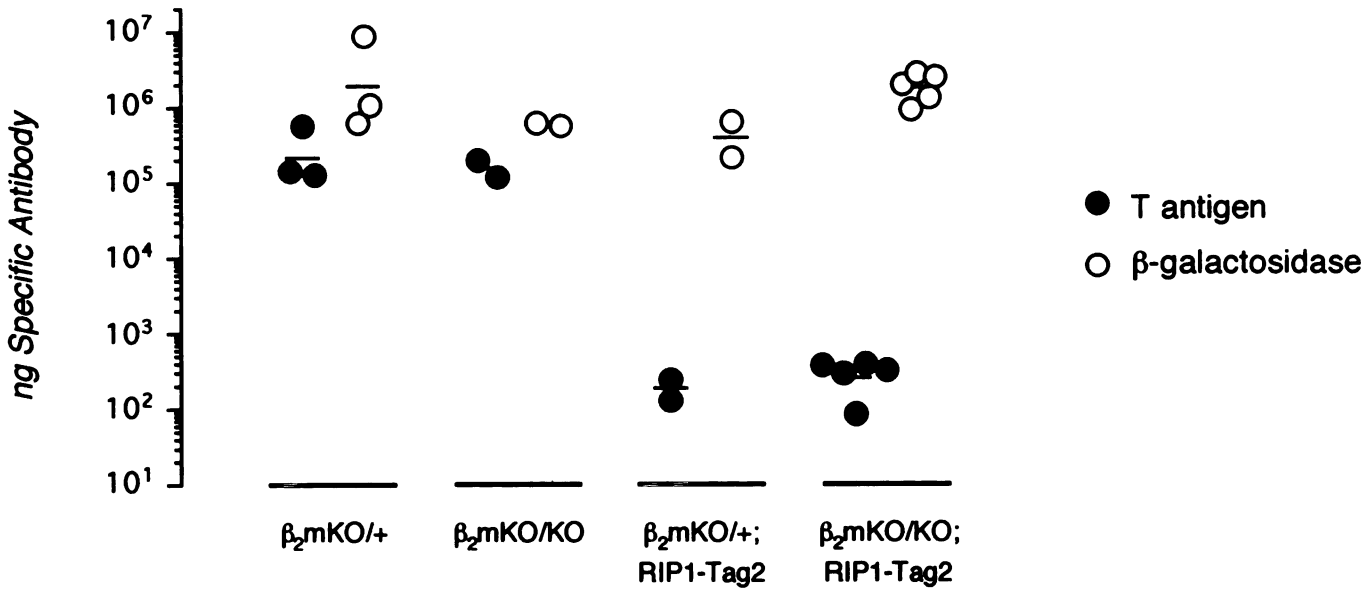

ng Specific Antibody
10^7
10^6
10^5
10^4
10^3
10^2
10^1
β2mKO/+
β2mKO/KO
β2mKO/+; RIP1-Tag2
β2mKO/KO; RIP1-Tag2
T antigen
β-galactosidase

Figure 8

Figure 9: *In vitro proliferation of ß2-microglobulin deficient RIP1-Tag2 lymphocytes.* Draining lymph node cells from immunized littermates were cultured in the presence of dilutions of T antigen (*A*) or of *M. tuberculosis* (*B*). The mean response and standard error of all animals in each group is shown: ß2mKO/+ (■, dashed line, n=2); ß2mKO/+; RIP1-Tag2 (○, dashed line, n=2); ß2mKO/KO (▲, solid line, n=2); and ß2mKO/KO; RIP1-Tag2 (▽, solid line, n=3). The antigen dependent proliferation was measured after 72 hours in culture by including ^{3}H-thymidine during the last 6 hours.

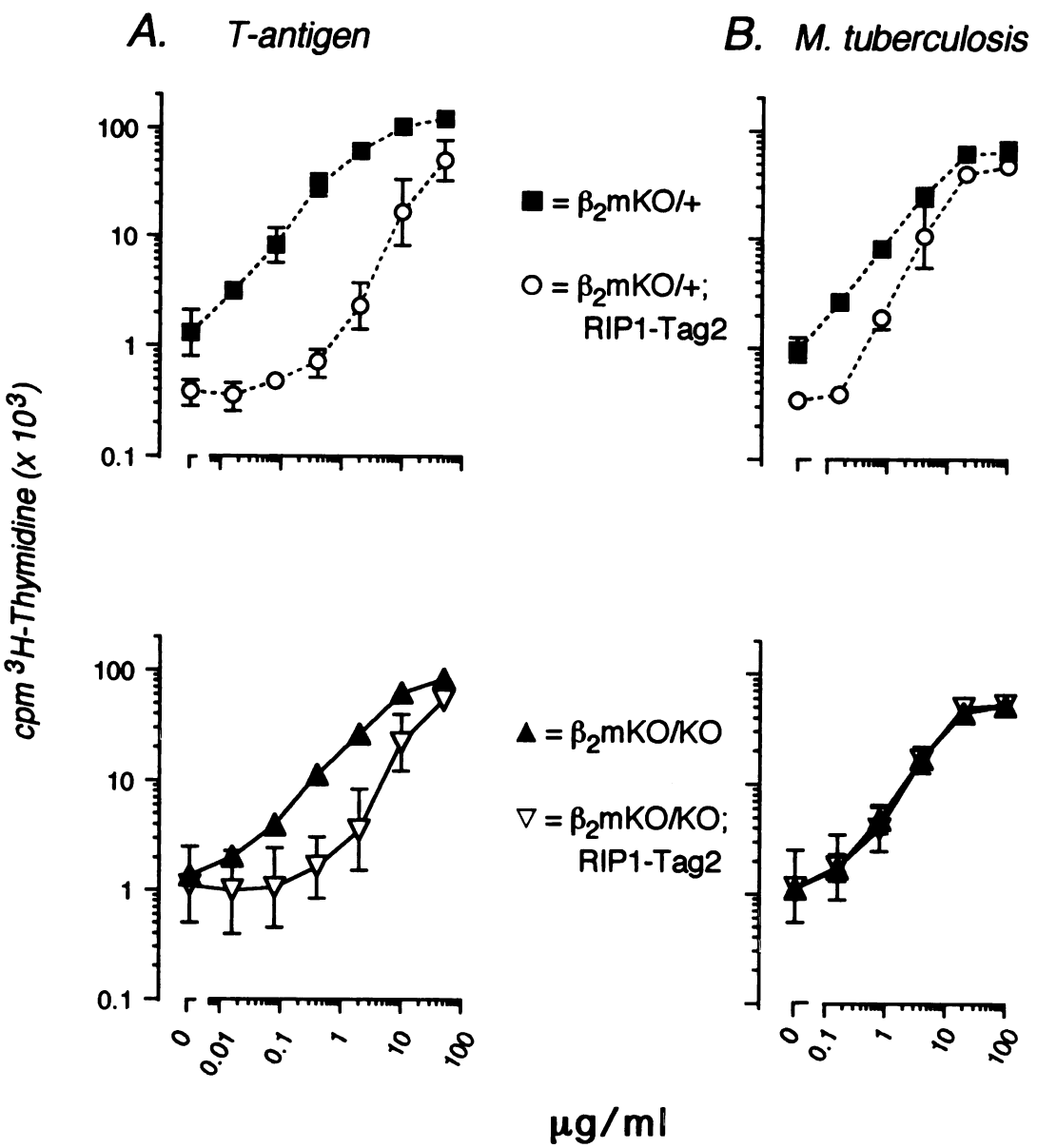

A. T-antigen
B. M. tuberculosis
cpm ^{3}H-Thymidine (x 10^3)
100
10
1
0.1
■ = β2mKO/+
○ = β2mKO/+; RIP1-Tag2
▲ = β2mKO/KO
▽ = β2mKO/KO; RIP1-Tag2
0
0.01
0.1
1
10
100
µg/ml

Figure 9

Tumor development and longevity in ß$_2$-microglobulin negative RIP1-Tag2 mice.

T antigen has been extensively studied as a tumor rejection antigen (reviewed by Tevithia, 1990). Since RIP1-Tag2 mice are tolerant of T antigen, it is unlikely that immune surveillance of nascent tumors plays a role in limiting tumor growth. However, in cell lines derived from preneoplastic and neoplastic islets of RIP1-Tag2 mice, preneoplastic ß cells express more MHC class I molecules on their surface and serve as better targets in cytotoxic T cell assays than the fully transformed ß tumor cells (Radvanyi et al., 1993). Thus, tumors in RIP1-Tag2 mice appear to down regulate class I MHC during tumor progression. Since natural killer cells recognize class I MHC molecules and are only able to kill cells which have down regulated its expression, natural killer cells may play a role in tumor surveillance in RIP1-Tag2 mice. ß$_2$-microglobulin deficient mice, in addition to the deficiency of CD8$^+$ cytotoxic T cell function, also lack functional natural killer cells (Liao et al., 1991; Höglund et al., 1991). Therefore, RIP1-Tag2 mice with a deficiency in class I expression may have an increased tumor burden or decreased longevity.

To explore this possibility, the life span of ß$_2$-microglobulin negative and positive RIP1-Tag2 mice, and their littermates was determined. As can be seen in Table 4, there is no difference in longevity in the two groups of RIP1-Tag2 mice, while their non-T antigen expressing littermates have a normal life span. Incidence of tumors in the two groups of mice was also explored. Gross inspection of pancreases from seven ß$_2$mKO/+ and nine ß$_2$mKO/KO RIP1-Tag2 mice revealed no obvious difference in tumor

Table 4: *Longevity of RIP1-Tag 2 mice deficient for the expression of β_2-microglobulin.* Average life span ± the standard deviation was calculated for all animals in each group. The number of animals in each group (n) is included in the table.

Genotype	***n***	***Average Lifespan in weeks***
β_2mKO/+	12	≥43
β_2mKO/KO	10	≥43
β_2mKO/+; RIP1-Tag2	21	14.8 ± 2.2
β_2mKO/KO; RIP1-Tag2	9	15.6 ± 3.2

Table 4

number or size (data not shown). Thus, the immune system does not appear to limit the development of tumors in RIP1-Tag2 mice.

Autoimmune Phenotype of RIP1-Tag5 Mice Deficient for Expression of Class I MHC

CD8+ T lymphocytes have been implicated in the autoimmune recognition and destruction of pancreatic ß cells in delayed onset RIP-Tag mice (Skowronski et al., 1990) and in other models of autoimmunity, such as the NOD mouse. To examine the role that CD8+ T cells play in the initiation and progression of spontaneous autoimmunity in our model, RIP1-Tag5 mice were bred to $ß_2$-microglobulin negative mice which had been backcrossed to C3HeBFe mice for 12 generations. It was necessary to backcross the $ß_2$-microglobulin mutation into C3HeBFe for 12 generations, since a gene influencing the expression of the RIP-Tag transgene in the pancreatic ß cells of RIP1-Tag5 mice appeared to be linked to the $ß_2$-microglobulin locus (data not shown). This was apparent when mice from the eighth backcross generation were crossed to RIP1-Tag5 mice and the expression of T antigen in the pancreas decreased as the number of copies of the wild type allele decreased: $ß_2$m+/+ mice had the highest level of T antigen expression, $ß_2$mKO/+ an intermediate level, and $ß_2$mKO/KO the lowest level. It is likely that this gene came from the parental strain of the $ß_2$mKO mice, 129. Mice intercrossed at the twelfth backcross generation showed no such gene dosage effect (data not shown).

RIP1-Tag5 mice develop spontaneous autoantibodies and infiltration of their T antigen expressing islets. Therefore, both phenotypes were examined in mice retaining and lacking $CD8^+$ T cells. First, mice were bled and analyzed for the appearance of spontaneous autoantibodies to T antigen (Figure 10). $ß_2$mKO/+; RIP1-Tag5 mice developed a similar incidence and titer of autoantibodies to T antigen as inbred RIP1-Tag5 mice. However, in $ß_2$mKO/KO; RIP1-Tag5 the appearance of autoantibodies was retarded. This delay manifests as a decreased incidence and titer relative to age matched $ß_2$mKO/+; RIP1-Tag5 mice. A linear regression analysis of the data reveals the overall trend in the two groups of mice.

RIP1-Tag5 mice begin expressing T antigen at nine to ten weeks of age in a few scattered ß cells, in a few of their islets. As the mice get older, the percentage of ß cells expressing T antigen increases, and by 17 weeks of age over 50% of the islets contain T antigen expressing ß cells. By this age the mice have developed significant lymphocytic infiltration of their T antigen expressing islets. To determine the degree of infiltration of these T antigen expressing islets, pancreatic sections from 17 and 20 week old RIP1-Tag5 mice, of all three $ß_2$-microglobulin genotypes, $ß_2$m+/+, $ß_2$mKO/+, and $ß_2$mKO/KO, were immunostained for T antigen, $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and MHC class I and class II. Since not all the islets in RIP1-Tag5 mice express T antigen, first the number of islets, then the number of T antigen expressing islets, and finally the number of T antigen expressing islets infiltrated with $CD4^+$ T and B lymphocytes was determined for each mouse. On average the percentage of islets expressing T antigen in $ß_2$m+/+; RIP1-Tag5, $ß_2$mKO/+; RIP1-Tag5, and $ß_2$mKO/KO; RIP1-Tag5 was 61.9 ± 3.4, 50.7 ± 8.9, and 49.4 ± 10.0 at 17 weeks of age and 62.1 ± 21.6, 79.5 ±

Figure 10: *Development of spontaneous autoantibodies in RIP1-Tag5 β_2-microglobulin negative mice.* Sera from littermates were tested in an ELISA for the presence of spontaneous autoantibodies recognizing T antigen and the ng of anti-T antigen autoantibody plotted against the age at which the mouse was bled. The data were used to perform a linear regression analysis for all animals in each group: ß$_2$mKO/+; RIP1-Tag5 (△, dashed line); ß$_2$mKO/KO; RIP1-Tag5 (●, solid line); ß$_2$mKO/+ (◇) and ß$_2$mKO/KO (O). The data for ß$_2$mKO/+ and ß$_2$mKO/KO are pooled for the linear regression analysis (dotted line).

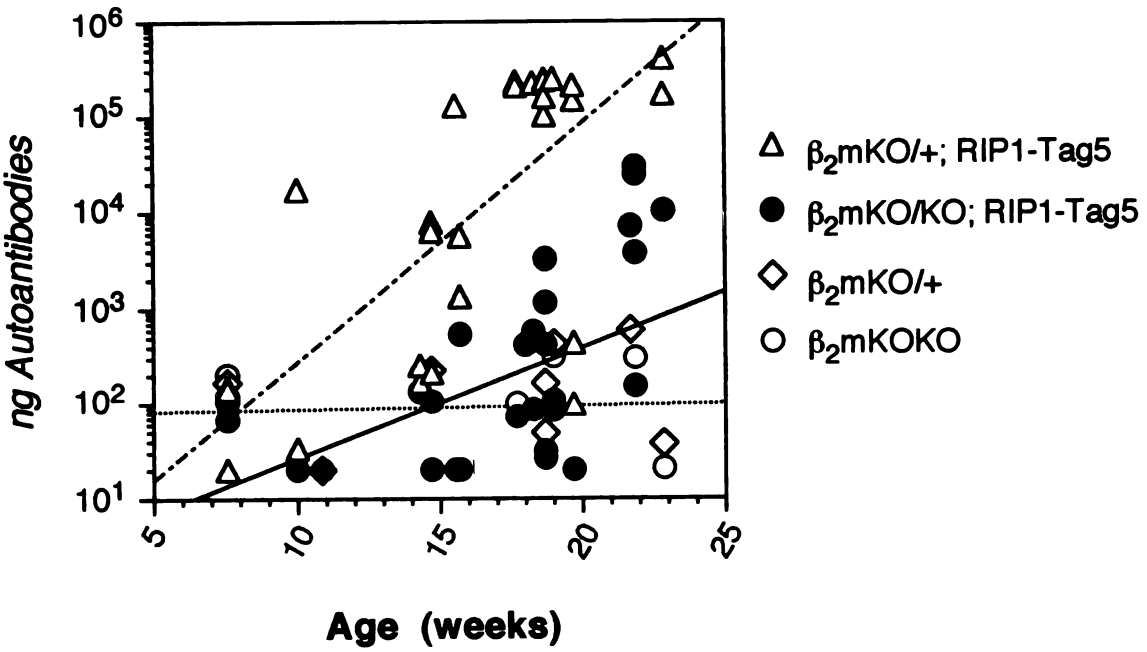

ng Autoantibodies
10^6
10^5
10^4
10^3
10^2
10^1
5
10
15
20
25
Age (weeks)
β2mKO/+; RIP1-Tag5
β2mKO/KO; RIP1-Tag5
β2mKO/+
β2mKOKO

Figure 10

19.3, and 52.5 ± 1.4 at 20 weeks of age, respectively. The percentage of T antigen expressing islets infiltrated with lymphocytes for each individual, and the mean and standard deviation of all animals in each group is plotted in Figure 11. A significant amount of variability is observed in the percentage of islets infiltrated within each group. Nonetheless, both groups of ß$_2$-microglobulin expressing RIP1-Tag5 mice, ß$_2$m+/+ and ß$_2$mKO/+, clearly show higher indices of infiltration of their T antigen positive islets at both ages than their ß$_2$mKO/KO; RIP1-Tag5 littermates. Only one 20 week old ß$_2$mKO/KO; RIP1-Tag5 mouse has more than 15% of its islets infiltrated, 25.7%, which is lower than all of the 20 week old class I MHC expressing individuals studied. In addition, the severity of the islet infiltration was less in the ß$_2$-microglobulin deficient RIP1-Tag5 mice than in the class I expressing RIP1-Tag5 mice (data not shown). Of the islets which were infiltrated with lymphocytes in ß$_2$-microglobulin deficient RIP1-Tag5 mice, a greater percentage had a mild form of infiltration, peri-islet infiltration, rather than the severe form, intra-islet infiltration. Therefore, both the severity and incidence of islet infiltration is reduced in the absence of functional MHC class I molecules and CD8$^+$ T cells. Moreover, it should be noted that despite the lack of CD8$^+$ T lymphocytes the islets can still be recognized by and infiltrated with both CD4$^+$ T and B lymphocytes. Thus, CD8$^+$ T cells are not necessary for recognition of islet cell antigens in RIP1-Tag5 mice, although the extent of infiltration is reduced in their absence. (This study was performed in collaboration with Christine Jolicoeur who performed the immunohistochemical analysis.)

Figure 11: *Infiltration of T antigen expressing islets is decreased in RIP1-Tag5 ß2-microglobulin negative mice.* The percentage of the T antigen expressing islets that are infiltrated with $CD4^+$ T and B lymphocytes for individual 17 week old and 20 week old RIP1-Tag5 mice is plotted: ß2m+/+; RIP1-Tag5 (◇); ß2mKO/+; RIP1-Tag5 (△); and ß2mKO/KO; RIP1-Tag5 (●). The mean percentage for all animals in each group is shown by a bar and the standard deviation is indicated by the error bars. The immunohistochemical analysis represented here was performed by Christine Jolicoeur.

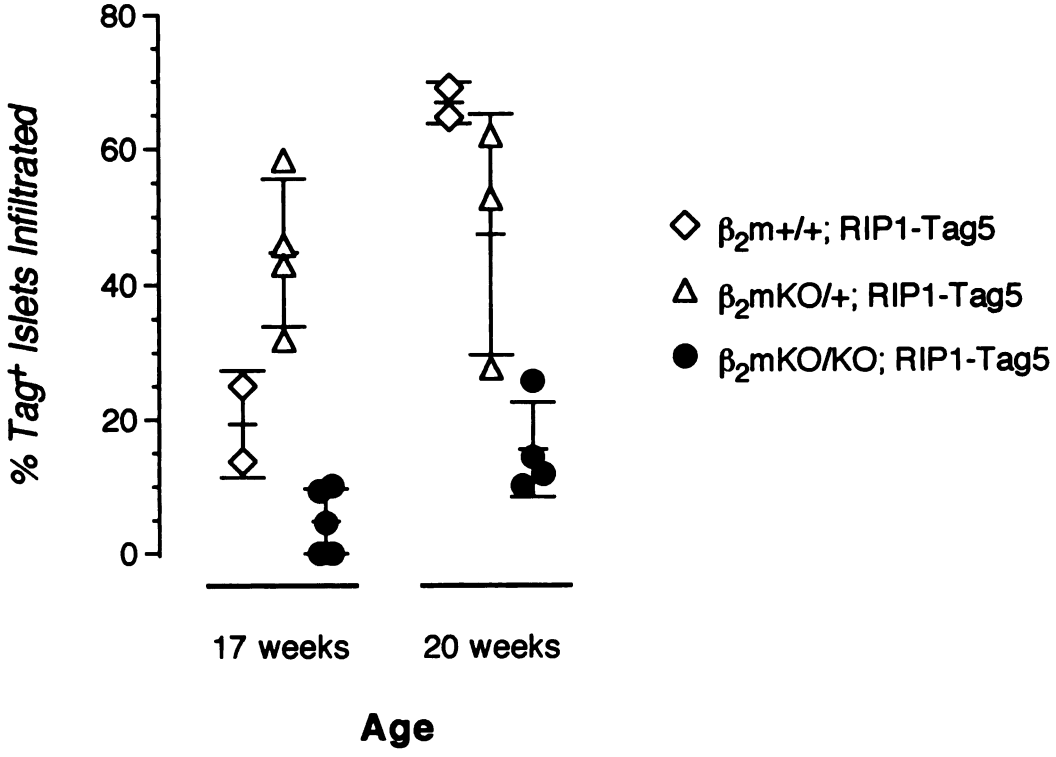

80
60
40
20
0
% Tag+ Islets Infiltrated
17 weeks
20 weeks
Age
β2m+/+; RIP1-Tag5
β2mKO/+; RIP1-Tag5
β2mKO/KO; RIP1-Tag5

Figure 11

Tumor incidence in ß2-microglobulin negative RIP1-Tag5 mice.

RIP1-Tag5 mice, despite the autoimmune attack against their T antigen expressing ß cells, still develop insulinomas. Since autoimmune infiltration of the islets is reduced in RIP1-Tag5 mice deficient for class I MHC expression and $CD8^+$ T cells, these mice may develop tumors more quickly than fully immunocompetent RIP1-Tag5 mice. To test this prediction of a more aggressive tumor progression, the number of tumors in pancreases of ß2-microglobulin deficient and proficient RIP1-Tag5 mice between 21 and 30 weeks of age, was determined. Tumors from collagenase digested pancreases were collected and counted (Christofori et al., 1994). The number of tumors in each pancreas is plotted against the age of the mouse in Figure 12. Contrary to the prediction of a more vigorous tumor development in these immunocompromised mice, similar or even fewer numbers of tumors developed in RIP1-Tag5 mice deficient for ß2-microglobulin expression as compared to age matched ß2-microglobulin proficient RIP1-Tag5 mice. A linear regression analysis of the data also indicates that the average number of tumors which developed in the absence of functional MHC class I expression is decreased relative to controls. (This study was performed in collaboration with Christine Jolicoeur who performed the tumor progression analysis.)

Figure 12: *Tumor incidence is decreased in RIP1-Tag5 β_2-microglobulin negative mice.* Collagenase digestion of pancreases was performed to free tumors from exocrine tissue and the number of tumors in each mouse counted. The age of each mouse is plotted against the number of tumors present in the pancreas, Linear regression analysis of the data was performed for all animals in both groups: ß$_2$mKO/+; RIP1-Tag5 (△, dashed line) and ß$_2$mKO/KO; RIP1-Tag5 (●, solid line). This analysis was executed by Christine Jolicoeur and planned by both of us.

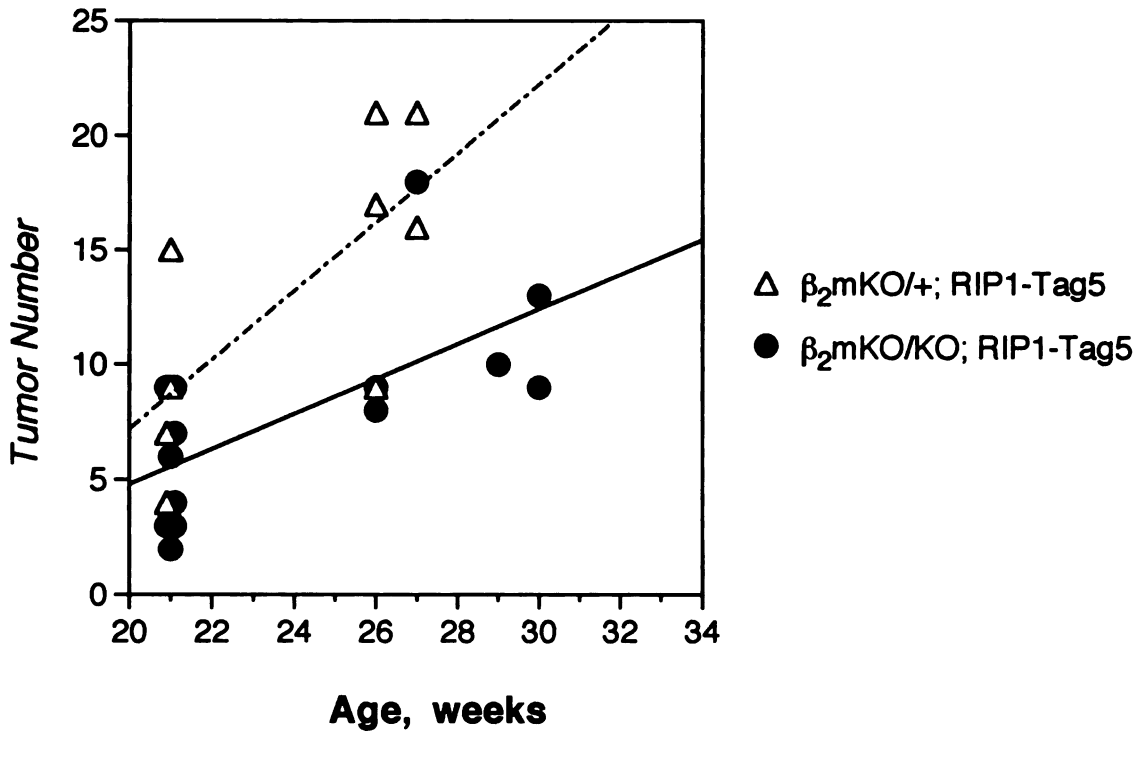

25
20
15
10
5
0
20
22
24
26
28
30
32
34
Tumor Number
Age, weeks
Δ β2mKO/+; RIP1-Tag5
● β2mKO/KO; RIP1-Tag5

Figure 12

Mapping the RIP1-Tag2 transgene insertion site.

In my initial attempts to generate RIP1-Tag2 mice homozygous for the targeted disruption of ß$_2$-microglobulin, ß$_2$mKO/+; RIP1-Tag2 mice were crossed to ß$_2$mKO/KO animals. From this cross one would predict the progeny to be 25% ß$_2$mKO/+, 25% ß$_2$mKO/+; RIP1-Tag2, 25% ß$_2$mKO/KO, and 25% ß$_2$mKO/KO; RIP1-Tag2, if the genes assort randomly. But this was not the case. The results from 59 such crosses are summarized in Table 5. Of the 413 pups analyzed, 79.6% were of the parental types, ß$_2$mKO/KO and ß$_2$mKO/+; RIP1-Tag2, and 20.4% were of the non-parental types, ß$_2$mKO/+ and ß$_2$mKO/KO; RIP1-Tag2. This distribution suggests that the wild type ß$_2$m allele and the RIP1-Tag2 transgene segregate together. The p value from a χ^2 analysis is $<<0.005$, indicated that the two genes are linked. Since ß$_2$m is know to lie at map position 54 on chromosome 2 (Figure 13), the RIP1-Tag2 transgene insertion site can be localized to chromosome 2, 20.3 cM from the ß$_2$m locus.

If the above distribution of progeny is due to linkage of the two genes and not a toxic gene combination, one would predict that once the RIP1-Tag2 and ß$_2$mKO alleles become linked on the same chromosome by a cross-over event, they would then cosegregate. To test this prediction ß$_2$mKO/KO; RIP1-Tag2 mice were bred to ß$_2$mKO/+ mice (Table 5). Of the 205 progeny of such crosses, 48.3% were of the parental types, ß$_2$mKO/+ and ß$_2$mKO/KO; RIP1-Tag2, and 51.7% were of the non-parental types, ß$_2$mKO/KO and ß$_2$mKO/+; RIP1-Tag2. This distribution of genotypes confirms that the distribution observed in the original crosses was not due to a toxic gene combination.

Table 5: *Linkage analysis of RIP1-Tag2 transgene insertion site and β_2m and agouti loci.* Crosses were performed as indicated in the subheadings. A^w is the dominant agouti allele contributed by the embryonic stem cells used to generate the β_2m deficient mice. 'a' is the recessive agouti allele contributed from C57BL/6 mice.

CROSS:

I. β_2mKO/+; RIP1-Tag2 x β_2mKO/KO

Genotype	Pups	% of Total	% Parental	% Non-Parental
β_2mKO/+	54	13.1%	-	13.1%
β_2mKO/KO	148	35.8%	35.8%	-
β_2mKO/+; RIP1-Tag2	181	43.8%	43.8%	-
β_2mKO/KO; RIP1-Tag2	30	7.3%	-	7.3%
Total:	413	100.0%	79.6%	20.4%

II. β_2mKO/KO; RIP1-Tag2 x β_2mKO/+

Genotype	Pups	% of Total	% Parental	% Non-Parental
β_2mKO/+	54	26.3%	26.3%	-
β_2mKO/KO	55	26.8%	-	26.8%
β_2mKO/+; RIP1-Tag2	51	24.9%	-	24.9%
β_2mKO/KO; RIP1-Tag2	45	22.0%	22.0%	-
Total:	205	100.0%	48.3%	51.7%

III. a/a; -/- x A^w/a; -/RIP1-Tag2

Genotype	Pups	% of Total	% Parental	% Non-Parental
a/a	5	9.8%	9.8%	-
A^w/a	17	33.3%	-	33.3%
a/a; -/RIP1-Tag2	16	31.4%	-	31.4%
A^w/a; -/RIP1-Tag2	13	25.5%	25.5%	-
Total:	51	100.0%	35.3%	64.7%

Table 5

Figure 13: *Genetic map of mouse chromosome 2.* This abbreviated map shows the some landmarks for orientation. Loci relevant to the genetic mapping studies are in bold. The map position where the RIP1-Tag2 transgene has inserted is indicated by a vertical bar. Adapted from Hillyard and coworkers (1993).

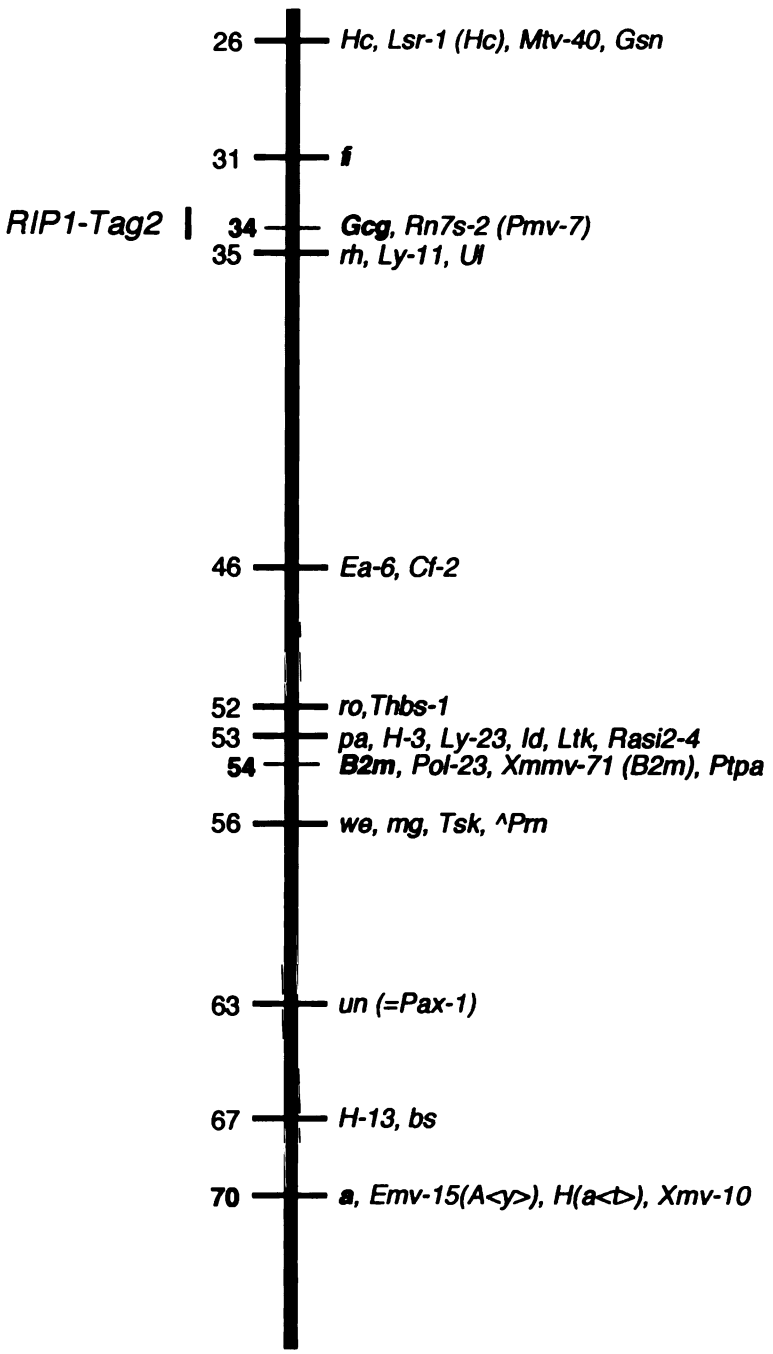

RIP1-Tag2
26
Hc, Lsr-1 (Hc), Mtv-40, Gsn
31
fi
34
Gcg, Rn7s-2 (Pmv-7)
35
rh, Ly-11, Ul
46
Ea-6, Cf-2
52
ro,Thbs-1
53
pa, H-3, Ly-23, Id, Ltk, Rasi2-4
54
B2m, Pol-23, Xmmv-71 (B2m), Ptpa
56
we, mg, Tsk, ^Prn
63
un (=Pax-1)
67
H-13, bs
70
a, Emv-15(A<y>), H(a<t>), Xmv-10

Figure 13

A similar analysis of RIP1-Tag3 transgene insertion site and ß$_2$m was also performed as a control (Table 6). When ß$_2$mKO/+; RIP1-Tag3 mice were crossed to ß$_2$mKO/KO, no linkage was revealed. Reciprocal crosses of ß$_2$mKO/KO; RIP1-Tag3 mice to ß$_2$mKO/+ showed similar distributions.

Although I had mapped the RIP1-Tag2 insertion site to chromosome 2, it could lie 20 cM either centromeric or telomeric of ß$_2$m. Since the agouti locus, a, a marker that I could easily follow, is also on chromosome 2, 16 cM from the ß$_2$m locus at map position 70, I could easily determine whether the RIP1-Tag2 transgene lies centromeric or telomeric of ß$_2$m. If RIP1-Tag2 is centromeric of ß$_2$m it will show very weak linkage with the recessive agouti allele, a. If RIP1-Tag2 is telomeric of ß$_2$m, then it will be tightly linked to a, about 4 cM away. To test the linkage, agouti RIP1-Tag2 mice, which had the recessive agouti allele, a, and RIP1-Tag2 on one chromosome 2 and the dominant agouti allele, A^w, on their other chromosome 2, were bred to non-transgenic C57BL/6J mice, which carry two copies of the recessive agouti allele, a (Table 5). If the two genes are tightly linked, the recessive agouti allele would segregate with RIP1-Tag2, and only four percent of the progeny would have agouti coat color, that is carry the A^w allele, and carry the RIP1-Tag2 transgene. Instead, twenty-five percent, the expected distribution for randomly assorting genes, possessed this gene combination. Of the 51 progeny analyzed, 35.3% were of the parental types and 64.7% were of the non-parental types. A χ^2 analysis was performed and the p value is between 0.1 and 0.05. This analysis provides evidence that the two genes are unlinked. To prove this hypothesis unambiguously it would take a much larger sample size than the one examined here. Nevertheless, the above analysis clearly indicates that RIP1-Tag2 and agouti are not closely linked,

Table 6: *Linkage analysis of RIP1-Tag3 transgene insertion site and $\beta_2 m$ locus.* **Crosses were performed as indicated in the subheadings.**

CROSS:

IV. $ß_2$mKO/+; RIP1-Tag3 x $ß_2$mKO/KO

Genotype	Pups	% of Total	% Parental	% Non-Parental
$ß_2$mKO/+	23	35.9%	35.9%	-
$ß_2$mKO/KO	15	23.4%	-	23.4%
$ß_2$mKO/+; RIP1-Tag3	13	20.3%	-	20.3%
$ß_2$mKO/KO; RIP1-Tag3	13	20.3%	20.3%	-
Total:	64	100.0%	56.3%	43.8%

V. $ß_2$mKO/KO; RIP1-Tag3 x $ß_2$mKO/+

Genotype	Pups	% of Total	% Parental	% Non-Parental
$ß_2$mKO/+	9	22.0%	22.0%	-
$ß_2$mKO/KO	7	17.1%	-	17.1%
$ß_2$mKO/+; RIP1-Tag3	13	31.7%	-	31.7%
$ß_2$mKO/KO; RIP1-Tag3	12	29.3%	29.3%	-
Total:	41	100.0%	51.2%	48.8%

Table 6

thus placing the RIP1-Tag2 transgene insertion site 20 cM centromeric of ß$_2$m, at map position 34. The glucagon gene has been previously mapped to this site. This raises the possibility that the RIP1-Tag2 transgene, which is under the control of the rat insulin promoter, has integrated adjacent to a locus which is normally expressed by another pancreatic endocrine cell type. Linkage analysis of RIP1-Tag2 and markers known to lie near map position 34 would confirm that the RIP1-Tag2 transgene insertion site is tightly linked to the glucagon locus.

SUMMARY AND CONCLUSIONS

The use of ß$_2$-microglobulin deficient mice in conjunction with RIP-Tag transgenic mice has allowed the investigation of the role of CD8$^+$ T cells and class I MHC expression in the induction of tolerance in RIP1-Tag2 mice, in the development of autoimmunity in RIP1-Tag5 mice, and in tumor progression in both families of transgenic mice. While the absence of class I MHC and CD8$^+$ T cells did not alter the induction of tolerance in RIP1-Tag2 mice, it did result in altered kinetics and reduced severity of autoimmunity in RIP1-Tag5 mice. The lack of CD8$^+$ T cells and functional natural killer cells in RIP1-Tag2 ß$_2$-microglobulin null mice did not alter tumor number, tumor size, or longevity. In contrast, RIP1-Tag5 ß$_2$-microglobulin null mice developed fewer tumors than their class I MHC expressing littermates. Unexpectedly, the cross of RIP1-Tag2 to ß$_2$-microglobulin null mice revealed that the RIP1-Tag2 transgene insertion site is 20 cM from the ß$_2$-microglobulin locus. Additional mapping studies demonstrated that the

RIP1-Tag2 transgene had integrated adjacent to the glucagon locus, another endocrine specific gene. The integration of the RIP1-Tag2 transgene adjacent to another neuroendocrine gene may explain the observation that T antigen is expressed by several neuroendocrine cell types, embryonic brain and neuroendocrine cells of the small intestine, as described in chapter 3.

The tolerance which develops in RIP1-Tag2 mice in the absence of MHC class I and $CD8^+$ T cells is indistinguishable from that in fully immunocompetent RIP1-Tag2, as assessed by production of T antigen specific antibody and in vitro proliferation of lymphocytes to T antigen. Since both of these assays rely on the function of $CD4^+$ T cells for their read-out, $CD8^+$ T cells do not appear to exert a regulatory role on the T antigen reactive $CD4^+$ T cells. The analysis performed here does not pertain to any role that $CD4^+$ T cells might play in regulating the $CD8^+$ T cells which recognize T antigen presented by MHC class I. The cytotoxic T cell activity was not measured in these mice, since $ß_2$-microglobulin deficient mice lack measurable cytotoxic T cell responses (Zijlistra et al., 1990). The induction and maintenance of tolerance to T antigen in the absence of MHC class I and $CD8^+$ T cells underscores the importance of $CD4^+$ T cells in limiting the immune response to T antigen. This tolerance must be induced by T antigen which has gained access to the class II MHC antigen processing compartment of specialized antigen presenting cells. The site of this tolerance induction and the cell type inducing it will be addressed in chapters 6 and 7.

In contrast to the inability to detect an altered phenotype in RIP1-Tag2 mice deficient in $ß_2$-microglobulin, the development of an autoimmune response to T antigen in RIP1-Tag5 mice lacking MHC class I expression is delayed. Both the incidence and titer of anti-T antigen autoantibodies, as

well as both the incidence and severity of infiltration of T antigen expressing islets, were reduced relative to β_2-microglobulin expressing RIP1-Tag5 mice. In addition, both phenotypes also appeared with delayed kinetics. This implicates $CD8^+$ T cells as important effectors in the induction of autoimmunity directed at the pancreatic ß cells. However, these findings also demonstrates that $CD8^+$ T lymphocytes are not required for the initiation of infiltration of T antigen expressing islets.

Immune surveillance of tumors has been assessed in both RIP1-Tag2 and RIP1-Tag5 mice deficient for the MHC class I expression. Remarkably, the lack of $CD8^+$ T cells and functional natural killer cells in RIP1-Tag2 mice lacking class I does not alter tumor number, tumor size, or longevity of these mice. However, this deficiency does alter the tumor phenotype of RIP1-Tag5 mice. Since the autoimmune response directed at the T antigen expressing islets in RIP1-Tag5 β_2-microglobulin null mice exhibited delayed kinetics and reduced severity relative to their MHC class I positive RIP1-Tag5 mice, the immune surveillance of their developing tumors would be expected to be likewise reduced, resulting in an increased rate of tumor formation. Strikingly, tumor development in RIP1-Tag5 deficient for MHC class I expression is not increased, as predicted, but, in fact, is reduced relative to RIP1-Tag5 controls. One possible explanation is that the lymphocytes infiltrating the islets secrete cytokines or other stimulatory factors which actually promote tumor progression. An observation that bears on this point is the greater number of tumors that develop in RIP1-Tag5 mice, which develop lymphocytic infiltration of their islets, than in embryonic onset RIP1-Tag2 mice, which are unable to mount an immune response against their T antigen expressing ß cells. Although there is no direct

evidence to support this hypothesis, if confirmed, it could have an important impact on the current efforts to harness the immune system to control cancer in humans.

CHAPTER 5:

Tolerance of Lymphocyte Subsets

INTRODUCTION

The tolerance of embryonic onset RIP-Tag mice has been characterized using three T cell dependent assays: i) production of antibody recognizing T antigen, ii) in vitro proliferation of primed lymph node cells, and iii) a cytotoxic T cell assay. However, these assays do not necessarily measure tolerance of specific lymphocyte types, since each of these response requires the interaction of several different cell types, B cells and $CD4^+$ and $CD8^+$ T lymphocytes, as well as antigen presenting cells, to produce the final outcome.

The ability of B cells to make antibody to T antigen requires that they receive help from $CD4^+$ T helper cells. Thus, their inability to make T antigen specific antibody may reflect tolerance of the $CD4^+$ T cells, not tolerance of the B cells themselves. In chapter 2, it was argued that the amount of T antigen protein produced by the pancreatic ß cells, even if released in its entirety into the bloodstream, is theoretically too low to tolerize the B cells. In fact, this level may be insufficient to tolerize the T cells. For a soluble protein, as little as 10^{-9} M in the bloodstream appears adequate to induce tolerance of T cells (Adelstein et al., 1990), while a protein level of at least 10^{-6} M is required for tolerization of B cells (Mitchison et al., 1984). Although it is unlikely that the B cells are tolerant in RIP1-Tag2 and RIP3-Tag2 mice, this has not been formally addressed.

The cytotoxic T cell assay measures the ability of cytotoxic $CD8^+$ T cells, generated by immunization in vivo and an in vitro boost, to lyse T antigen expressing fibroblasts. In this assay, $CD4^+$ and $CD8^+$ T lymphocytes from whole spleen are co-cultured to generate the cytotoxic effectors. Thus, the inability of embryonic onset RIP-Tag mice to generate T antigen specific cytotoxic T cells may reflect direct and complete tolerization of $CD8^+$ T cells, the product of partial tolerization of both $CD4^+$ and $CD8^+$ T cells, or simply lack of antigen specific $CD4^+$ T cell help during the generation of T antigen specific $CD8^+$ cytotoxic effectors. The cytotoxic T cell responses against some viruses and against allogeneic cells can be generated by $CD8^+$ T cells in the absence of help from $CD4^+$ T cells, both in vivo and in vitro (Doherty et al., 1992; Kosaka and Sprent, 1993). However, to generate helper-independent cytotoxic T cells in all of these systems, IL-2, a growth factor which is produced by both $CD8^+$ T and $CD4^+$ Th1 type lymphocytes and is required for the proliferation of the $CD8^+$ T lymphocytes, must be added to cultures during the in vitro restimulation.

The in vitro proliferation assay, which revealed a reduced capacity of transgenic T lymphocytes to proliferate in response to the soluble T antigen protein presented by class II MHC molecules in the culture, demonstrates that the $CD4^+$ T cells are tolerant of T antigen. Nonetheless, these cultured lymphocytes from tolerant RIP-Tag mice still exhibit a residual proliferative response to T antigen at high antigen concentration. Moreover, $CD4^+$ T cells are not a homogeneous population, but include immature T helper cells, Th0 cells, which can differentiate into Th1 or Th2 type effectors. Th1 cells produce a set of cytokines, which include IL-2 and IFN-γ, that promote inflammatory type immune responses, such as generation of cytotoxic T

cells, activation of macrophages, and production of complement fixing antibody. Release of cytokines such as IFN-γ also inhibits the function of Th2 type cells. In turn, Th2 type cells make a different set of cytokines, which include IL-4 and IL-10. These promote generation of non-complement fixing antibody and allergic type reactions, and conversely inhibit immune functions mediated by Th1 cells (O'Garra and Murphy, 1993; Finkelman, 1995). Thus, Th1 and Th2 cells not only produce distinct sets of cytokines which promote divergent types of immune reactions, they suppress each others T helper function. Therefore, the residual $CD4^+$ T cells in tolerant RIP-Tag mice, which can respond to T antigen, may have developed into either Th1 or Th2 type effectors, which could then provide some help for immune responses involving B cells and $CD8^+$ T cells.

In this chapter I will examine the relative tolerance induction of each of these lymphocyte populations in RIP1-Tag2 and RIP3-Tag2 mice. First, to assess tolerance of the B cells, a classical hapten-carrier experiment will be performed. Next, to determine whether the residual $CD4^+$ T cell response is predominated by Th1 or Th2 type effectors, the isotypes of the anti-T antigen antibodies produced by tolerant RIP-Tag mice will be examined, since Th1 and Th2 cells promote isotype switching to different IgG subclasses. Additionally, the ability of alum, which induces IgG_1 synthesis by B cells, an isotype promoted by Th2 type cells, will be compared to that of complete Freund's adjuvant, which stimulates production of multiple IgG isotypes, for their ability to induce T antigen specific antibody. Third, to ask if tolerant RIP-Tag mice can generate T antigen specific $CD8^+$ cytotoxic T cells, IL-2 will be added to the cytotoxic T cell assay during the in vitro restimulation to provide an exogenous source of this essential T cell growth factor.

MATERIALS AND METHODS

Hapten-Carrier experiment. The T antigen-KLH (keyhole limpet hemocyanin) conjugate was prepared by CalTag (South San Francisco, CA) using 4 mg T antigen protein and 1 mg of KLH. Six week old mice were immunized with 15 μg of conjugate and 5 μg of KLH emulsified in complete Freund's adjuvant HR37a, and boosted twice with the same amount of protein, two and four weeks later, in incomplete Freund's adjuvant. Mice were bled six day after each boost. Sera were tested in an ELISA as described in chapter 2, except that titers were calculated for each sample. The titer was defined as the serum dilution which gave a half maximal response. The tertiary response is shown.

Isotype Analysis. Six week old mice were immunized with a mixture of 10 μg of T antigen and 5 μg of ß-galactosidase emulsified in complete Freund's adjuvant, and boosted twice, two and four weeks later, with the same amount of both antigens in incomplete Freund's adjuvant. Sera were tested in an ELISA, as described in Chapter 2, except that the isotypes of the antigen specific IgG were determined, as well as the total antigen specific IgG response. Biotinylated anti-mouse IgG_1, anti-mouse IgG_{2a}, anti-mouse IgG_{2a}, anti-mouse IgG_3, and anti-total IgG (Fisher Scientific) were used as secondary reagents. A titer was calculated for each response to both antigens at the lowest dilution to give a positive response. The mean titer of the non-transgenic mice for each antigen specific response was calculated. The titer for each animal's antigen-specific, IgG isotype response was then divided by mean response of the control mice to get its fractional response to T antigen

and to ß-galactosidase for each isotype. The fractional response to T antigen for each animal was then divided by its fractional response to ß-galactosidase to get the T antigen/ß-galactosidase ratio for each animal. This calculation was performed for each isotype. This calculation normalizes the T antigen response for each animal to its response to ß-galactosidase and defines the mean isotype specific response of the controls as one.

Comparison of Alum and Freund's Adjuvant. Two groups of six week old transgenic and non-transgenic mice were immunized with a mixture of 10 µg of T antigen and 5 µg of KLH using either alum, group one, or complete Freund's , group 2, as an adjuvant. Mice were boosted twice with the same amount of protein using either alum, group one, or incomplete Freund's, group 2, as the adjuvant. The sera were tested as described for the hapten carrier experiment.

Cytotoxic T cell Assay. The cytotoxic T cell assay was performed as described in Chapter 2, except that the splenocytes were cultured with irradiated C57SV either with or without added murine IL-2 at 10 U/ml (Boehringer Mannheim, Indianapolis, IN). After five days of culture splenocytes were tested for their capacity to lyse either T antigen expressing fibroblasts C57SV (Knowles et al., 1979) or MC57G (Trinchieri, et al., 1976), a syngeneic H-2^b fibroblastic cell line derived from a methylcholanthrene-induced tumor of a C57BL/6 mouse, kindly provided by B. B. Knowles.

RESULTS

B cells from Embryonic Onset RIP-Tag Mice Are Not Tolerant

In the previous humoral response assays, RIP1-Tag2 and RIP3-Tag mice were shown to produce on average 350-fold less antibody to T antigen than controls (Chapter 2). To answer the question of whether the B cells are tolerant or not, a classical hapten-carrier experiment was performed. B cells express antigen specific Ig receptors on their cell surface. These receptors efficiently bind and internalize their cognate antigen. This internalized antigen is processed and then presented by MHC class II molecules at the cell surface. If a $CD4^+$ T cell is present which can recognize this particular peptide-class II MHC complex presented by the B cell, it provides help to the B cell, which can then produce a secreted form of the antibody found on its cell surface. If no antigen specific $CD4^+$ T cell help is available to the B cell, no antibody is produced. To ask if B cells are unable to produce antibody to a specific antigen, in this case the 'hapten', the hapten antigen can be covalently coupled to another antigen, a 'carrier', to which the $CD4^+$ T cells are not tolerant. In this circumstance, when the B cell internalizes its cognate antigen, the 'hapten', it also internalizes the carrier antigen. The B cell will then present peptides generated from both antigens on class II MHC molecules on its cell surface and $CD4^+$ T cells specific for the carrier antigen will help the B cells to make antibody to the hapten. In this way, the lack of antigen specific help can be circumvented and the ability of the B cells to produce antibody of a given specificity can be uncovered.

To test the tolerance of the B cells in embryonic onset RIP-Tag mice, two groups of mice, each containing transgenic and non-transgenic animals, were tested for their ability to produce antibody to T antigen and, this time as a control, keyhole limpet hemocyanin (KLH), instead of ß-galactosidase. One group of animals was immunized with a mixture T antigen and KLH, and the other group was immunized with T antigen covalently coupled to KLH, a hapten carrier conjugate. If the B cells are tolerant, then the transgenic mice in neither group should produce much antibody to T antigen. However, if the B cells are not tolerant and rather the T cells are tolerant, then the transgenic mice in second group, the one which was immunized with the T antigen-KLH conjugate, should make as much T antigen specific antibody, as the control mice in that group, since the KLH specific T cells can provide help to the T antigen specific B cells.

As shown in Figure 14, the majority of the transgenic mice immunized with the T antigen-KLH conjugate made an antibody responses to T antigen as strong as that of the control mice in the same group. Overall, this group of mice made weak antibody responses to KLH and shows more variability than is typically observed with when mice are immunized either with T antigen and ß-galactosidase or a mixture of T antigen and KLH. The poor response to KLH may be due to denaturation of the protein during conjugate formation. Nevertheless, the fact that most of the transgenic mice produced as much antibody recognizing T antigen as the non-transgenic mice indicates that the transgenic B cells are not tolerant.

Surprisingly, the transgenic mice in the control group, the one immunized with a mixture of T antigen and KLH, made stronger antibody responses to T antigen than is typically seen when mice are immunized with

Figure 14: *RIP-Tag B cells are not tolerant of T antigen.* Non-transgenic and transgenic mice were immunized and boosted with T antigen and KLH or a T antigen-KLH conjugate. Antigen specific response to T antigen (●) and KLH (O) were determined in an ELISA. The mean response of all animals in each group is shown by a bar.

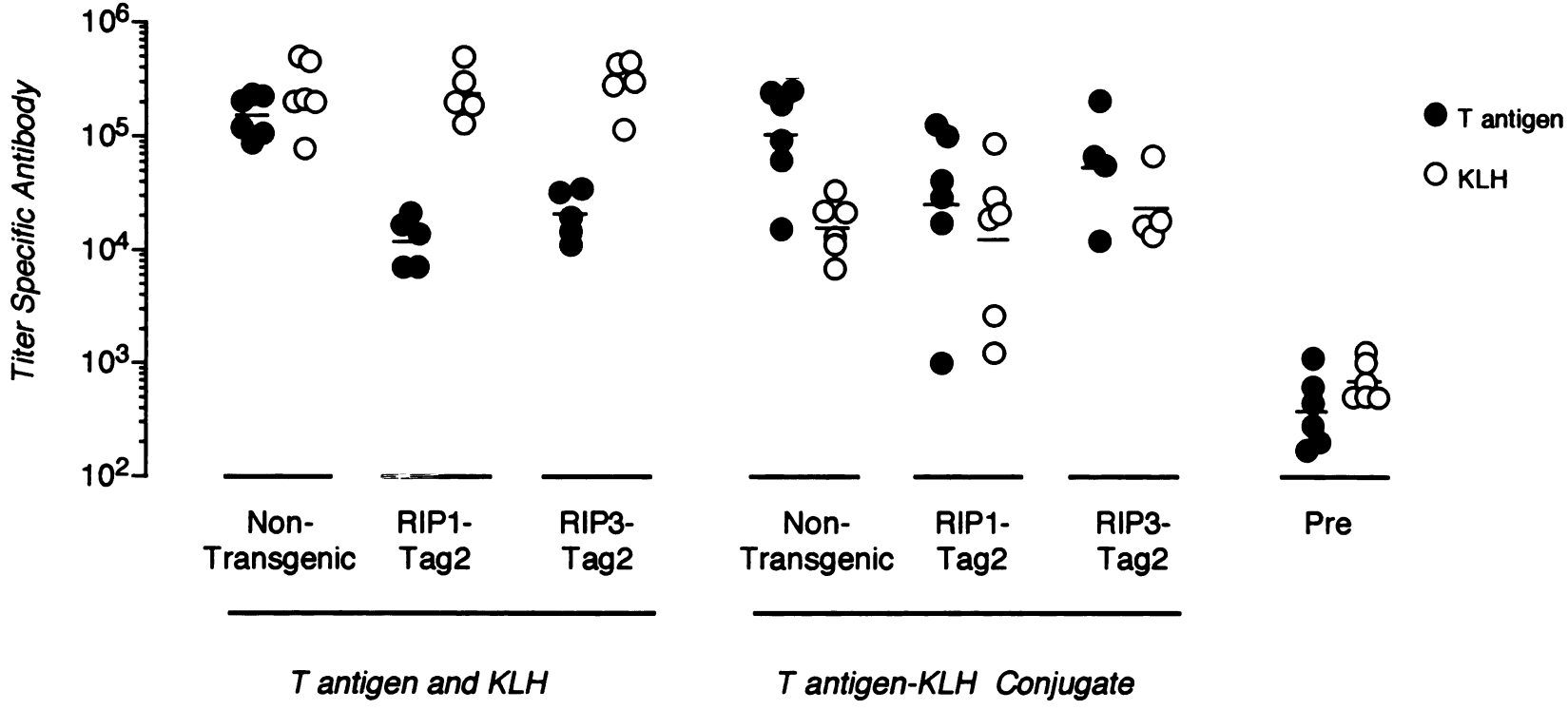

Titer Specific Antibody
10^6
10^5
10^4
10^3
10^2
T antigen
KLH
Non-Transgenic
RIP1-Tag2
RIP3-Tag2
Non-Transgenic
RIP1-Tag2
RIP3-Tag2
Pre
T antigen and KLH
T antigen-KLH Conjugate

Figure 14

T antigen and ß-galactosidase. The mean responses were 12.6 and 7.4 fold lower than those of the non-transgenic mice, for RIP1-Tag2 and RIP3-Tag2, respectively, versus the typical 350 fold reduction. One possible explanation is that the KLH itself has a non-specific stimulatory effect on the immune system. A second possibility is that the KLH is very sticky and has bound some T antigen non-covalently, resulting in a carrier-like effect. A third possibility is that the phenotype of the mice has drifted upon further backcrossing to C57BL/6. This is unlikely, since both independent lines of transgenic mice exhibit this effect. To assess these alternative explanations, two groups of mice, each with transgenic and non-transgenic mice, were immunized either with T antigen and KLH or T antigen and ß-galactosidase (data not shown). The transgenic mice immunized with T antigen and ß-galactosidase responded as previously observed, while some the transgenic mice immunized with T antigen and KLH made strong responses to T antigen. This suggests that KLH has a potent immunostimulatory effect on the cells producing antibody to T antigen. Whether this effect is due to a non-specific stimulatory effect or the ability of KLH to strongly bind T antigen is not known.

Th1 Type CD4$^+$ T Cells Develop More Readily Than Th2 Cells in Tolerant RIP-Tag Mice

Recent experiments have shown that CD4$^+$ Th1 and Th2 type cells can be differentially tolerized. The majority of these studies have focused on the induction of tolerance in T cell clones, but a few have focused on this

phenomenon in vivo (De Wit et al., 1992; Burstein et al., 1992; Romball and Weigle, 1993). In each of these studies, adult mice were pretreated with soluble antigen, as a tolerogen, and then challenged with the same antigen in adjuvant. Since Th1 cells produce IFN-γ which promotes IgG_{2a} production by B cells, while Th2 cells make IL-4 which promotes IgG_1 production (Finkelman, 1995), the isotype profiles of the antibody response, as well as cytokine production, were examined. The pretreatment of mice with soluble antigen resulted in a decreased Th1 response, measured as a deficiency of IgG_{2a} in vivo and a decreased production of IFN-γ in vitro. In the same animals, the Th2 response, as measured by the ability to produce IgG_1 and IgE in vivo and secrete IL-4 in vitro is affected to a lesser extent, or even increased. These studies suggest that the pretreatment of adult mice with soluble antigens may result in a preferential differentiation of immature antigen specific Th0 cells to Th2 type cells after immunization.

In the humoral response assay, RIP1-Tag2 and RIP3-Tag2 mice where shown to produce low titers antibody to T antigen after immunization. The mean response was decreased by approximately 350-fold in mice co-immunized with a mixture of T antigen and ß-galactosidase. Some animals were unable to make any antibody to T antigen, while others produced measurable antibody. To investigate whether the T cells providing help to the B cells are of the Th1 or Th2 type, the isotype profiles of a group of non-transgenic and transgenic mice which had made antibody to T antigen was examined. Since the mice had been immunized with both T antigen and ß-galactosidase, the antigen specific isotype profiles could be determined for both antigens. Figure 15 shows the relative production of the four IgG isotypes to T antigen and ß-galactosidase. All four transgenic mice examined

Figure 15: *Relative isotype specific antibody responses of RIP-Tag mice.* Sera from mice immunized with T antigen and ß-galactosidase, were tested in an ELISA for the presence of antigen specific, IgG isotypes. The antigen specific titer for total IgG, IgG1, IgG_{2a}, IgG_{2b}, and IgG_3 was determined for each animal by ELISA. The antigen specific, isotype specific response for each animal was divided by the mean response of the controls for the same response. The T antigen specific response divided by the response to ß-galactosidase for each animal is shown for non-transgenic (●), RIP1-Tag2 (△), and RIP3-Tag2 (O). This calculation serves to normalize the T antigen specific response of each animal to its response to ß-galactosidase and defines the mean response of the non-transgenic mice as one. The mean response for the non-transgenic and all transgenic mice is shown by a bar. The two major isotypes produced are IgG_1 and IgG_{2a}.

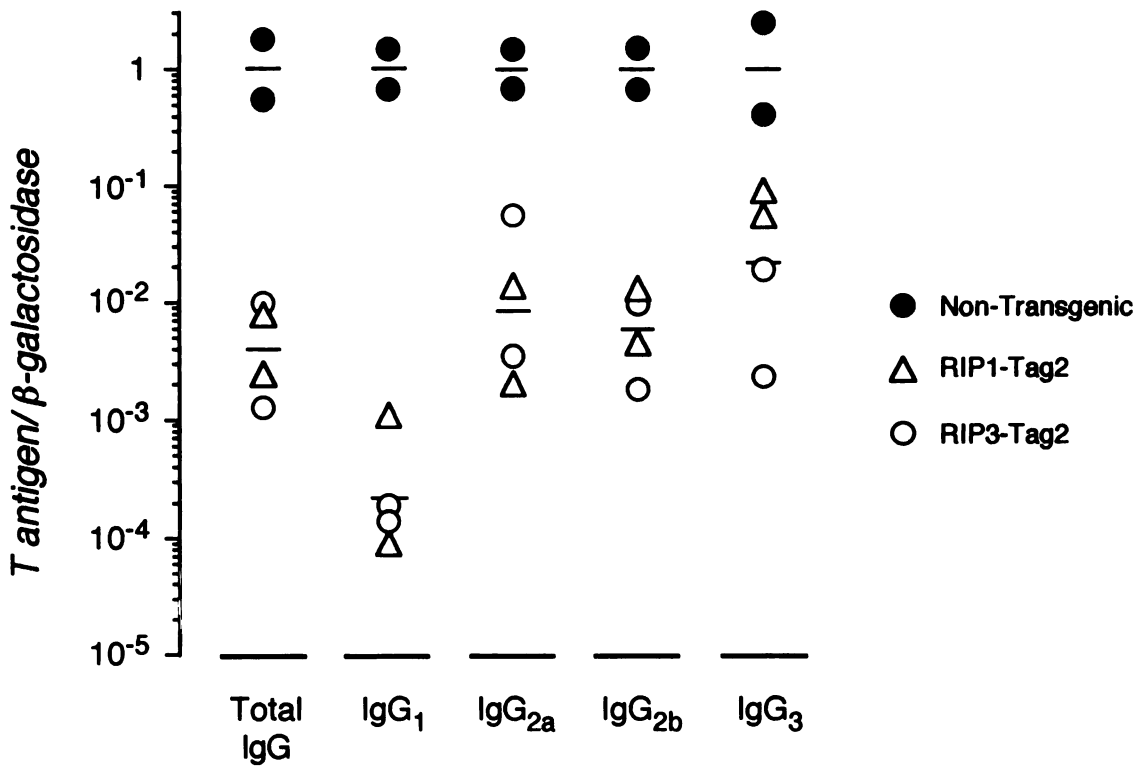

T antigen/β-galactosidase
1
10-1
10-2
10-3
10-4
10-5
Total IgG
IgG1
IgG2a
IgG2b
IgG3
Non-Transgenic
RIP1-Tag2
RIP3-Tag2

Figure 15

showed a deficiency of T antigen specific IgG_1, relative to the other isotypes. The isotype profiles of the anti-ß-galactosidase response were similar for non-transgenic and transgenic mice. These findings suggests that the T antigen specific $CD4^+$ T cells in RIP1-Tag2 and RIP3-Tag2 mice tend to differentiate into Th1 type effectors, which support cellular immunity, rather than Th2 type cells under these experimental conditions.

The mice in the previous experiment were immunized with a mixture of T antigen and ß-galactosidase emulsified in complete Freund's adjuvant, and then given two boosts of the same antigen combination in incomplete Freund's adjuvant. Freund's adjuvant is a potent stimulator of the immune system, and promotes production of multiple immunoglobulin isotypes. In contrast, a second adjuvant, alum, predominantly promotes the generation of IgG_1, a Th2 supported isotype (Enyon and Parker, 1992). Thus, by using alum as an adjuvant instead of Freund's, RIP-Tag mice could be tested under a second set of experimental conditions for their ability to generate T antigen specific Th2 type cells. As mentioned in the previous section, co-immunization of transgenic mice with T antigen and KLH results in a stronger anti-T antigen antibody response by RIP-Tag mice than co-immunization with T antigen and ß-galactosidase. Thus, by using KLH as the co-immunogen instead of ß-galactosidase, reduced responsiveness to T antigen when using alum as an adjuvant could be more readily observed. Therefore, two groups of mice, both containing non-transgenic and transgenic mice, were immunized with T antigen and KLH using either Freund's or alum as the adjuvant, and tested for the production of antigen specific IgG (Figure 16). The non-transgenic mice, immunized using alum as the adjuvant, made a more robust antibody response to T antigen than those

Figure 16: *Ability of alum and CFA to induce T antigen specific antibodies.* Non-transgenic and transgenic mice were immunized and boosted with T antigen and KLH using either Freund's adjuvant (CFA) or alum as an adjuvant. Titers to T antigen (●) and KLH (O) were calculated for each animal. The tertiary response of transgenic and non-transgenic mice is shown, as well as the background level detected in the preimmune sera (Pre) of both non-transgenic and transgenic mice. The control animals are the same as shown in Figure 14. The mean response of all animals in each group is shown by a bar.

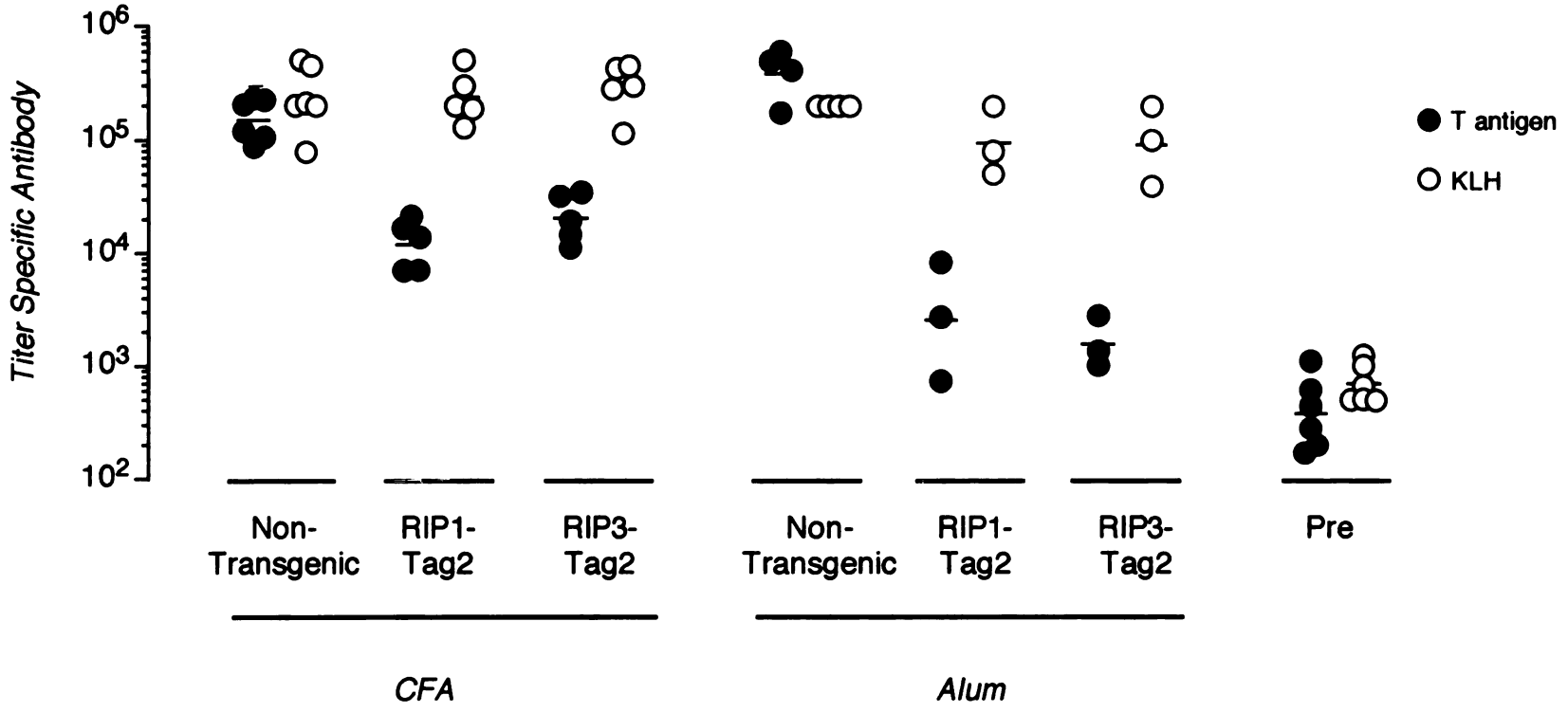


Figure 16

immunized with Freund's adjuvant. In contrast, the transgenic mice immunized with alum produced less T antigen antibody than those immunized with Freund's adjuvant. Overall, the difference in the response to T antigen of the non-transgenic and transgenic mice in the group immunized with alum is 20 times greater than that in the group immunized with Freund's, 250 times less versus 12.5 times less, respectively. Although the sera were assayed for the presence of antigen specific total IgG, alum promotes the almost exclusive production of IgG_1 (Enyon and Parker, 1992). Hence, it can be inferred that the reduced ability of alum, relative to Freund's adjuvant, to promote T antigen specific IgG in embryonic onset RIP-Tag mice, is attributable to decreased production of IgG_1. This result provides further support for the notion that differentiation of immature T antigen specific $CD4^+$ Th0 cells into Th2 type cells is more severely impaired than the differentiation into Th1 type cells in embryonic onset RIP-Tag mice.

Cytotoxic T Cell Tolerance Is Partially Overcome by IL-2

From the above analysis of the T antigen specific IgG isotype production, it can be inferred that under certain conditions $CD4^+$ Th1 cells develop more readily than Th2 cells in RIP1-Tag2 and RIP3-Tag2 mice. However, the Th1 cells still appear to be tolerant relative to those in the control mice. $CD4^+$ Th1 type cells promote cellular immune responses and secrete high level IL-2, which is required for the proliferation of $CD8^+$ cytotoxic precursors. Yet, many cytotoxic T cell responses can be generated in the absence of $CD4^+$ T cells (Doherty et al., 1992; Kosaka and Sprent, 1993;

Doherty, 1993; Bodmer et al., 1993). Therefore, the inability of embryonic onset RIP-Tag mice to mount a cytotoxic T cell response against T antigen may be due to complete tolerance of the $CD8^+$ T cells, or lack sufficient T cell help. To ask if any T antigen cytotoxic T cells can be generated from tolerant RIP-Tag mice, a cytotoxic T cell assay was set up and IL-2 included during the in vitro restimulation. The addition of IL-2 to the cultures should allow the proliferation of cytotoxic precursors which recognize T antigen and reveal any helper-independent $CD8^+$ T cells in RIP-Tag mice which can recognize T antigen expressing targets. Splenocytes isolated from immunized RIP1-Tag2 and non-transgenic control mice were cultured in the presence or absence of IL-2 and assayed for their ability to kill fibroblasts expressing T antigen or not (Figure 17). Lymphocytes from transgenic mice, which had been cultured in the presence of exogenous IL-2, lysed T antigen expressing targets at the two highest effector to target ratios tested. As expected, untreated splenocytes from RIP1-Tag2 mice demonstrated no killing of the same targets. Culturing in IL-2 did not significantly alter the killing of the T antigen expressing fibroblasts by lymphocytes from non-transgenic mice. Neither did treatment of spleen cells with IL-2 from transgenic or non-transgenic mice result in recognition of syngeneic T antigen negative fibroblasts. Thus, RIP1-Tag2 mice possess some T antigen reactive helper-independent $CD8^+$ T cells whose presence can be revealed by the addition of exogenous IL-2.

Figure 17: *Cytotoxic T cell tolerance is partially overcome by IL-2.* Splenocytes from immunized mice were cultured for five days with irradiated C57SV either without (■, non-transgenic; O, RIP1-Tag2) or with (▲, non-transgenic; ▽, RIP1-Tag2) added IL-2, and then tested for their ability to kill syngeneic fibroblasts either expressing *(A)* or not expressing *(B)* T antigen in a ^{51}Cr release assay. The mean and standard error of three animals in each group is shown.

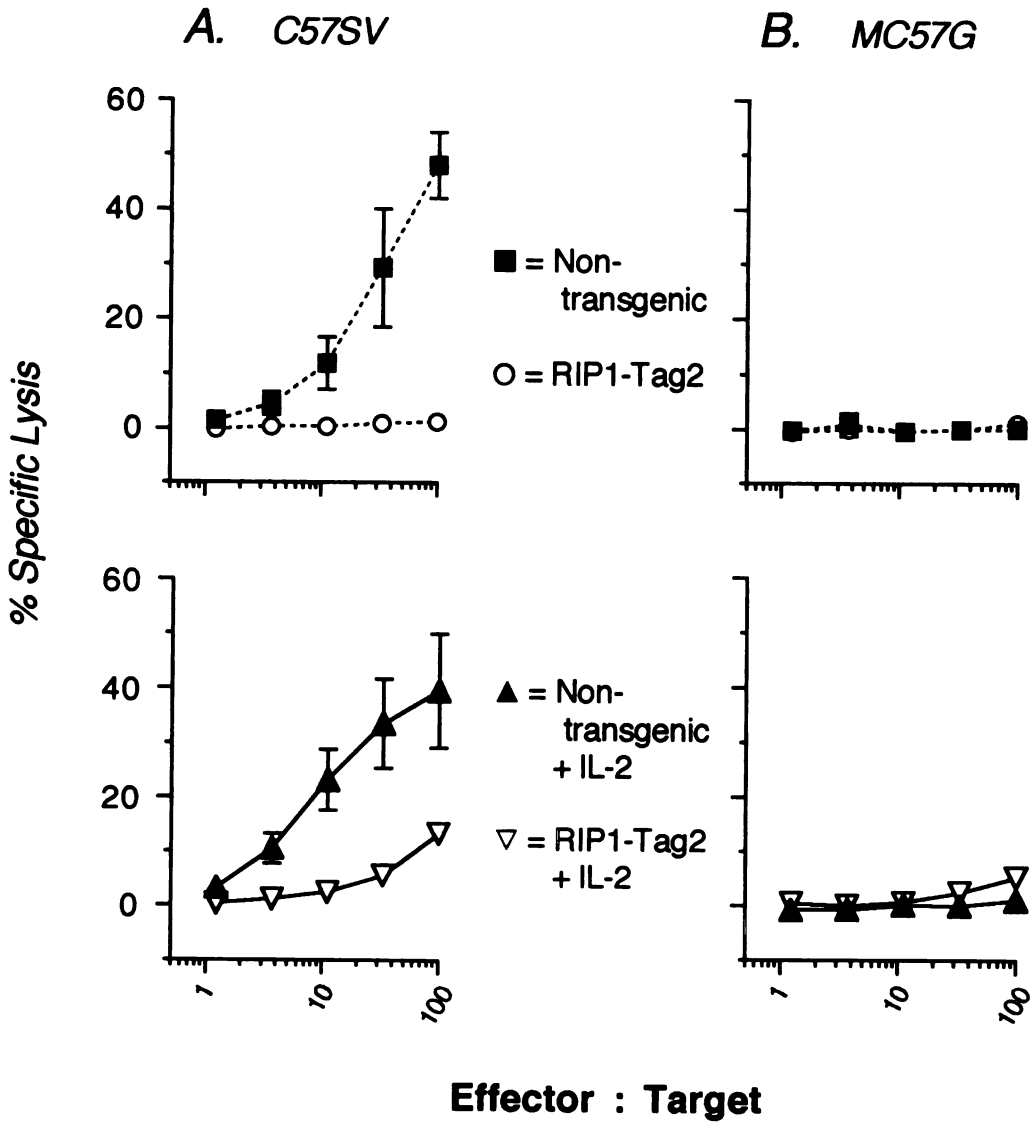

A. C57SV
B. MC57G
% Specific Lysis
60
40
20
0
■ = Non-transgenic
○ = RIP1-Tag2
▲ = Non-transgenic + IL-2
▽ = RIP1-Tag2 + IL-2
1
10
100
Effector : Target

Figure 17

SUMMARY AND CONCLUSIONS

In the initial characterization of the non-responsive phenotype of embryonic onset RIP-Tag mice, three responses to T antigen were examined: antibody, cytotoxic, and in vitro T cell proliferation. I have shown that two of these responses, impaired production of T antigen specific antibody and decreased proliferation of lymphocyte in response to stimulation by T antigen protein in vitro, are limited by tolerant $CD4^+$ helper T cells. The third response, the inability to generate cytotoxic T cells recognizing T antigen, may result from tolerance induction of both $CD4^+$ and $CD8^+$ T cells.

B cells in RIP1-Tag2 and RIP3-Tag2 mice fail to produce significant quantities of antibody to T antigen when immunized with T antigen protein. Here I have shown that these B cells can be induced to make antibody to T antigen by performing a hapten-carrier experiment. When transgenic mice are immunized with T antigen covalently coupled to a carrier molecule, KLH, they made similar levels of antibody to T antigen as their non-transgenic littermates. Thus, the B cells in embryonic RIP-Tag mice are not affected by the expression of T antigen in the pancreatic ß cells or thymus.

$CD4^+$ T cells are implicated in limiting the antibody response to T antigen in tolerant RIP-Tag mice. Naive $CD4^+$ T cells, after encountering antigen, can differentiate into one of two types of effectors, Th1 or Th2, which secrete different patterns of cytokines and promote different types of immune responses. Many immune responses, against both pathogens and autoaggessive reactions, are predominated by either a Th1 or Th2 type response (Finkelman, 1995). In RIP1-Tag2 and RIP3-Tag2 mice immunized with T antigen protein, the development of T antigen specific Th2 cells, as

assessed by their ability to promote class switching to IgG_1, is more severely impaired than that of the Th1 cells, as assessed by their ability to promote class switching to IgG_{2a}. Although the actual cytokine production by the T cells was not examined, it is well established that IL-4, a Th2 type cytokine promotes class switching to IgG_1 and inhibits class switching to IgG_{2a}, while IFN-γ, a Th1 type cytokine, does the opposite (De Wit et al., 1992; Burstein et al., 1992; Romball and Weigle, 1993; Finkelman, 1995). In tolerant RIP-Tag mice the majority of the T antigen reactive $CD4^+$ T cell activity which is detected, albeit significantly less than that detected in non-transgenic control mice, can be attributed to the development of Th1 type cells, which promote cellular immunity.

The preferential development of Th1 type cells in RIP-Tag mice differs from the preferential development of Th2 type cells observed in adult mice tolerized with soluble proteins (De Wit et al., 1992; Burstein et al., 1992; Romball and Weigle, 1993). In these models mice were treated with large quantities of tolerogen, 0.2 to 2.5 mg, enough to tolerize both the T and the B lymphocytes (Mitchison et al., 1984). Additionally, different strains of mice were employed in these studies than the C57BL/6 strain studied here. The genetic background of mice has been shown to influence the development of Th1 and Th2 cells in response to infectious agents (Sher and Coffman, 1992) and in the development of autoimmunity (Scott et al., 1994). In the tolerant RIP-Tag mice, the generation of Th1 and Th2 type T antigen specific responses was measured relative to the response of the same mice to a second control antigen, ß-galactosidase. Thus, this preferential development is not induced solely by the immunization procedure, since it is antigen specific. The dose of immunogen has been shown to influence the

development of Th1 and Th2 type responses. Low doses of antigen favor a Th2 type response, while Th1 type responses are favored with high doses of antigen (Seder and Paul, 1994). Although the dose of both antigens, T antigen and ß-galactosidase, given to tolerant RIP-Tag mice were low, 10 and 5 μg respectively, these two proteins may be presented at different levels by class II molecules on antigen presenting cells. Moreover, the site of tolerance induction and cell type inducing it may influence the ability of the $CD4^+$ T cells to differentiate into Th1 or Th2 effectors (Finkelman, 1995). Nonetheless, this study does demonstrate that a Th1 type response to T antigen can predominate in tolerant RIP-Tag mice.

$CD8^+$ T cells from RIP1-Tag2 and RIP3-Tag2 mice fail to kill T antigen expressing targets in a standard cytotoxic T cell assay. Yet, when splenocytes from immunized transgenic mice are cultured in the presence of IL-2, all mice tested were able to lyse the same targets, albeit only at the two highest effector to target ratios tested. This impaired response may reflect lack of sufficient T cell help from the tolerant $CD4^+$ Th1 type cells, or simply a direct, yet incomplete tolerization of the T antigen specific $CD8^+$ T lymphocytes. $CD8^+$ T cells can generate helper-independent response to alloantigens in vitro (Kosaka and Sprent, 1993) and to viruses both in vivo and in vitro (Doherty et al., 1992). Mice rendered genetically deficient of CD4 or of class II MHC are also able to generate cytotoxic T cells in vivo and in vitro (Doherty, 1993, Bodmer et al., 1993). However, to generate helper-independent cytotoxic T cells after in vitro restimulation, IL-2 is typically added during the in vitro boost. This is unlikely to reflect a requirement for help from $CD4^+$ Th1 type cells, which secrete IL-2, since virus specific cytotoxic activity can be detected in lymphocytes taken directly from influenza infected MHC class II

deficient mice without in vitro restimulation. Furthermore, $CD8^+$ T cells also produce their own IL-2. At this time it is not know why it is necessary to add exogenous IL-2 to these cultures during the in vitro boost to recover cytotoxic activity. It may be that under the in vitro restimulation conditions that all the IL-2 produced is consumed, while in vivo other factors allow for expansion of the $CD8^+$ cytotoxic effectors. Although many different cytotoxic response can be generated in the absence of $CD4^+$ T cell help, cytotoxic responses to the male specific antigen HY and to the non-classical class I MHC molecule Qa have been shown to require T cell help (Guerder and Matzinger, 1992). Therefore, it is not possible to determine from the experiment performed here whether insufficient T cell help limits the generation of T antigen specific cytotoxic T cells in RIP-Tag mice. However, this experiment does indicate that not all the T antigen reactive $CD8^+$ T cells have been rendered tolerant.

CHAPTER 6:

The Role of Thymic T antigen Expression in Tolerance Induction

INTRODUCTION

Although the pancreatic ß cells are the main site of T antigen synthesis in both RIP1-Tag2 and RIP3-Tag2 mice, the persistent expression of T antigen RNA in the thymus, described in chapter 3, raises the possibility that the thymus is involved in inducing tolerance to T antigen. From the RNA-PCR analysis, it was not possible to distinguish whether a few cells express significant levels of T antigen or many cells express very low levels. The level of T antigen message present in the thymus was estimated to be 3000 to 10,000 fold lower than in the pancreatic ß cells. If many cells were expressing low levels of T antigen, it would be difficult to imagine how these cells could possibly tolerize the developing T cells. Alternatively, if few cells were expressing higher levels, it would be easier to reconcile this pattern of expression with a immunological function for these T antigen expressing cells. However, initial efforts to identify T antigen expressing cells in transgenic thymus have failed.

T antigen is a nuclear protein which should be easily shunted into the MHC class I presentation pathway. As mentioned earlier, it is more difficult to imagine how T antigen produced in the thymus could gain access to the MHC class II presentation pathway. Thus, one may expect to see a stronger tolerization of the $CD8^+$ class I MHC restricted T cells than the $CD4^+$ class II MHC restricted T cells by thymic cells expressing low levels of T antigen protein. Several other investigators using insulin promoted transgenes

have found that their neo-antigen is expressed in the thymus. By performing thymic transplants into immunodeficient hosts, these investigators have shown that thymic expression of the transgene contributes to reduced responsiveness of $CD8^+$ T cells to the novel self antigen (Heath et al., 1992; von Herrath et al., 1994).

In this chapter I examine the contribution of thymic T antigen expression to the development of tolerance by transplanting newborn RIP-Tag thymus under the renal capsule of syngeneic athymic mice. These chimeric mice, whose only source of T antigen is their transgenic thymus, are tested for tolerance to T antigen in the three assays employed throughout this work: production of T antigen specific antibody, ability of primed lymph node cells to proliferate in response to T antigen in vitro, and the capacity to generate T antigen specific cytotoxic T cells.

MATERIALS AND METHODS

Thymic Transplants. C57BL/6J nu/nu male mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred to C57BL/6J female mice to generate nu/+ females. These were bred to nu/nu male mice to generate a colony of nu/nu mice. Both male and female nu/nu mice were used as hosts for transplantation. Thymus donors were progeny of RIP1-Tag2 and RIP3-Tag2 males, backcrossed for at least 15 generations to C57BL/6, bred to C57BL/6 females. Thymi were removed from newborn mice, p0 and p1 (p0 being the day of birth), and placed in 24 well plates in RPMI supplemented with 10% fetal calf serum, and penicillin/streptomycin. Prior to removal of

the thymus, animals were marked with a number, their tails clipped and digested with proteinase K in buffer. Tail tip samples were tested for presence or absence of the transgene by PCR. Some of the thymi were cultured overnight, but the majority were transplanted the same day. No difference in the phenotype was observed in mice receiving fresh or cultured thymi. Four thymic lobes were transplanted under the kidney capsule of 5 to 16 week old nu/nu mice. Mice were allowed to reconstitute for 10 to 21 weeks before analysis.

Immunological Assays. The antibody, proliferation, and cytotoxic T cell assays were performed as described in chapter 2. All experiments included i) nu/nu mice transplanted with non-transgenic thymus, ii) nu/nu mice transplanted with transgenic thymus, iii) non-transgenic mice, and iv) transgenic mice. In addition, untransplanted nu/nu were also used as controls for the antibody and cytotoxic assays. Untransplanted nu/nu mice were not included in the proliferation assay, since they lack large lymph nodes which are the source of the cells for this assay.

RESULTS

T antigen Expressing Thymus Induces Tolerance of $CD4^+$ T cells

To assess the immunological function of thymic T antigen expression, mice were created whose only potential source of T antigen was the thymus. Thymi from newborn RIP1-Tag2, RIP3-Tag2, and non-transgenic littermates

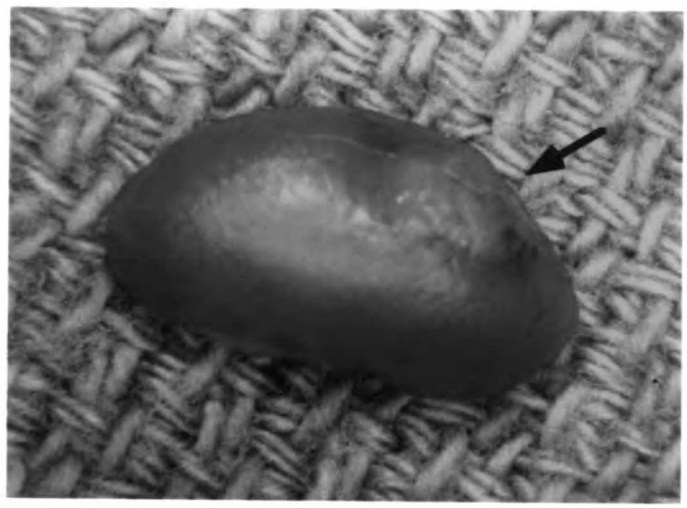
were transplanted under the kidney capsule of C57BL/6 nu/nu mice. The immune system of these transplanted mice was allow to develop for a minimum of 10 weeks, typically 12. Thymi placed under the kidney capsule become plump and well vascularized during this time (Figure 18). These reconstituted mice were then tested in each of the three assays previously employed to characterize the tolerance in RIP-Tag mice.

First, nude mice, transplanted with non-transgenic or RIP1-Tag2 thymus, were tested for their ability to produce antibody to T antigen and ß-galactosidase (Figure 19). As controls, non-transgenic, RIP1-Tag2, and untransplanted nude mice were included. Since the B cells in nude mice are not tolerant of T antigen, this assay is a measure of the tolerance of $CD4^+$ T helper cells. As expected, nude mice transplanted with non-transgenic thymus, much like unmanipulated non-transgenic mice, made strong antibody responses to both antigens. Nude mice transplanted with RIP1-Tag2 thymus looked strikingly similar to RIP1-Tag2 mice, generating strong antibody responses to ß-galactosidase, but little antibody to T antigen. Nude mice which had not received a thymus transplant failed to make antibody to either antigen.

Next, lymphocytes from immunized nude mice transplanted with RIP1-Tag2 or non-transgenic thymus were tested for their capacity to proliferate to T antigen in vitro (Figure 20). Once again, the nude mice transplanted with non-transgenic thymus responded similarly to non-transgenic mice, while the nude mice transplanted with RIP1-Tag2 thymus behaved similarly to RIP1-Tag2 mice. Impressively, the two sets of curves are superimposable. Lymph node cells from all animals proliferated well to the control antigen, *M. tuberculosis*. This result has been confirmed in

Figure 18: *The thymus develops under the renal capsule.* Thymic lobes were placed under the kidney capsule of nu/nu mice *(A).* The thymus is the small mass on the top right side of the kidney (see arrow). After 12 week in vivo, the thymus has become well vascularized, plump, and increased greatly in size (see arrow) *(B).*

A



B

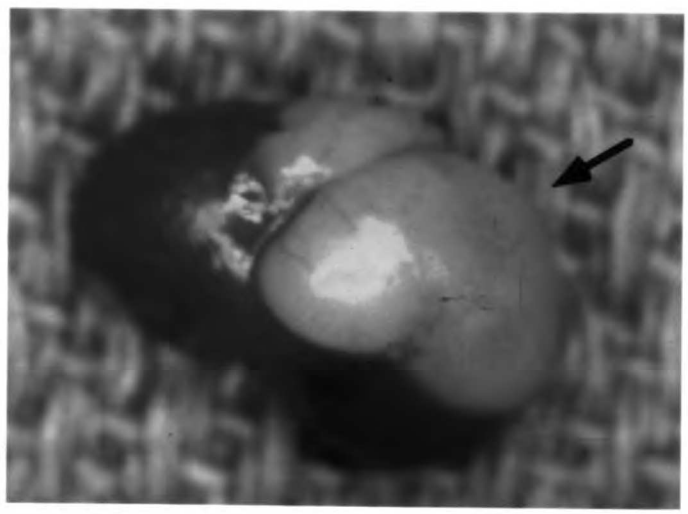


Figure 18

Figure 19: *Antibody response of nu/nu mice with RIP1-Tag2 thymus.* Non-transgenic, RIP1-Tag2, nu/nu mice transplanted with non-transgenic thymus, nu/nu mice transplanted with RIP1-Tag2 thymus, and unmanipulated nu/nu mice were immunized and boosted with T antigen and ß-galactosidase in Freund's adjuvant. Sera were tested for the presence of antibody recognizing T antigen (●) and ß-galactosidase (O). The mean response of all animals in the group is shown by a bar. The tertiary antibody response is shown.

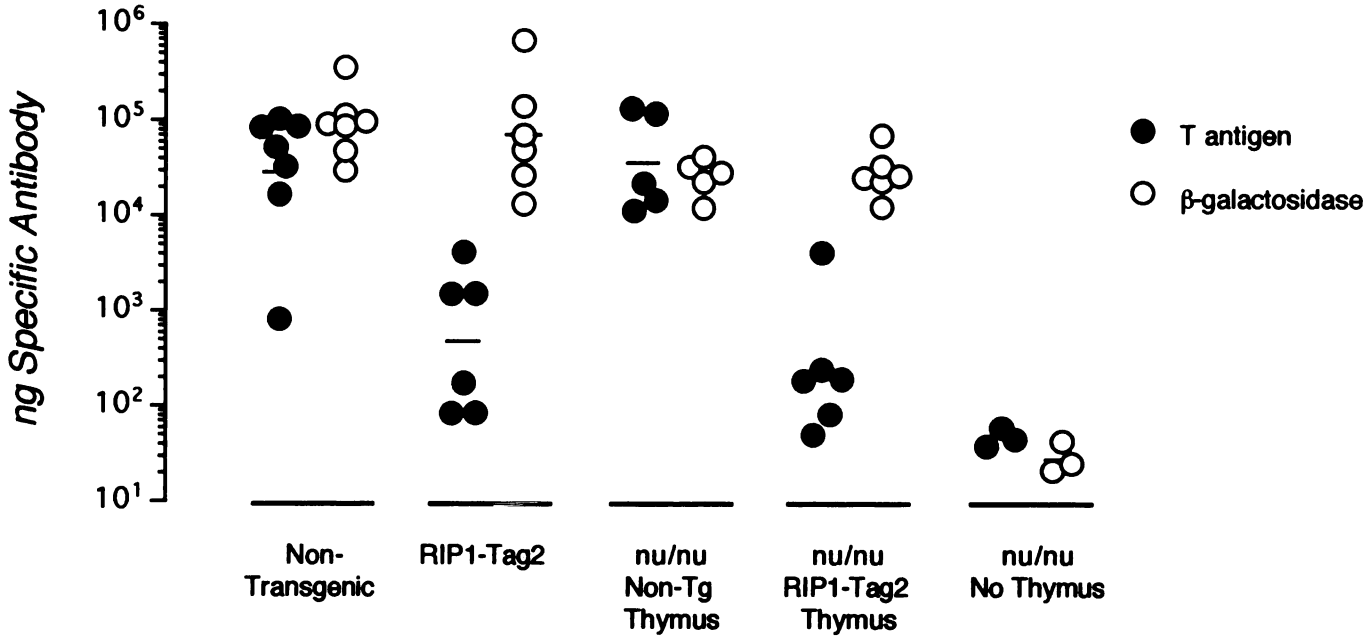

ng Specific Antibody
10^6
10^5
10^4
10^3
10^2
10^1
T antigen
β-galactosidase
Non-Transgenic
RIP1-Tag2
nu/nu Non-Tg Thymus
nu/nu RIP1-Tag2 Thymus
nu/nu No Thymus

Figure 19

Figure 20: *In vitro proliferation of lymphocytes from nu/nu mice transplanted with RIP1-Tag2 thymus.* Lymph node cells from immunized non-transgenic (■, dashed line, n=3), RIP1-Tag2 (O, dashed line, n=3), nu/nu mice transplanted with non-transgenic thymus (▲, solid line, n=5), and nu/nu mice transplanted with RIP1-Tag2 thymus (▽, solid line, n=5) were cultured with dilutions of T antigen *(A)* and the control antigen M. tuberculosis *(B)*. The antigen dependent proliferation was measured after 96 hours of culture by pulsing the wells with ^{3}H-thymidine for the last 6 hours. The mean and standard error of all animals in each group is shown.

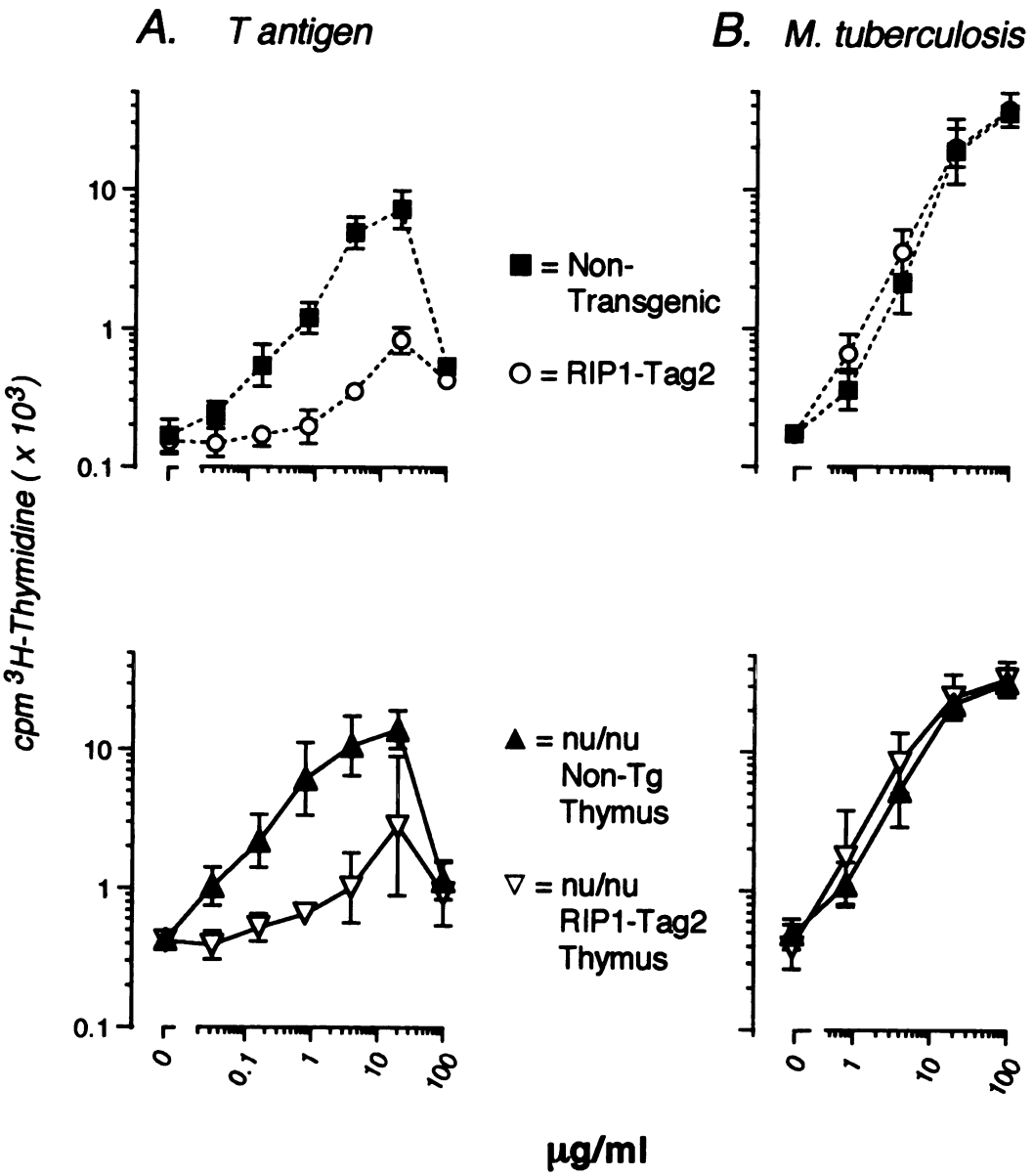

A. T antigen
B. M. tuberculosis
cpm ³H-Thymidine (x 10³)
10
1
0.1
■ = Non-Transgenic
○ = RIP1-Tag2
▲ = nu/nu Non-Tg Thymus
▽ = nu/nu RIP1-Tag2 Thymus
0
0.1
1
10
100
µg/ml

Figure 20

another set of nude mice transplanted with RIP1-Tag2 thymus. In a separate experiment, nude mice transplanted with RIP3-Tag2 thymus, and all the appropriate controls, were tested (Figure 21). nu/nu mice transplanted with RIP3-Tag2 thymus are similarly tolerant to those transplanted with RIP1-Tag2 thymus. Thus, using two assay which measure the tolerance induction of $CD4^+$ T cells, the low level of T antigen expression in the thymus of RIP1-Tag2 and RIP3-Tag2 mice is sufficient to confer tolerance, indistinguishable from that observed in unmanipulated transgenic mice.

Transgenic Thymus is Not Sufficient to Confer Complete Cytotoxic T cell Tolerance.

Next, mice whose only source of T antigen was the thymus were tested for their ability to generate cytotoxic T cells recognizing T antigen. Splenocytes from immunized nude mice, transplanted with non-transgenic or RIP1-Tag2 thymus, and non-transgenic, RIP1-Tag2, and unmanipulated nude mice were restimulated in vitro, and assessed for their ability to kill T antigen expressing and allogeneic targets (Figure 22). As expected, lymphocytes from non-transgenic mice and nude mice transplanted with non-transgenic thymus lysed the T antigen expressing targets efficiently, while splenocytes from RIP-Tag2 mice failed to recognize them. Remarkably, the spleen cell cultures from the nude mice transplanted with RIP1-Tag2 thymus gave variable killing of the T antigen expressing targets. This variability is reflected by the large error bars shown for the mean response of the group. Of the eight animals in this group, two of the mice

Figure 21: *In vitro proliferation of lymphocytes from nu/nu mice transplanted with thymus from the second transgenic line, RIP3-Tag2* . Lymph node cells from immunized non-transgenic (■, dashed line, n=3), RIP3-Tag2 (O, dashed line, n=3), nu/nu mice transplanted with non-transgenic thymus (▲, solid line, n=2), and nu/nu mice transplanted with RIP3-Tag2 thymus (▽, solid line, n=4) were cultured with dilutions of T antigen *(A)* and the control antigen M. tuberculosis *(B)*. The antigen dependent proliferation measured after 72 hours of culture by pulsing the wells with ^{3}H-thymidine for the last 6 hours. The mean and standard error of all animals in each group is shown.

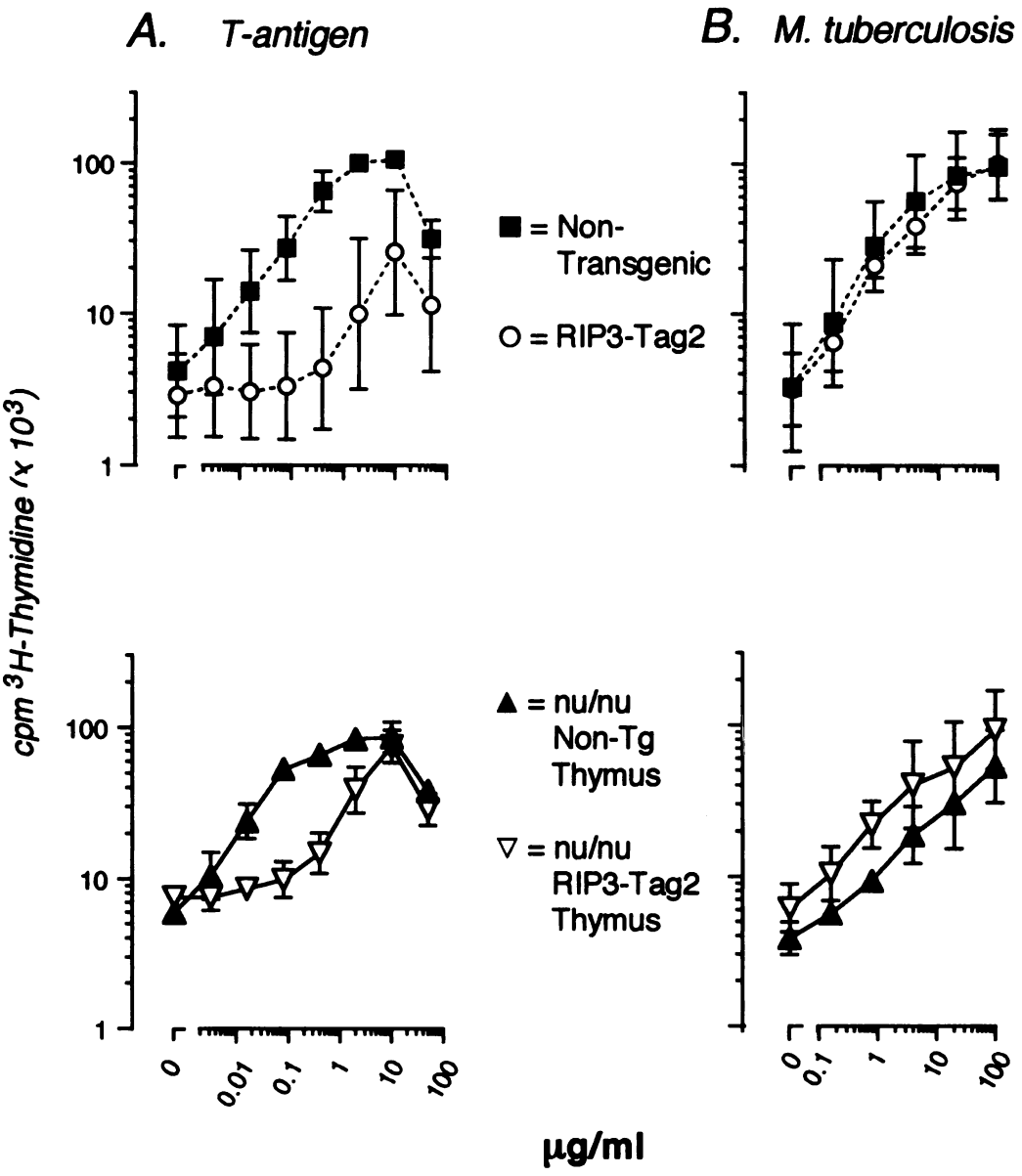

A. T-antigen
B. M. tuberculosis
cpm ³H-Thymidine (x 10³)
100
10
1
■ = Non-Transgenic
○ = RIP3-Tag2
▲ = nu/nu Non-Tg Thymus
▽ = nu/nu RIP3-Tag2 Thymus
0
0.01
0.1
1
10
100
µg/ml

Figure 21

Figure 22: *T antigen specific cytotoxic response of nu/nu mice transplanted with RIP1-Tag2 thymus.* Splenocytes from immunized non-transgenic (■, dashed line, n=5), RIP1-Tag2 (O, dashed line, n=8), nu/nu mice transplanted with non-transgenic thymus (▲, solid line, n=8), nu/nu mice transplanted with RIP1-Tag2 thymus (▽, solid line, n=8), and unmanipulated nu/nu mice (◇, dotted line, n=2) were cultured either with irradiated C57SV (2500 rads) or BALB/c spleen cells (3000 rads). After five days cultured cells were tested for their ability to kill ^{51}Cr loaded T antigen expressing fibroblasts C57SV *(A)*, syngeneic T antigen negative fibroblasts MC57G (data not shown), or P815 *(B)*, allogeneic targets in a 4 hour killing assay. The mean and standard error of all animals in each group are shown.

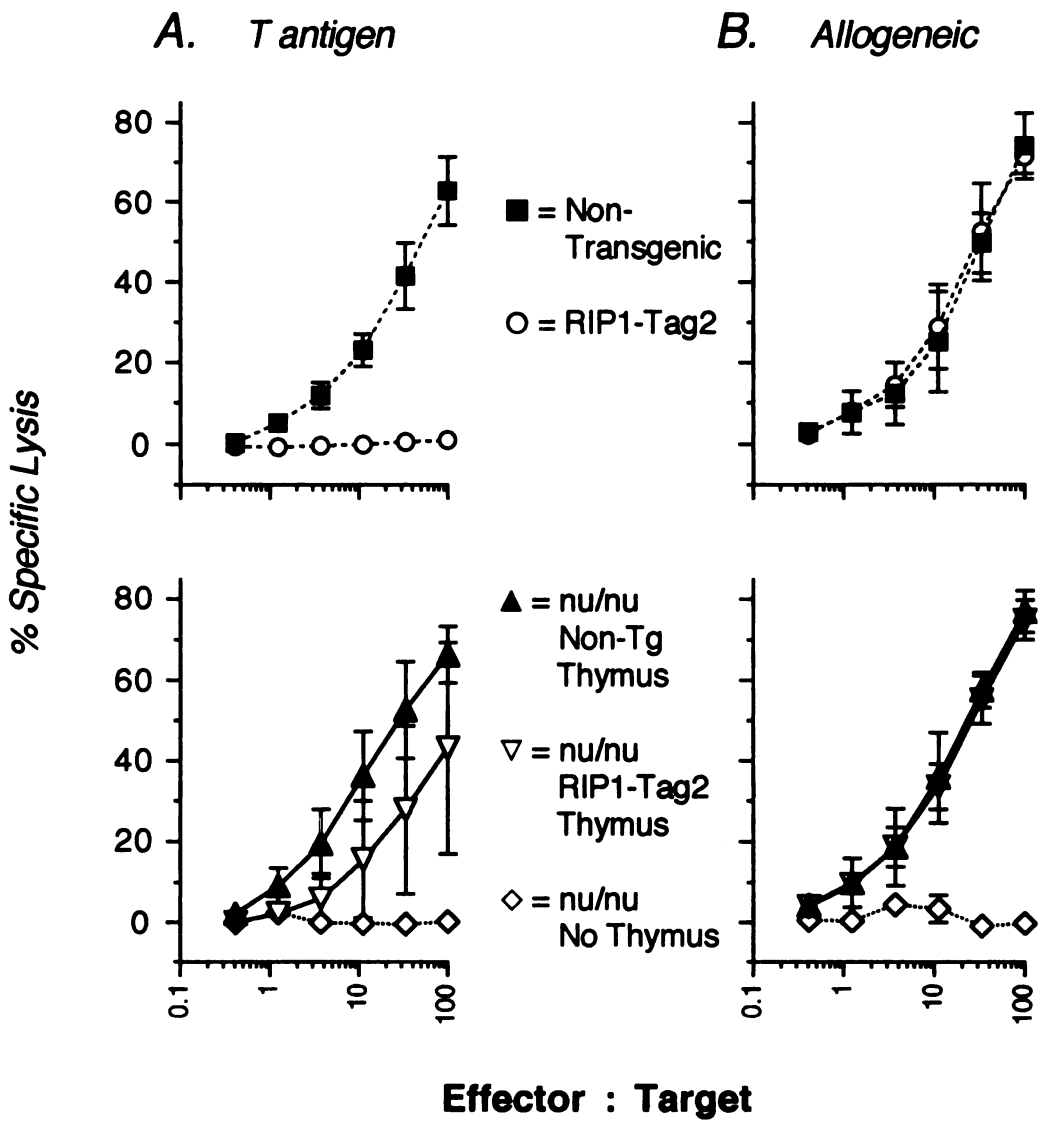

A. T antigen
B. Allogeneic
% Specific Lysis
80
60
40
20
0
■ = Non-Transgenic
○ = RIP1-Tag2
▲ = nu/nu Non-Tg Thymus
▽ = nu/nu RIP1-Tag2 Thymus
◇ = nu/nu No Thymus
0.1
1
10
100
Effector : Target

Figure 22

failed to generate cytotoxic T cells recognizing T antigen, three were indistinguishable from their non-transgenic littermates, and three generated an intermediate response. This variable killing is unlikely to be due to an unusual natural killer cell activity in nude mice, since nude mice which had not received a thymic transplant did not lyse either the T antigen expressing fibroblasts or the allogeneic targets. Additionally, all nude mice which had been transplanted with thymus were capable of generating cytotoxic T cells recognizing allogeneic targets, indicating that this variability is not a reflection of poor reconstitution of immune system. Finally, this variable activity was not seen in nude mice transplanted with non-transgenic thymus nor in mice transplanted with transgenic thymus but analyzed in one of the other two assays. This experiment has been repeated with similar results.

To confirm this result, nude mice transplanted with RIP3-Tag2 thymus were also tested for their ability to generate cytotoxic T cells recognizing T antigen (Figure 23). Again, a similar result was observed. Non-transgenic and nude mice transplanted with non-transgenic thymus both generated strong cytotoxic T cell responses to T antigen, while RIP3-Tag2 mice were unable to do so. Nude mice transplanted with RIP3-Tag2 thymus, like the nude mice transplanted with RIP1-Tag2 thymus, produced variable responses. Cultured spleen cells from two mice were unable to lyse T antigen expressing targets, two were indistinguishable from the controls, and one gave an intermediate response. This experiment was also repeated with similar results. Although a thymus from an embryonic onset RIP-Tag mouse can induce tolerance of $CD4^+$ T cell, it is insufficient, in most cases to induce tolerance of the $CD8^+$ cytotoxic T cells.

Figure 23: *T antigen specific cytotoxic response of nu/nu mice transplanted with thymus from the second transgenic line, RIP3-Tag2* . Splenocytes from immunized non-transgenic (■, dashed line, n=4), RIP3-Tag2 (O, dashed line, n=4), nu/nu mice transplanted with non-transgenic thymus (▲, solid line, n=3), and nu/nu mice transplanted with RIP3-Tag2 thymus (▽, solid line, n=5) were cultured either with irradiated C57SV (2500 rads) or BALB/c spleen cells (3000 rads). After five days cultured cells were tested for their ability to kill ^{51}Cr loaded T antigen expressing fibroblasts C57SV *(A)*, syngeneic T antigen negative fibroblasts MC57G (data not shown), or P815 *(B)*, allogeneic targets in a 4 hour killing assay. The mean and standard error of all animals in each group are shown.

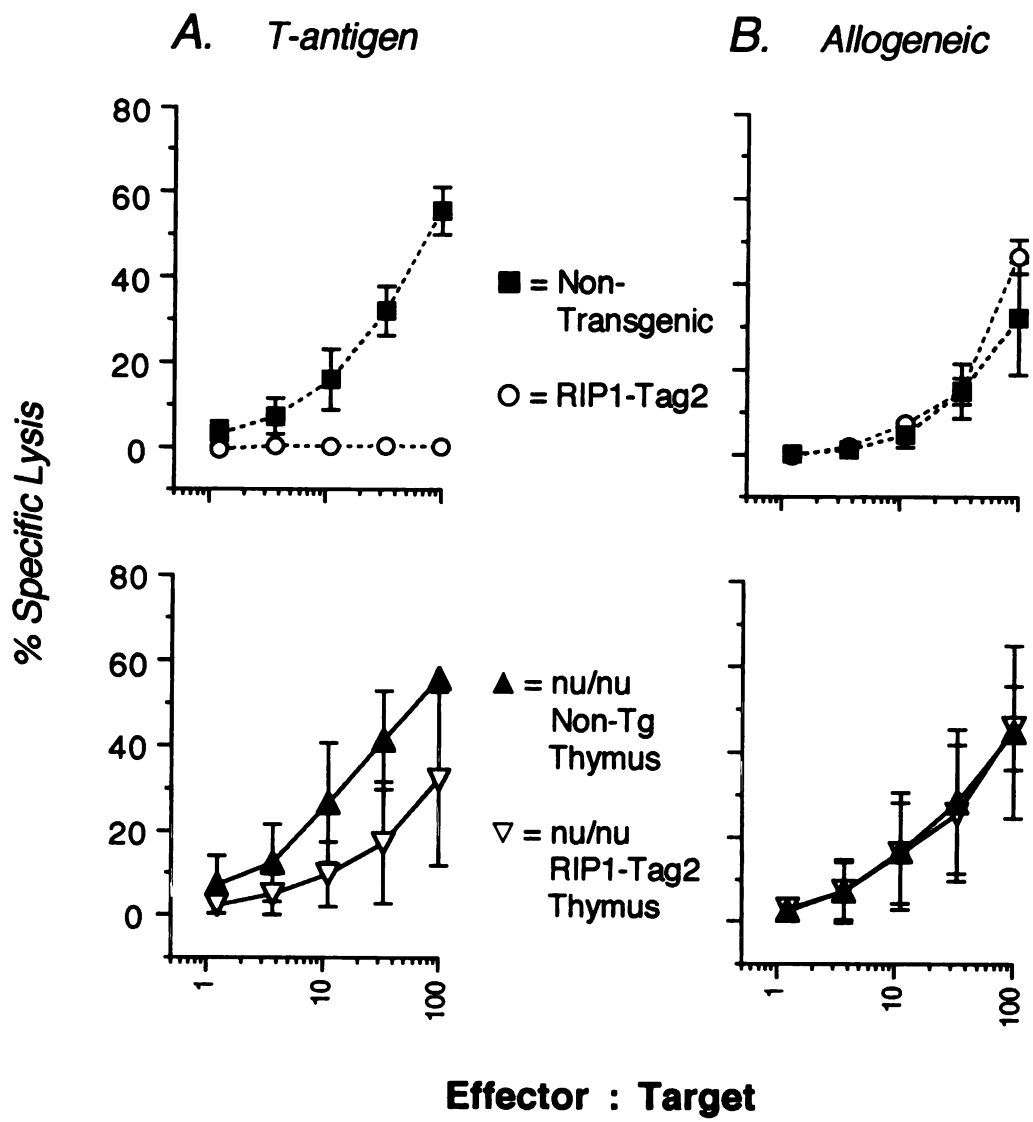

A. T-antigen
B. Allogeneic
% Specific Lysis
80
60
40
20
0
■ = Non-Transgenic
○ = RIP1-Tag2
▲ = nu/nu Non-Tg Thymus
▽ = nu/nu RIP1-Tag2 Thymus
1
10
100
Effector : Target

Figure 23

SUMMARY AND CONCLUSIONS

In this chapter the capacity of transgenic thymus from RIP1-Tag2 and RIP3-Tag2 mice to confer tolerance of T lymphocytes was assessed. The immune system of nude mice was allowed to develop after transplantation of non-transgenic or transgenic thymus under the renal capsule. Nude mice which had received a transgenic thymus were indistinguishable from unmanipulated transgenic mice when assessed for their ability to make T antigen specific antibody and for primed lymph node cells to proliferate in response to T antigen. Surprisingly, when these mice, whose only source of T antigen protein was their transgenic thymus, were tested for their ability to generate a cytotoxic T cell responses to T antigen, the splenocytes from the majority of these mice, unlike those from the transgenic controls, lysed T antigen expressing targets.

Transplantation of thymus from newborn RIP1-Tag2 and RIP3-Tag into athymic hosts allows for development of an immune system in which the $CD4^+$ T cells are tolerant of T antigen, as demonstrated in the antibody and in vitro proliferation assays. It is puzzling as to how T antigen gains access to the class II antigen processing pathway. For T antigen to be presented on class II molecules MHC, it must reach the endosome, the compartment in which loading of class II molecules with peptide occurs. This must happen in a specialized antigen capturing cell such as a macrophage or a dendritic cell. Two possibilities mechanisms for T antigen presentation by class II MHC molecules can be proposed. The first possibility is that T antigen could be released from the cells expressing it and be endocytosed by antigen presenting cells. Although only a small amount of T

antigen is produced in the thymus, this material could be released to adjacent cells, for example upon cell death. Christofori and coworkers (1994) have recently shown that T antigen induces apoptosis of pancreatic ß cells in RIP-Tag mice. This could potentially occur in the thymus. Alternatively, small amounts of T antigen protein may reach the cell surface. T antigen has been detected at the surface of cells (Klockmann and Deppert, 1983; Butel and Jarvis, 1986), although this extracellular material has only been detected in cells lines expressing high levels of T antigen. The local release of this T antigen in the thymus either by cell death or by shedding from cells may account for its ability to be presented by MHC class II molecules. Yet, this released material would still have to compete with the universe of antigens found in the extracellular environment of the thymus for presentation. A second more attractive hypothesis is that the cell type expressing T antigen expresses high levels of class II MHC and is specialized for tolerance induction.

In contrast to the complete $CD4^+$ T cell tolerance in chimeric mice whose only source of T antigen is their transgenic thymus, the majority of nude mice transplanted with RIP-Tag thymus are able to generate T antigen specific cytotoxic T cells, although this ability varies from mouse to mouse. How is it that the $CD8^+$ T lymphocytes from these mice are able to generate T antigen specific cytotoxic T cells? One possibility is that the $CD4^+$ T cells which develop in the chimeric mice provide additional help in the generation of cytotoxic $CD8^+$ effectors. As discussed in Chapter 5, it is not known whether T antigen specific $CD8^+$ T cells require help from $CD4^+$ T cells to generate cytotoxic T effectors. Moreover, the $CD4^+$ T cells in the chimeric mice are as tolerant of T antigen as those in unmanipulated RIP1-

Tag2 and RIP3-Tag2 mice. Thus, they would be unable to provide additional T cell help to the $CD8^+$ T cells in the production of T antigen specific cytotoxic effectors, even if they were required. A second potential explanation is that the T antigen specific killing observed in the transplanted nude mice is due to natural killer cells. This is also unlikely, since splenocytes from untransplanted nude mice did not kill the T antigen expressing C57SV fibroblasts, allogeneic targets, or syngeneic fibroblasts which do not express T antigen (data not shown). A third possible reason is that endogenous $CD8^+$ T cells, which develop in older nude mice (Kennedy et al., 1992), are responsible for the observed killing. However, the nude mice transplanted with RIP-Tag thymus were transplanted at various ages and allowed to develop varying amounts of time. No correlation was observed between the age at which the mouse was transplanted or the length of time which it was allowed to reconstitute, and its phenotype (data not shown). One final hypothesis can be suggested. T antigen reactive $CD8^+$ T cells may require an interaction with T antigen outside the thymus to become tolerant. In this model, T antigen presented by class I MHC molecules in the thymus would provide an initial tolerization signal to the $CD8^+$ T cells. If this signal is not confirmed in the periphery, once the $CD8^+$ T cells have left the thymus, then they remain at least partial responsive to T antigen. Whether this initial antigen encounter in the thymus would render all T antigen reactive $CD8^+$ T cells partially tolerant, or only tolerize the highest affinity T cells is open for speculation. The finding that a few of the chimeric mice with transgenic thymus were unable to generate T antigen specific T cells, while the majority could, may be explained by the stochastic nature of the interactions of the developing T cells with antigen presenting

cells which induce tolerance in thymus. However, in the majority of chimeric mice this initial tolerance inducing signal in the thymus would be insufficient to induce complete tolerance of the $CD8^+$ T cells, thus allowing for the development of T antigen specific cytotoxic T cells upon immunization. Several investigators have postulated that tolerance induction of $CD8^+$ T cells is a multistep process, a initial signal being delivered to the developing T cell in the thymus which is later confirmed in the periphery, to explain their findings (Kosaka and Sprent, 1993; Arnold et al., 1993). Although this is the most intriguing possibility, the other more trivial explanations, although unlikely, have not been completely excluded.

CHAPTER 7:

Characterization of the Thymic Cell Type Expressing T antigen and Endogenous Pancreatic Genes

INTRODUCTION

In the previous chapter, chimeric mice were produced whose only source of T antigen was a RIP1-Tag2 or RIP3-Tag2 thymus. These mice were created by transplanting RIP-Tag thymus into athymic hosts. After allowing the T lymphocytes to develop through the transgenic thymus, these chimeric mice were compared to unaltered RIP-Tag mice for their ability to make an immune response to T antigen. In the two assays which measure tolerance of the $CD4^+$ T cells, a similar level of tolerance, as that which develops in intact RIP1-Tag2 and RIP3-Tag2 mice, was observed in the chimeras. Thus, thymic expression of T antigen is sufficient to confer tolerance of the $CD4^+$ T cells in embryonic onset RIP-Tag mice. It is remarkable that such a small amount of protein, produced in the thymus, can have such a profound effect.

Other investigators have also shown that insulin gene directed transgenes, when expressed intrathymically, can induce non-responsiveness to the novel self protein (Heath et al., 1992; von Herrath et al., 1994). Although these investigators have demonstrated that thymic expression of their transgene of interest can tolerize $CD8^+$ T cells, they provided no evidence that this expression pattern can be generalized to endogenous "tissue specific" genes or to $CD4^+$ T cells. Thymic expression of transgenes, could, in general, be a phenomenon related to the promoter-reporter gene combination or the particular transgene insertion site. In chapter 2, I showed

that this is not the case. The thymic expression of T antigen, in RIP-Tag mice, recapitulates that of the endogenous insulin genes. Moreover, I have detected message for several endogenous genes, normally expressed in various different pancreatic cell types, in the thymus. These findings indicate that expression of T antigen can be used as a marker for tolerance induction to endogenous genes, which is difficult to study, since many endogenous proteins are essential for viability.

The identification of the cell type expressing T antigen may provide information about how tolerance to endogenous "tissue specific" proteins is achieved. The candidates are cortical or medullary epithelial cells, and bone marrow derived thymocytes, macrophages or dendritic cells. Bone marrow derived dendritic cells have been postulated to be the major cell type responsible for deleting self reactive cells in the thymus (Ramsdell and Fowlkes, 1990). In addition, studies employing T cell receptor transgenic mice have provided evidence that the medullary epithelium can induce both deletional and non-deletional forms of tolerance (Ramsdell and Fowlkes, 1990; Arnold et al., 1993; Robey and Fowlkes, 1994). The majority of these investigations have examined the response of abundant classes of T cells, bearing transgenic T cell receptors or specific V_β genes, which interact with MHC molecules. Although these studies demonstrate that these different populations of thymic cells can induce tolerance, they do not address the question of which cells induces tolerance to endogenous, processed peptide antigens. The role of peptide, presented in the groove of MHC molecules, in both positive and negative selection is becoming increasingly clear (Robey and Fowlkes, 1994).

In this chapter the identity of thymic cells producing T antigen, as well as the endogenous pancreatic genes expressed in the thymus, is investigated using two complementary approaches. First, immunohistochemical staining of thymic sections demonstrates that T antigen expressing cell possess morphological and positional characteristics of dendritic cells. Cell fractionation and immunodepletion studies of cells prepared from young adult thymus also supports the identification of the thymic cell type producing T antigen as a dendrtric cell. Finally, transcripts for several other pancreatic genes are found in the same cell fraction which contains the T antigen mRNA.

MATERIALS AND METHODS

Immunohistochemistry. Paraffin embedded thymi from newborn RIP1-Tag2 mice or from mice which had been transplanted under the renal capsule, were sectioned. Sections were treated with Antigen Retrieval Solution (Biogenix, San Ramon, CA). T antigen staining was visualized using a polyclonal rabbit anti-T antigen antibody, generated in our lab, and developed with an ABC-Elite kit (Vector, Burlingame, CA). The substrate was DAB. Sections were counterstained either with hematoxylin and eosin or methyl green.

Preparation of Low Density Cells from Thymus. Cells were prepared according to the protocol of Crowley and coworkers (1989; Swiggard et al., 1992). Briefly, individual thymic lobes were collected from 3.5 to 6 week old

mice, and perfused with collagenase, 100U/ml (Type III, Worthington Biochemicals, Freehold, NJ) in Hank's buffered saline. The perfusate was collected separately (fraction 1). Thymic lobes were torn into small pieces with forceps. The cells released by this step were collected, while the larger pieces of tissue were further digested in 400 U/ml collagenase, at 37°C for 15 minutes. The cells released by mechanical disruption of the thymus and those released by digestion with collagenase at 37°C, were pooled and passed through a wire mesh (fraction 2). The cells were then pelleted at 1000 rpm in a Sorvall RT6000B, and resuspended in dense BSA. Five to 6 ml were placed in a Sorvall (18 x 100 polycarbonate) tube, and overlaid with 1.5 ml of RPMI. The cells were spun for 15 minutes at 9000g (7000 rpm) in a Sorvall HS-4 rotor, without a brake. The pellet (fraction 3) and the fluffy cells at the interface (fraction 4) were collected separately. This procedure is diagrammed in Figure 25.

Preparation of Dense BSA. To prepare dense BSA, 186 ml PBS, 65 ml dH_2O, and 29 ml 1 M NaOH were added together in the bottom of a 1 liter beaker, taking care not to splash the liquid on the sides. The liquid was overlaid with 106 g BSA (Cohn fraction V, #3220-75, Intergen, NY) and allowed to dissolve overnight at 4°C. The next day, the refractive index of the solution was measured and adjusted to 1.384 to 1.385 at 25°C. The solution was then sterile filtered, using a prefilter and a 0.45 mm Nalgene filter unit, and stored at 4°C.

Flow Cytometry. FACS analysis was performed on aliquots of all fractions to confirm their composition following the protocol of Crowley and coworkers

(1989). Briefly, 10^5 to 10^6 cells were resuspended in 1% BSA-PBS-0.02% azide in U bottom 96-well plates. Fc receptors on cells were blocked with 20% mouse serum, except for the samples labeled with antibody recognizing CD32. Cells were then incubated for 45 minutes to one hour with antibodies recognizing F4/80 (ATCC), CD32, B220, Thy-1 (Pharmingen, San Diego, CA), M5/114 (Boehringer Mannheim, Indianapolis, IN), and NLDC145 (Kraal et al., 1986, a kind gift from L. Jerabek in I. Weissman's lab). All antibodies were rat IgG. Antibodies purchased were directly conjugated to FITC, so they could be used in double staining with biotinylated NLDC145 and SA-PE (Beckton Dickinson, Milpitas, CA). For unconjugated antibodies anti-rat IgG-FITC (Jackson Immunoresearch, West Grove, PA) was employed as a secondary. Both single and double stainings were performed. Cells were washed with the same buffer and pelleted in a Sorvall RT6000B centrifuge. After washing three times, cell were fixed with 2% paraformaldehyde for 10 minutes, washed once more, and then stored in the dark until the next day. Forward scatter and fluorescence 3 channels where monitored, and compensation adjusted, to separate macrophages from dendritic cells, and both populations from small cells, mostly thymocytes. Cells were analyzed on a FACSort (Becton-Dickinson, Milpitas, CA).

RNA-PCR. RNA-PCR analysis was performed as described in chapter 3, using primers described in chapter 2.

Antibody Depletion of Low Density Cells. Unconjuated monoclonal antibodies used in the depletion experiment were titrated on low density cells (fraction 4) and analyzed by FACS to determine a concentration which

gave specific staining, with low non-specific background. Biomag anti-rat IgG beads (Advanced Magnetics, Cambridge, MA) were used according to the manufactures' recommendations. Beads were washed twice with Hank's buffered saline, 10% fetal calf serum, 7.5 mM EDTA. Then, 8 x10^8 particles, which gives a ratio of 50 beads/cell, were preincubated with titrated monoclonal antibodies, 120 mg Thy-1, 60 mg B220, 240 mg CD32, 60 mg NLDC145, 360 mg F4/80, and 25 mg M5/114, for 20 minutes in a total volume of 900 μl. Beads were washed twice and added to 1.6 x 10^7 low density cells in 10 ml and incubated on ice for 20 minutes, with gentle swirling. The cells bound to the beads were the separated from the cells remaining in solution with a magnet (Advanced Magnetics). Both bound and free fractions were collected, and RNA made as described in Chapter 2. The PCR analysis was performed as described in Chapter 3. The PCR signals were quantitated by scanning the autoradiograms of the PCR gels, using a BioRad densitometer (Richmond, CA).

RESULTS

Clues about Thymic Cell Type Expressing T antigen

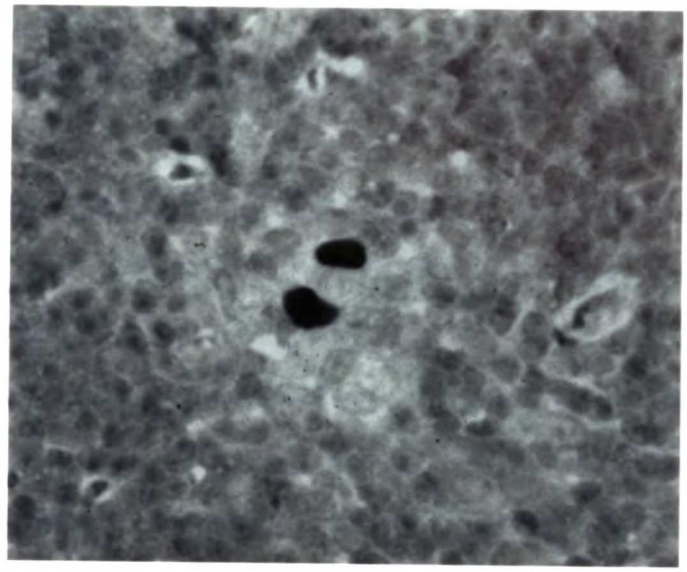
Immunohistology provides the unique opportunity to obtain information about the morphology and anatomical location of the T antigen expressing cells, which may provide clues about their identity. Since the amount of T antigen expressed in the thymus of both lines of mice is sufficient to induce tolerance of the $CD4^+$ T cells, when assessed 12 weeks

after transplantation into a nude mouse, the T antigen expressing cells must be present in these transplanted thymi. Therefore, thymi from nude mice, transplanted with RIP1-Tag2 and RIP3-Tag2 thymus, were examined for T antigen expression.

As shown in Figure 24, T antigen expressing cells were found in tissue sections from RIP1-Tag2 (panel A) and RIP3-Tag2 (panel B) thymi that had developed under the renal capsule for 12 to 14 weeks. The labeled nuclei are indented and quite large. Panel 24A, a tissue section from a transplanted RIP1-Tag2 thymus, shows a pair of T antigen staining cells. These doublets were often observed, and were also seen in newborn RIP1-Tag2 and transplanted RIP3-Tag2 thymic sections (data not shown). Tissue sections were counterstained with two different dyes to reveal distinct types of information about the T antigen expressing cells. When counterstained with hematoxylin and eosin, a large pale area, most likely the cytoplasm of this pair of cells, is seen surrounding the T antigen staining nuclei (panel 24A). The second counterstain, methyl green, allowed the cortex and the medulla to be easily distinguished. The cortex, which is packed with small thymocytes stains more heavily than the medulla. In tissue sections counterstained with methyl green, T antigen expressing cells are typically found adjacent to the cortical-medullary junction, sometimes at the junction (panel 24B) and sometimes in the medulla. This immunohistochemical analysis demonstrates that the T antigen expressing cells have large, indented nuclei, surrounded by a plentiful, clear cytoplasm, and are found at the cortical-medullary junction and in the medulla. The cell type that best fits this morphological and anatomical description is the interdigitating dendritic cell, which has been implicated in tolerance induction (Fairchild

Figure 24: *T antigen expression persist in transplanted transgenic thymus.* Paraffin sections from RIP1-Tag2 *(A)* and RIP3-Tag2 *(B)* thymi, which had been allowed to develop under the kidney capsule of a nude mouse, were immunostained for T antigen. T antigen staining cells often appeared as pairs *(A)*. Counterstaining with hematoxylin and eosin reveals a large, clear cytoplasm around the T antigen staining nuclei *(A)*. When counterstained with methyl green, which allows for easy identification of the cortex (C) and medulla (M), T antigen staining cells can be localized to the cortical-medullary junction *(B)*. Immunohistochemistry was performed by Ryo Hirose and Phil Galante.

A



B

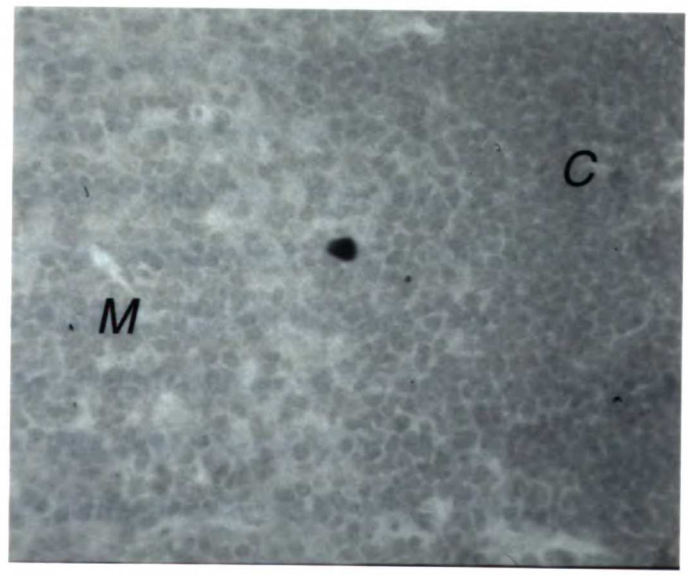


Figure 24

and Austyn, 1990). This study was performed in collaboration with Ryo Hirose and Phil Galante who performed the immunohistochemical analysis. These experiments were planned and evaluated by all three of us.

Fractionation of T antigen Expressing Cells from the Thymus

The above analysis suggests that the thymic cell type expressing T antigen is a dendritic cell. Although this immunohistochemical study offers suggestive evidence supporting such an interpretation, it is not conclusive. Another approach to identify the cells producing T antigen is to purify different thymic cell populations based on their physical characteristics and assay them for the presence of T antigen. Protocols to isolate thymic dendritic cells (Crowley et al., 1989) and epithelial cells (Izon et al., 1994) have been developed. The fractionation scheme used in these studies is outlined in Figure 25. Briefly, thymi from young three to six week old mice were collected and perfused with collagenase. This perfusion serves to infuse the thymus with collagenase and release thymocytes. The perfusate was collected separately (fraction 1). Thymic lobes were then torn into small pieces, and further digested with collagenase to release epithelial cells and resident dendritic cells. The cells released by this treatment were then collected by centrifugation (fraction 2). These released cells were resuspended in dense BSA, overlaid with RPMI, and fractionated by centrifugation. The low density cells (fraction 4), enriched for dendritic cells and macrophages (Crowley et al., 1989), and the pellet (fraction 3), containing the bulk of the thymocytes and epithelial cells (Izon et al., 1994), and some

Figure 25: *Fractionation scheme for low density cells.* Thymi from young mice were collected and perfused, treated with collagenase, and pelleted. This pellet is referred to as the total released cells (fraction 2). The total released cells were resuspended in dense BSA, overlaid with RPMI, and spun at 9000g. The perfusate (fraction 1), final pellet (fraction 3), and low density cells (fraction 4) were separately collected.

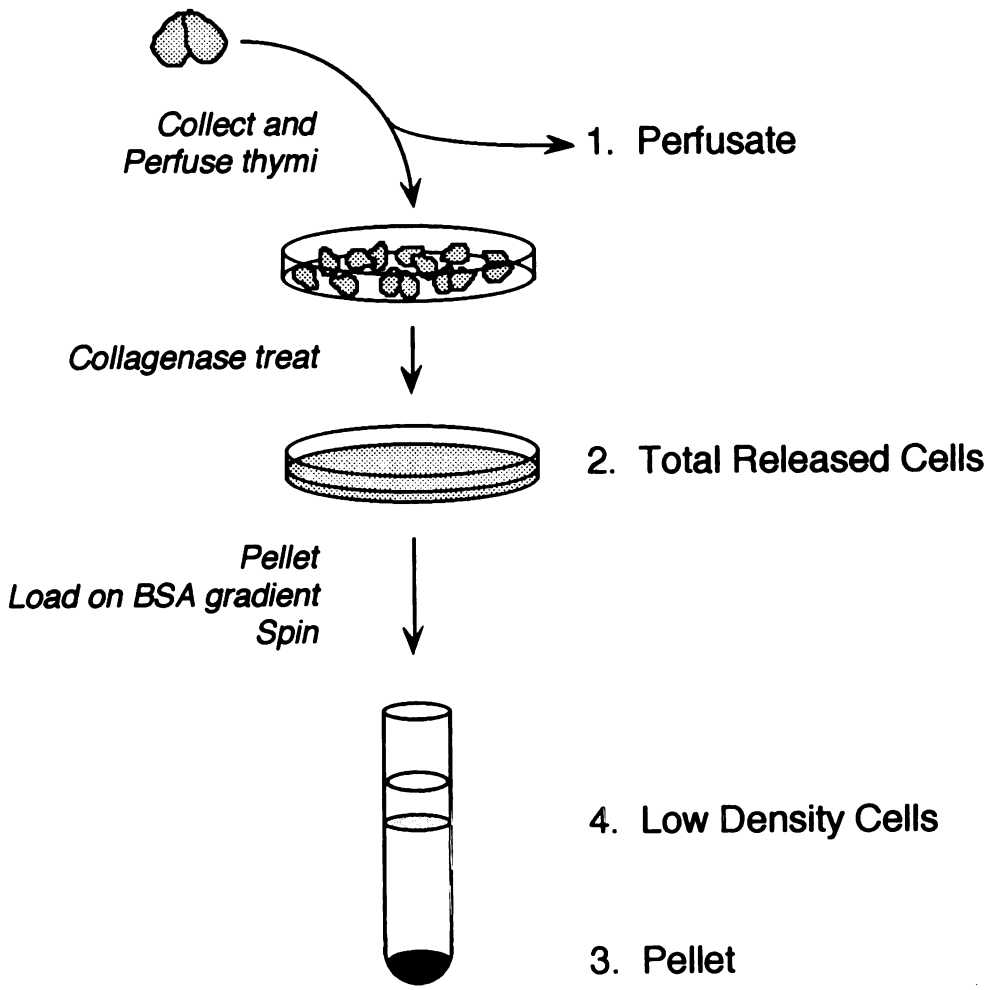

Collect and
Perfuse thymi
1. Perfusate
Collagenase treat
2. Total Released Cells
Pellet
Load on BSA gradient
Spin
4. Low Density Cells
3. Pellet

Figure 25

macrophages, from this spin (fraction 3), were collected separately. The low density fraction contains approximately 1% of the total cells (Table 7).

To confirm the identity of the cells in the various populations, aliquots from each of the fractions were analyzed on the FACS for their size, as measured by forward scatter, side scatter, and for their autofluorescence, as measured in the fluorescence 3 channel (Figure 26). The majority of cells in fractions 1, 2, and 3 had low forward and side scatter, and minimal autofluorescence. These characteristics are properties of small cells with few cytoplasmic granules, such as thymocytes. In contrast, the cells in the low density fraction, contained a high percentage of cells with higher forward and side scatter, and autofluorescence. At least 27% of the cells in the low density fraction exhibited these characteristics. Thymic dendritic cells range in size, exhibiting high forward scatter, intermediate side scatter and some autofluorescence, consistent with their being large cells, larger than thymocytes and even some macrophages, with few cytoplasmic granules. Macrophages typically display high forward and side scatter, as well as high levels of autofluorescence, indicative of their large size and numerous cytoplasmic granules (Crowley et al., 1989; Vremec et al., 1992). This analysis shows that the low density fraction of the cells is greatly enriched for these two bone marrow derived populations. Cells from the pellet and low density fractions were also inspected under the microscope after being cultured for 90 minutes (Figure 27). The majority of the cells from the pellet fraction are small and round, while many of the low density cells were large, adhered to plastic, and spread out processes during the culture period. It is difficult to distinguish the macrophages from the dendritic cells in this culture, since thymocytes, which copurify with these cells, adhere to their

Table 7: *Enrichment of low density cells from collagenase treated thymus.* **Thymi from nine, four to six week old RIP1-Tag2 mice were collected and the cells fractionated according to the scheme shown in Figure 25. Aliquots from each fraction were counted and the yield of cells determined.**

Fraction		***# Cells***	***% of Total***
1	Perfusate	7.02×10^8	11.1%
2	Total released	2.82×10^9	44.4%
3	Pellet	5.57×10^9	87.8%
4	Low density	7.17×10^7	1.1%

Table 7

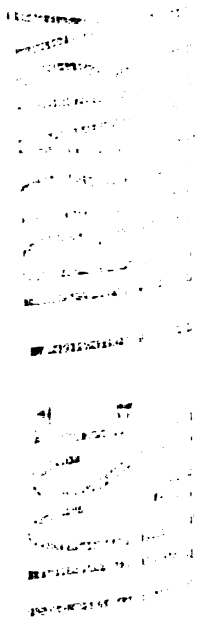


Figure 26: *FACS analysis of fractionated thymic populations.* Cells from all four fractions were analyzed for size (forward scatter), granularity (side scatter), and autofluoresence (Fluorescence 3). The percentage of cells in eac of the quadrants if shown.

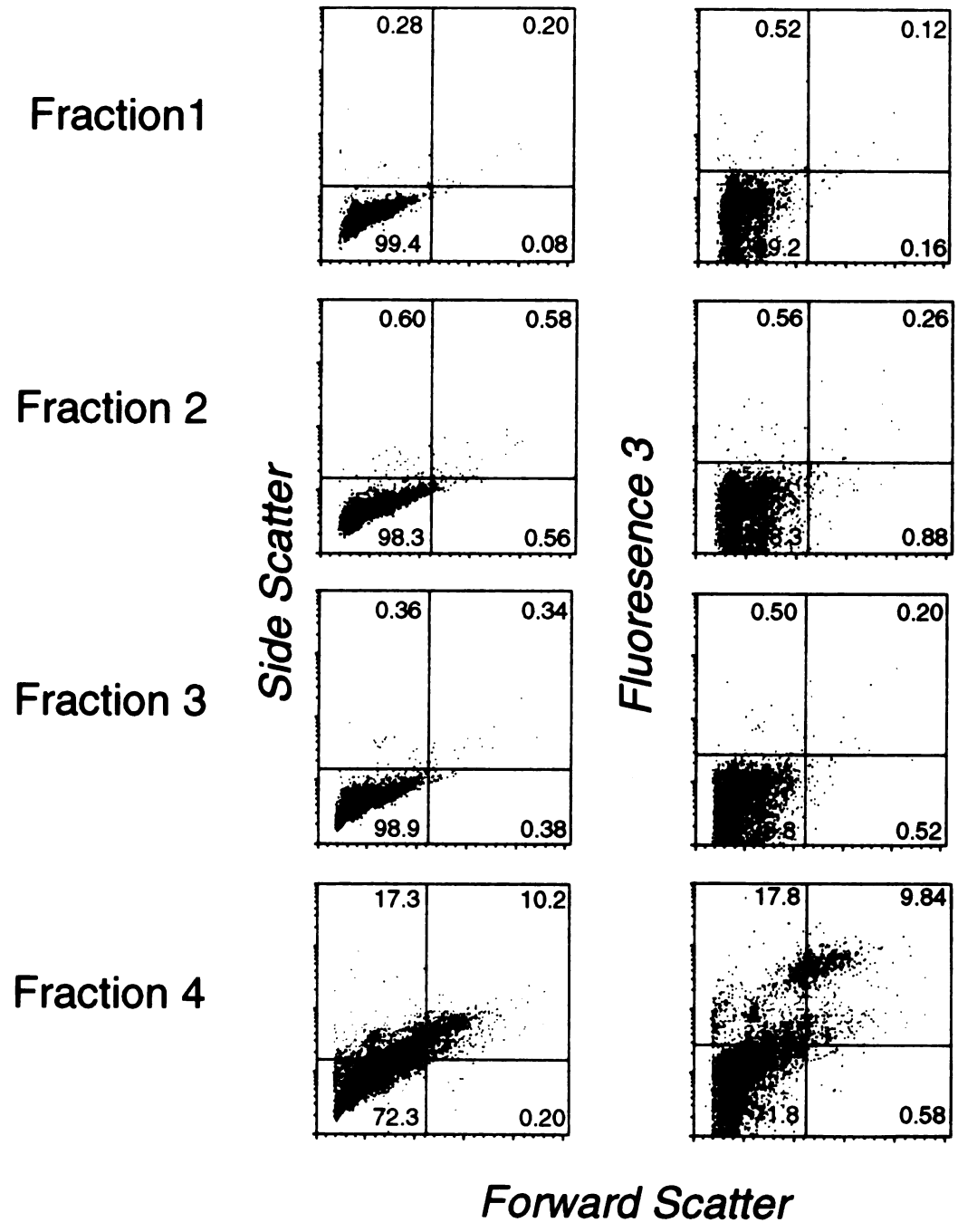
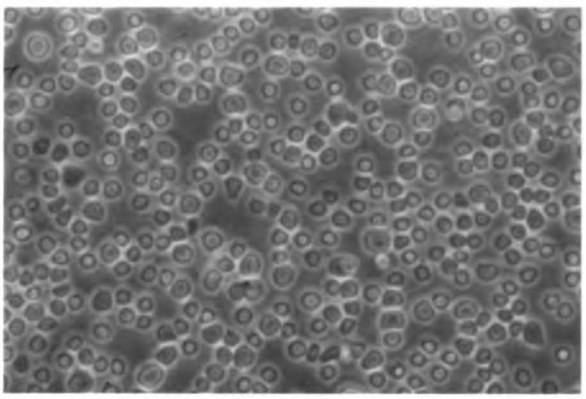

Fraction1
Fraction 2
Fraction 3
Fraction 4
0.28
0.20
99.4
0.08
0.52
0.12
9.2
0.16
0.60
0.58
98.3
0.56
0.56
0.26
0.88
0.36
0.34
98.9
0.38
0.50
0.20
0.52
17.3
10.2
72.3
0.20
17.8
9.84
0.58
Side Scatter
Fluoresence 3
Forward Scatter

Figure 26

Figure 27: *Morphology of cells present in pellet and low density cells fractions.* Thymic cells were fractionated according to the scheme in Figure 24 and placed in culture for ninety minutes. The majority of the cells from the pellet, fraction 3, are small and round, and are most likely thymocytes *(A).* Many of the low density cells are large and adhere to the tissue culture dish during the culture period *(B).* The small cells sitting on top of the adherent cells are thymocytes.

A



B

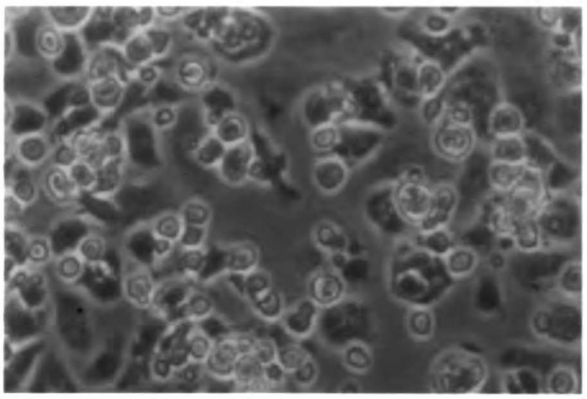


Figure 27

surface. Once again, adherence to tissue culture plastic is a property of both dendritic cells and macrophages (Crowley et al., 1989).

Next, the thymic cell fractions were tested for the presence of cells expressing T antigen. RNA was prepared from aliquots of the four different fractions, reverse transcribed, and tested by RNA-PCR for the enrichment of T antigen and, as a positive control, $ß_2$-microglobulin message (Figure 28). Spliced T antigen RNA was only detected in the low density fraction. Analysis of the precursor fraction, fraction 2, failed to show any message for T antigen. As shown previously (Table 3), T antigen RNA cannot always be detected in thymic RNA from adult mice. Thus, T antigen is expressed by a cell type enriched in the low density fraction, which contains a high percentage of macrophages and dendritic cells.

mRNAs for Other Pancreatic Genes Are Also Enriched in the Low Density Fraction.

If the cells present in the low density fraction are involved in induction of tolerance to endogenous "tissue specific" proteins, message for the pancreatic genes expressed in the thymus should also be detected in the low density fraction. Therefore, cDNAs from the four different fractions, prepared from RIP1-Tag2 thymus, were tested for the presence of insulin and glucagon, produced by two different islet endocrine cell types, as well as amylase and elastase, both produced by the exocrine pancreas. Insulin, glucagon, and elastase had previously been shown to be expressed intrathymically, while RNA-PCR had failed to detected message for amylase

Figure 28: *T antigen message is present in the low density fraction of cells.* cDNA was prepared from each of the four thymic fractions, prepared from thymi taken from nine four- to six- week old RIP1-Tag2 mice. Aliquots were tested for the presence of spliced T antigen and $ß_2$-microglobulin ($ß_2$m) message. This analysis was performed on the same preparation of cells shown in Figure 26 and in Table 7. cDNA prepared from a newborn (nb) RIP1-Tag2 thymus was included as a positive control.

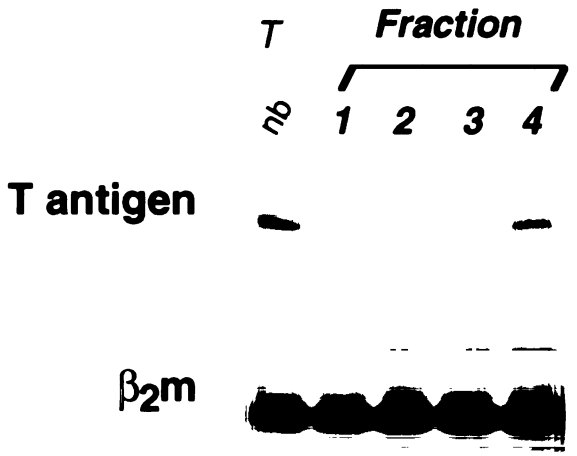


Figure 28

(see Table 1). mRNA for all four genes was detected in the low density fraction (Figure 29). The signal for insulin was clearly present in fraction 2, and was clearly enriched in the low density population. Signals for glucagon and amylase, in addition to being enriched in fraction 4, were present in the perfusate. The signal for elastase was also present in the low density fraction, while a low level was detected in fraction 3, but not the precursor fraction, fraction 2. In sum, T antigen, insulin, glucagon, amylase, and elastase are all expressed by cells present in the same low density cell population.

Antibody Depletion of Low Density Cells.

The cells in the low density fraction are not a homogeneous population and include dendritic cells, macrophages, B cells, and thymocytes (Crowley et al., 1989). These cells can be separated by their ability to differentially adhere to plastic. When the low density cells are plated out on plastic, macrophages and dendritic cells adhere after 90 minutes of culture. Thymocytes, as well as a population of non-adherent dendritic cells, can then be washed off. After overnight culture at 37°C, the loosely adherent dendritic cells lift off the dish, leaving the bulk of the macrophages behind. In this way, non-adherent, loosely adherent, and adherent populations can be isolated, each which is enriched for a given population of cells. When such populations were prepared and analyzed for the presence of T antigen message, no signal was obtained (data not shown), suggesting that the overnight culture resulted in the down regulation of T antigen expression. It is well established that overnight culture of freshly prepared dendritic cells

Figure 29: *mRNA for endogenous pancreatic genes present in low density cells.* cDNA from the four thymic cell fractions, prepared from four to six week old RIP1-Tag2 mice, was tested for enrichment of message for endogenous pancreatic genes by RNA-PCR. Presence or absence of reverse transcriptase (RT) in the reverse transcription reaction is indicated by a + or -. The amount of RNA, in μg, reverse transcribed is indicated over the lane. Included as controls are pancreas (P) from six week old RIP1-Tag2, thymus (T) from newborn (nb) RIP1-Tag2, and thymus from adult non-transgenic mouse.

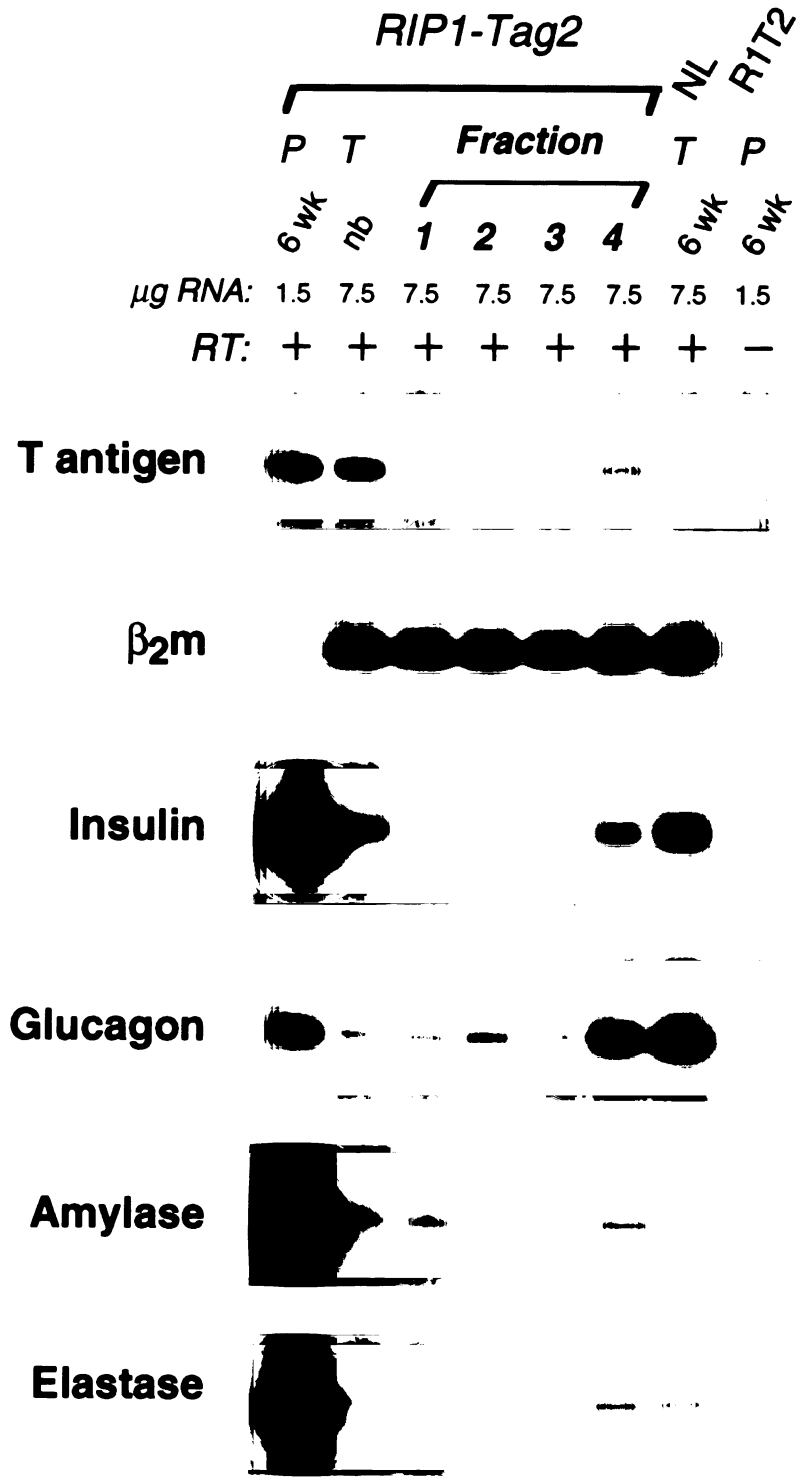

RIP1-Tag2
NL
R1T2
P
T
Fraction
T
P
6wk
nb
1
2
3
4
6wk
6wk
µg RNA: 1.5 7.5 7.5 7.5 7.5 7.5 7.5 1.5
RT: + + + + + + + −
T antigen
β2m
Insulin
Glucagon
Amylase
Elastase

Figure 29

greatly reduces their ability to present antigens (Steinman and Swanson, 1995). The effects on culture on gene expression may explain some of the variability seen in the levels of insulin and elastase mRNAs discussed above.

Another approach, antibody mediated magnetic bead depletion, has been used to isolate highly purified populations from low density cells prepared from thymus (Vremec et al., 1992). Before proceeding with the magnetic bead depletion, the composition of the low density cells was determined by two color FACS analysis. Cells were stained with antibodies recognizing cell surface markers present on different cell types represented in this population. Due to the high percentage of autofluorescent macrophages, it was difficult to distinguish stained cells in a typical FL1 vs. FL2 plot. Therefore, gates were drawn around the different populations, which could be identified on the forward scatter versus fluorescence 3 plot (Figure 30). These gated populations could then be analyzed separately for staining with various antibodies in a two color analysis. It should be noted that the bulk of the cells in fractions 1 and 3, are present in region 4 on the FACS plots, while the cells in this region only comprise 71.3% of the cells in the low density fraction (fraction 4).

A summary of the FACS analysis is shown in Table 8. Macrophages, which are large and autofluorescent, appear in region 1. They stain with antibodies recognizing F4/80, the Fc receptor II CD32, and MHC class II, and represent about 12.5% of the total cell population. Dendritic cells were present in several of the regions, due to the range of sizes exhibited by these cells. They vary in size between slightly larger than thymocytes to slightly larger than macrophages. The bulk of the dendritic cells are present in

Figure 30: *Percentage of large, autofluorescent cells in thymic cell fractions.* Cells from perfusate, pellet, and low density fractions, 1, 3, and 4, respectively, were stained with antibodies recognizing various thymic cell types and analyzed by FACS. Shown are the forward scatter versus the fluorescence 3 profiles for each of the three fractions, the regions used for gating during analysis of the staining, and the percentage of cells present in each of the regions, is shown for the unstained samples.

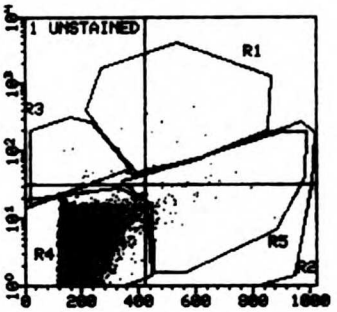
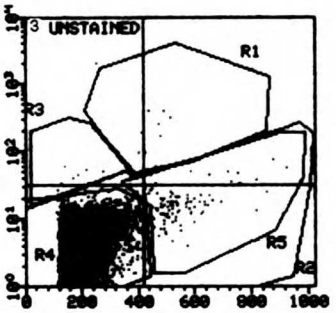
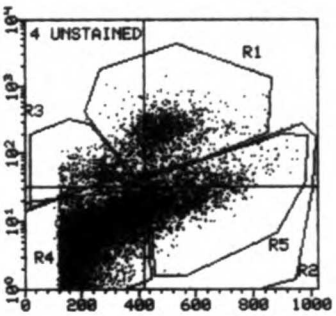
		R1	R3	R4	R5
Fraction1		0.09	0.13	99.0	0.63
Fraction 3		0.09	0.08	98.5	1.11
Fraction 4		13.0	4.3	71.3	17.8

Figure 30

Table 8: *Cellular composition of thymic cell fraction 4.* FACS analysis was used to determine the composition of low density cells. Cells were stained with the antibodies indicated, to identify the various cell types present in each of the regions. Regions are those shown in Figure 30.

Region and Characteristics		%	Cellular Composition	Markers
R1	Large, high autofluorescence	12.5%	11.5% macrophages	F4/80$^+$, CD32$^+$, Class II$^+$
			1.0% dendritic cells	NLDC145$^+$, CD32$^+$, Thy-1$^+$ Class II^{++}, F4/80^{lo}
R3	Small, some autofluorescence	4.6%	3.0% monocytes or resting macrophages	F4/80$^+$, CD32$^+$, Class II$^+$
			1.0% dendritic cells	NLDC145$^+$, CD32$^+$, Thy-1$^+$ Class II^{++}, F4/80^{lo}
			0.6% thymocytes	Thy-1$^+$ only
R4	Small, little autofluorescence	68.0%	48.2% thymocytes	Thy-1$^+$ only
			11.9% dendritic cells Class II^{++}, F4/80^{lo}	NLDC145$^+$, CD32$^+$, Thy-1$^+$
			7.9% other APC, may include monocytes and/or B cells	CD32$^+$, Class II$^+$, NLDC145$^-$, F4/80$^-$
R5	Large, some autofluorescence	14.5%	12.5% dendritic cells	NLDC145$^+$, CD32$^+$, Thy-1$^+$ Class II^{++}, F4/80^{lo}
			2.0% thymocytes	Thy-1$^+$ only

Table 8

regions 4 and 5. They express NLDC145, a molecule found on dendritic cells which is absent on the other cells present in the low density fraction. They also express very high levels of MHC class II, much higher than the levels found on the macrophages. In addition, the dendritic cells express low levels of F4/80 and CD32, which are typically found on macrophages. Thy-1 was also detected at low levels on the dendritic cells. These last three molecules have been reported to be expressed at low levels on dendritic cells by several groups (Crowley et al., 1989; Vremec et al., 1992). The bulk of the thymocytes, which stained with Thy-1, but not any other markers, are present in region 4, the dominant population in the other fractions. A few larger cells stained for Thy-1, these are likely T cells blasts. Another population emerged in the analysis. These cells are small, present in regions 3 and 4, and stain with antibodies recognizing CD32 and MHC class II. Some of them stained with F4/80, while none expressed NLDC145. These cells are likely other antigen presenting cells, which may include a population of thymic B cells, monocytes, or small resting macrophages.

Having characterized the surface phenotype of the various populations present in the low density fraction, it was now possible to use these antibodies to separate the different cell populations and analyze them for expression of insulin. Aliquots of low density thymic cells were treated with anti-rat IgG-magnetic beads precoated with antibodies recognizing thymocytes (Thy-1), B cells (B220), macrophages (CD32, F4/80, M5/114), and dendritic cells (NLDC145, M5/114, Thy-1, CD32, F4/80). Cells which bound to the beads were collected with a magnet, while those which remained in the supernatant were collected by centrifugation. cDNA was prepared from both the bound and free cells. These cDNAs were then tested for the presence of

insulin and, as a control, ß2-microglobulin message (Figure 31). Two antibodies, one expressed exclusively by the dendritic cells, NLDC145, and the other expressed at very high levels by dendritic cells, M5/114, which recognizes MHC class II, both depleted the low density cells of the population expressing insulin. The insulin signal now appeared in cells which were bound to the beads. Antibody to NLDC145 and class II depleted the low density cells of 82% and 96% of the insulin signal, respectively. Three other antibodies also depleted the low density cells of insulin message, although less efficiently than either NLDC145 and M5/114, approximately 35%. All three recognize molecules which were shown by FACS analysis to be expressed on dendritic cells at low levels, Thy-1, CD32, and F4/80. Neither antibody recognizing B cells, B220, nor magnetic beads which had not been precoated with monoclonal antibody, depleted the low density cells of significant amount of insulin message. These data are consistent, once again, with the T antigen expressing, insulin producing cell, being a dendritic cell.

SUMMARY AND CONCLUSIONS

Two lines of evidence suggest that the T antigen expressing cells in the thymus, which are capable of inducing tolerance of the developing $CD4^+$ T lymphocytes, are interdigitating dendritic cells. First, immunohistochemical analysis of thymic tissue sections indicates that the nuclei of the T antigen expressing cells are large and indented. These cells have a large diffuse cytoplasm and are situated close to the cortico-medullary junction and in the

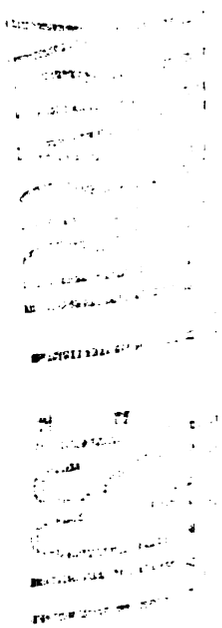


Figure 31: *Message for insulin is present in low density cells expressing MHC class II and a dendritic cell marker.* Low density cells, prepared from thymi of 43 three to six week old mice, were immunomagnetically depleted of various cell populations using monoclonal antibodies recognizing molecules expressed by different cell types present within the low density fraction. cDNA was prepared from cells which bound (Bound) to the beads and cells which remained in the supernatant (Free). Samples were analyzed for the presence of message for insulin, and as a control, $ß_2$-microglobulin. The percentage of the total insulin signal present in the bound fraction is indicated. The antibody used in the immunodepletions are indicated over the lanes. The lane marked with C, is a newborn RIP1-Tag2 thymus cDNA. Non-depleted samples from fractions 1, 3, and 4, from the same preparation, are also included as controls. Results of FACS analysis from this experiment are shown in Figure 30 and Table 8.

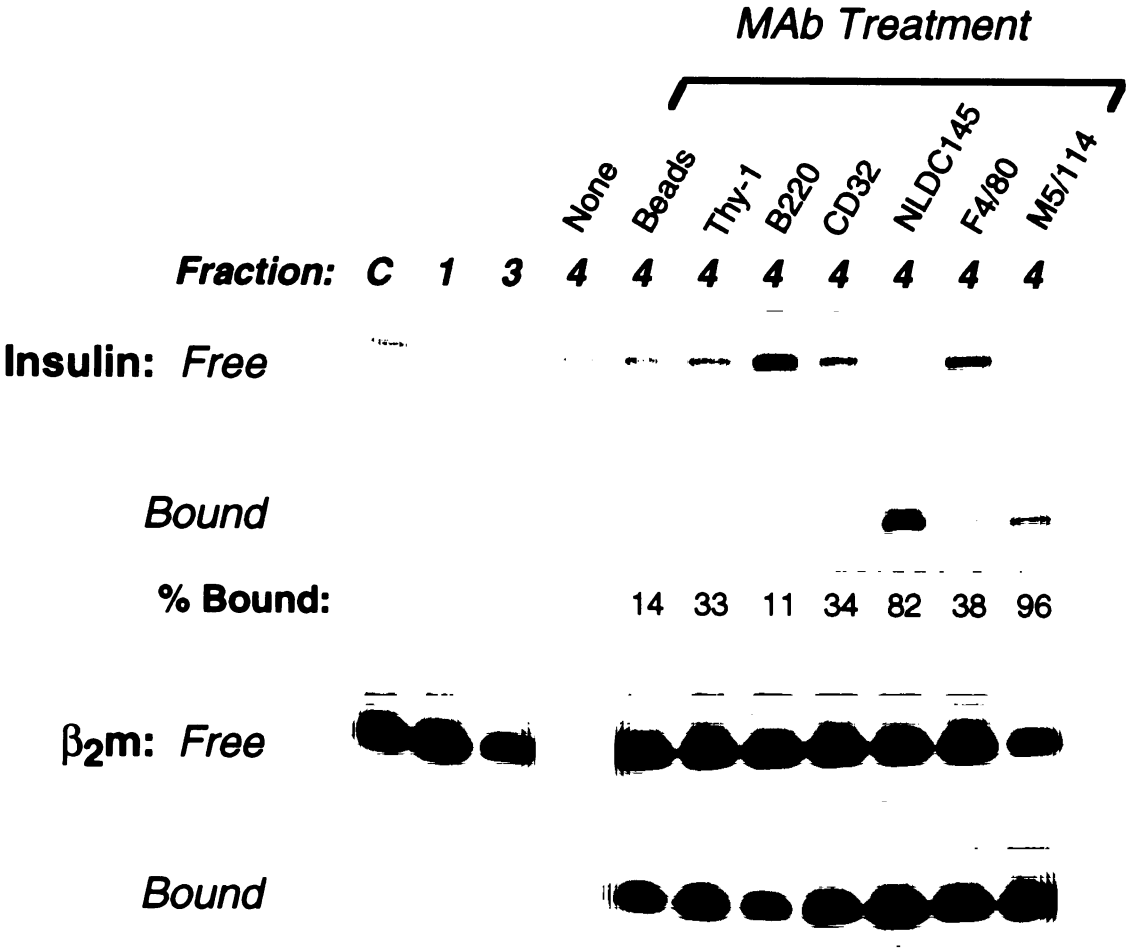

MAb Treatment
None
Beads
Thy-1
B220
CD32
NLDC145
F4/80
M5/114
Fraction: C 1 3 4 4 4 4 4 4 4 4
Insulin: Free
Bound
% Bound:
14 33 11 34 82 38 96
β2m: Free
Bound

Figure 31

medulla. These characteristics best describe the morphological and anatomical features of dendritic cells (Fairchild and Austyn, 1990). Secondly, cell fractionation studies indicate that the T antigen and insulin producing cells of the thymus have a low buoyant density. Dendritic cells and macrophages are present in this low buoyant density fraction, while the bulk of the epithelial cells have a high buoyant density and are found in the pellet fraction (Izon et al., 1994). Antibody depletion of the low density population demonstrated that insulin producing cells express a set of cell surface markers consistent with their identification as dendritic cells. The formal possibility exists that this cell is either a macrophage or an medullary epithelial cell. However, the bulk of the evidence does not support this contention.

In this chapter evidence is provided that the cell type expressing T antigen is a dendritic cell, a cell type which has been postulated to be involved in tolerance induction. In the previous chapter, thymic transplantation studies demonstrate that T antigen synthesized by the thymus efficiently induces tolerance of $CD4^+$ T cells. To induce this tolerance T antigen peptides have to be presented by class II MHC molecules. Thymic dendritic cells express exceptionally high levels of the class II gene products, higher than on dendritic cells from other source (Crowley et al., 1989; Vremec et al., 1992) or thymic macrophages (Crowley et al., 1989). Until recently little has been known about the manner in which dendritic cells process and present antigens. Recent progress is beginning to shed light on these mechanisms (Steinman and Swanson, 1995). It may be that thymic dendritic cells are specialized for presenting endogenously synthesized antigens on MHC class II molecules. This presentation could then result in

the induction of tolerance of developing $CD4^+$ T cells to the T antigen synthesized by the dendritic cells.

If this cell is specialized for the induction of tolerance to peripheral "tissue specific" proteins, it should also express the pancreatic genes previously identified as being expressed in the thymus. I have shown that messages for several genes normally expressed in both endocrine and exocrine pancreas can be detected in the low density fraction of cells. Dendritic cells make up about 25% of the cells in this population, while the rest of the fraction is composed of macrophages, thymocytes, and unspecified antigen presenting cells, most likely B cells, monocytes, or small resting macrophages. Therefore, it is possible that another cell type present in this fraction may express these pancreatic markers. However, the immunodepletion study provides strong evidence that at least insulin is expressed by dendritic cells. mRNAs for two of the pancreatic genes, glucagon and amylase, are not found exclusively in the low density fraction. Although messages for both are enriched in fraction 4, the low density fraction, they are also found in fraction 1, the perfusate. The bulk of the cells in fraction 1 are thymocytes, which are easily released from the thymus during perfusion. This fraction may be contaminated with low levels of dendritic cells, or another cell population. Although the co-expression of these "peripheral" proteins by a single cell type has not been proven, the current data is consistent with this interpretation and awaits confirmation.

CHAPTER 8:

Lessons Learned from RIP-Tag Mice

In this final chapter I will discuss the studies presented in this thesis on the immunological outcome of the expression of a novel self protein the SV40 T antigen (Tag) expressed under the control of the rat insulin promoter (RIP) in transgenic mice. First, I will review the following five topics: i) the characterization of the tolerant phenotype of embryonic onset RIP-Tag mice, ii) the characterization of the autoimmune phenotype of adult onset RIP-Tag mice, iii) immune surveillence of tumors in RIP-Tag mice, iv) a comparison of the requirements for the development of these two alternative phenotypes, v) the contribution of thymic expression of T antigen to the development of tolerance, vi) and the possible role of peripheral expression of T antigen in the development of tolerance. In conclusion, I will discuss the role that the thymus may play in inducing tolerance to "tissue specific" genes which are normally thought of as being peripheral.

Tolerance in Embryonic Onset RIP-Tag Mice

In an effort to understand how tolerance to proteins present in rare cell types is established, a transgenic model 'embryonic onset' RIP-Tag mice, that express a neo-self antigen have been studied. In these mice T antigen expression begins in the pancreatic ß cells at embryonic day 10, concomitant with the expression of the endogenous insulin genes (Alpert et al., 1988). The ß cells are a rare cell type consisting of about 5x 10^5 cell, distributed in 400

pancreatic islets. I have shown by RNA-PCR that the main source of T antigen protein, accessible to the immune system in these transgenic mice, is the pancreatic ß cell. Initial immunological characterizations demonstrated that these mice showed impaired responsiveness to T antigen: they had a reduced capacity to produce T antigen specific antibodies, their lymphocytes proliferated poorly to T antigen in vitro, and they were unable to generate T antigen specific cytotoxic T cells. In an effort to understand the role of antigen presentation by MHC class I molecules and $CD8^+$ T cells in establishing this tolerance, RIP1-Tag2 mice were crossed to mice deficient for the expression of $ß_2$-microglobulin. The $ß_2$-microglobulin negative progeny of this cross developed the same degree of tolerance as embryonic onset RIP-Tag mice which had this arm of their immune system intact. The tolerance of the $CD4^+$ T cells, as assessed by the in vitro proliferation assay, and the ability to generate T antigen specific antibodies were employed to characterize this tolerance. Since both of these assays depend on the ability of $CD4^+$ T cell function for their outcome, this result underscores the role that presentation of T antigen in a tolerogenic form by MHC class II molecules plays in the development of this phenotype.

In the course of my thesis research, the tolerance of the lymphocyte subsets has been examined in depth. During our initial characterization of the immunological phenotype of these mice, an in vitro proliferation assay was used to measure the tolerance of the $CD4^+$ T cells. The other two assays, the ability to produce T antigen specific antibody and the capacity to generate T antigen specific cytotoxic T cells, were not direct measures of the tolerance of the B cells or $CD8^+$ T cells, respectively, since these assays may have relied upon the $CD4^+$ T cells to provide help to these other lymphocyte

populations. Therefore, experiments were designed to more directly test the tolerance of the B cells and $CD8^+$ T cells.

First the tolerance of the B cells was addressed. From the quantitative assessment of the amount of T antigen protein produced in the pancreas shown in Chapter 2, it was estimated that if all the T antigen produced by the ß cells were quantitatively released into the bloodstream, the concentration would be insufficient to tolerize the B cells. Although this calculation allowed me to predict that the B cells were not tolerant of T antigen, this supposition required confirmation. To directly test this prediction, a classical hapten-carrier experiment was performed, where mice were immunized with T antigen, the hapten, covalently coupled to KLH, the carrier. The ability of the transgenic mice to produce as much anti-T antigen antibody as control mice when immunized with this covalently modified T antigen demonstrated that the B cells in embryonic onset RIP-Tag mice are not tolerant of T antigen.

Of the three assays used to study tolerance to T antigen in RIP-Tag mice, the cytotoxic T cell assay gave the most profound result. RIP-Tag mice failed to generate any T antigen specific cytotoxic T cell response. This inability may reflect direct tolerization of the $CD8^+$ T cells or lack of sufficient help from $CD4^+$ T cells. Cytotoxic T cells to many (Kosaka and Sprent, 1993; Doherty, 1993; Bodmer et al., 1993), but not all (Guerder and Matzinger, 1992), antigens can be generated in the absence of $CD4^+$ T cell help. However, to see helper-independent $CD8^+$ T cell activity, IL-2 must be added to the lymphocytes during the in vitro restimulation. To ask if the $CD8^+$ T cells in RIP-Tag mice are tolerant, splenocyte from immunized mice were cultured in the presence of IL-2 during the in vitro restimulation. When splenocytes

from transgenic mice which had been cultured in IL-2 were tested for their ability to lyse T antigen expressing targets, T antigen specific killing was now observed, although the lysis of targets was significantly less that of the control non-transgenic mice. This assay demonstrates that not all the $CD8^+$ T cells are tolerant and that at least some are helper-independent. However, it is not possible to determine from this experiment whether any $CD4^+$ helper-dependent $CD8^+$ T cells are present in tolerant RIP-Tag mice, since the $CD4^+$ T cells in these mice are tolerant. To assess whether any helper-dependent $CD8^+$ T cells are present, chimeric mice could be created by mixing $CD8^+$ T cells from tolerant RIP-Tag mice and $CD4^+$ T cells from non-tolerant mice and using these cells to reconstitute nude or RAG deficient hosts. These non-tolerant $CD4^+$ T lymphocytes could now provide help to the transgenic $CD8^+$ T cells. Assessment of the ability of these reconstituted mice to generate T antigen specific cytotoxic T cells would reveal the presence of helper-dependent T antigen specific $CD8^+$ T cells.

The $CD4^+$ T cells in embryonic onset RIP-Tag mice exhibit reduced responsiveness to T antigen, although some residual activity is observed. These $CD4^+$ T cells provide little help to B cells in the generation of T antigen specific antibody, proliferate poorly in response to T antigen protein in vitro, and may limit the generation of cytotoxic T cells recognizing T antigen. Naive $CD4^+$ T cells after encountering antigen differentiate into one of two types of effectors, Th1 and Th2 type helpers, which secrete different patterns of lymphokines and promote different types of immune responses. Th1 type cells secrete IL-2 and IFN-γ, which support cellular immune responses, such as generation of cytotoxic T cells, activation of macrophages, and production of complement fixing antibodies. Th2 type

cells secrete IL-4 and IL-10, which are associated with the production of non-complement fixing antibodies and allergic type responses (Finkelman, 1995). To ask if either of these populations develops preferentially in RIP-Tag mice, the IgG isotype profiles of the T antigen specific antibodies which do develop in RIP-Tag mice were assessed. IgG_1, an isotype whose production is supported by Th2 type cells, was underrepresented relative to the other IgG_{2a}, an isotype whose production is supported by Th1 type cells, as well as other IgG isotypes. Thus, Th1 type cells develop more readily upon immunization with T antigen. The inability of alum, which induces the almost exclusive development of IgG_1 (Enyon and Parker, 1992), to act as an adjuvant for the production of T antigen specific IgG also supports this conclusion.

In summary, antigen presentation by class I MHC and $CD8^+$ T cells are not required for the induction of tolerance to T antigen in embryonic onset RIP-Tag mice. B cells in these mice are not tolerant of T antigen, since they can produce antibody to T antigen under appropriate conditions. This inability to make antibody to T antigen is limited by the $CD4^+$ T lymphocytes. The residual $CD4^+$ T cell activity which develops after immunization of tolerant RIP-Tag mice has predominantly Th1 like activity, which supports cellular immune responses, and generation of complement fixing antibody. However, these $CD4^+$ Th1 type cells are still tolerant of T antigen. Some helper-independent $CD8^+$ T cell activity can be generated in tolerant RIP-Tag mice. However, whether the tolerant $CD4^+$ T cells prevent the generation of helper-dependent $CD8^+$ T cell is not known.

Autoimmunity in Delayed Onset RIP-Tag Mice Deficient for $ß_2$-microglobulin.

Mice from the delayed onset lineage RIP1-Tag5 first begin expressing T antigen in adulthood and fail to establish tolerance to this neo-self antigen. Instead, they develop an autoimmune response directed at their T antigen expressing ß cells. This autoimmunity is characterized by production of spontaneous autoantibodies directed against T antigen and an infiltration of T antigen expressing islets by $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and macrophages. To test whether the $CD8^+$ T cells are critical to the initiation of this autoimmune response, RIP1-Tag5 were bred to mice deficient for the expression of $ß_2$-microglobulin, which therefore lack functional expression of class I MHC molecules and fail to develop $CD8^+$ T cells. RIP1-Tag5 mice lacking $CD8^+$ T cells as a result of this deficiency develop autoimmunity, but with reduced severity. The appearance of spontaneous autoantibodies and infiltration of their T antigen expressing islets is delayed and both phenotypes develop more slowly, once initiated. These findings indicate that $CD8^+$ T cells are not essential for the initiation of disease, but may be important effectors in disease progression. A similar result has been observed in the induced autoimmune disease experimental allergic encephalomyelitis (Jiang et al., 1992; Koh et al., 1992). To confirm that this phenotype is due to the deficiency of the $CD8^+$ T cells, RIP1-Tag5 mice have been bred to mice deficient for the expression of CD8. These mice lack cytotoxic $CD8^+$ T cells, but express normal levels of class I MHC (Fung-Leung et al., 1991). The characterization of these mice is in progress.

The findings in RIP1-Tag5 mice are in stark contrast to phenotype of NOD/Lt mice, a model of insulin dependent diabetes, which were rendered genetically deficient for $CD8^+$ T cells (Katz et al., 1993; Serreze et al., 1994; Wicker et al., 1994). When NOD/Lt were crossed to $ß_2$-microglobulin deficient mice, they failed to develop diabetes or infiltration of their islets. These results imply an essential requirement for $CD8^+$ T lymphocytes in the initiation of the autoimmune response. This conclusion contrasts with that inferred from the cross of RIP1-Tag5 mice with $ß_2$-microglobulin deficient mice. It is likely that the contrasting results reflect distinctive requirements for the induction of islet infiltration in the two models. In NOD/Lt mice, 11 genetic loci have been mapped which influence disease progression (Theofilopoulos, 1995). Two of these Idd-3 and Idd-10 map to loci, IL-2 and Fc receptor I, respectively, which are important for the development of normal immune responses. In contrast, RIP1-Tag5 mice are inbred into C3HeBFe/J and the only genetic factor influencing the development of autoimmunity is the expression of the RIP-Tag transgene.

Immune Surveillance of Tumors in RIP-Tag Mice.

One feature common to all RIP-Tag mice, both those from families which establish tolerance and those from families which develop autoimmunity, is the eventual development of ß cell tumors, due to the expression of the oncogenic T antigen in their pancreatic ß cells. To determine whether immune surveillance by lymphocytes limits tumor progression in either tolerant RIP1-Tag2 or non-tolerant RIP1-Tag5 mice,

mice from both these lineages were bred to mice deficient for the expression of ß$_2$-microglobulin. ß$_2$-microglobulin null mice lack both cytotoxic T cells and natural killer cells, both which require the expression of functional class I MHC molecules for their maturation. These two types of lymphocytes have been previously implicated in the elimination of tumor cells by the immune system. RIP1-Tag2 mice deficient for the expression of ß$_2$-microglobulin showed no change in their tumor number, tumor size, or longevity, relative to their class I expressing siblings. Therefore, immune surveillance by lymphocytes does not normally limit tumor progression in RIP1-Tag2 mice. However, RIP1-Tag5 mice typically develop an autoimmune response directed at their T antigen expressing islets. Surprisingly, this immune response is inadequate to prevent the inevitable development of insulinomas in these mice. RIP1-Tag5 mice deficient for the expression of ß$_2$-microglobulin elaborate an immune response against their T antigen expressing islets, although this response is reduced in severity and its initiation is somewhat delayed, relative to class I expressing RIP1-Tag5 mice. These findings implicate the CD8$^+$ cytotoxic T cells as important effectors in the evolution of this autoimmune response. Thus, one may predict that tumor progression may be more aggressive in the absence of the cytotoxic T cells and natural killer cells in ß$_2$-microglobulin null RIP1-Tag5 mice. In contrast to this prediction, the rate of tumor progression in ß$_2$-microglobulin deficient RIP1-Tag5 mice is decreased, not increased, relative to the ß$_2$-microglobulin expressing RIP1-Tag5 mice. Thus, the autoimmune reaction appears to promote, not hinder, tumor development. The most likely explanation is that the autoimmune T cells, which home to the islets, secrete cytokines of other factors which promote tumor formation. The

elucidation of the mechanism by which these lymphocytes encourage tumor development may provide insight into potential therapies to inhibit tumor progression in humans.

Comparison of the Requirements for Establishing Tolerance and Inducing Autoimmunity.

The availability of mice which express the same transgene, but develop opposite immunological phenotypes, provides the rare opportunity to examine the requirements for the development of these two divergent outcomes. Embryonic onset RIP-Tag mice develop tolerance to T antigen, while delayed onset RIP-Tag mice are non-tolerant. Tolerance develops in embryonic onset RIP1-Tag2 mice in the absence of functional class I MHC molecules and $CD8^+$ T cells. This finding underscores the essential role that antigen presentation by class II MHC molecules, which present T antigen peptides to the $CD4^+$ T cells, play in establishing the tolerant phenotype. In tolerant RIP1-Tag2 and RIP3-Tag2 mice, the $CD4^+$ T cells proliferate poorly in response to T antigen in vitro, and limit the ability of the B cells, which are non-tolerant of T antigen, to respond to this novel self protein.

The autoimmune phenotype in delayed onset lineage RIP1-Tag5 also indicates that the $CD4^+$ T cells play an important role in the development of autoimmunity. $CD4^+$ T lymphocytes from immunized RIP1-Tag 5 mice are fully responsive to T antigen in an in vitro proliferation assay. In RIP1-Tag5 mice which lack functional MHC class I molecule and $CD8^+$ T cells, an autoimmune response to the T antigen expressing ß cells develops, although

with reduced severity, which manifests as a decreased incidence and titer of autoantibodies, as well as a reduced percentage of islets which become infiltrated with lymphocytes. Therefore, the $CD8^+$ T cells appear to play an important role in the progression, although they are not required for initiation of the autoimmune response in RIP1-Tag5 mice. Thus, in the development of these alternative phenotypes, tolerance and autoimmunity, the $CD4^+$ T cells play a pivotal role in the development of the immunological phenotype.

The profound systemic tolerance observed in embryonic onset RIP-Tag mice motivated the examination of the thymus, the site of T cell development and central tolerance induction, for the expression of T antigen. Mice from both embryonic onset families, RIP1-Tag2 and RIP3-Tag2, were shown to express T antigen in the thymus from birth until at least two weeks of age, and in the case of RIP1-Tag2, until immunological maturity is reached. This expression recapitulates that of the endogenous insulin genes which are also expressed intrathymically throughout development, indicating that this expression is not an artifact of the transgenic system. If this thymic expression contributes to the development of the tolerant phenotype in embryonic onset RIP-Tag mice, then a prediction can be made: delayed onset RIP-Tag mice should fail to express T antigen intrathymically. The expression of T antigen was examined in thymus from both young and older delayed onset lineage RIP1-Tag5. T antigen message was not detected at any time point examined. Thus, thymic expression of T antigen correlates with the development of tolerance to this novel self antigen, and failure to express T antigen in the thymus correlates with non-tolerance and the development of autoimmunity.

Contribution of Thymic T antigen Expression to Tolerance Induction

Thymic T antigen expression correlates with the development of tolerance and recapitulates that of the endogenous insulin genes, which are expressed intrathymically throughout development. To ask if other 'tissue specific' genes are produced in the thymus, the expression of other pancreatic genes normally synthesized in the both endocrine and exocrine pancreas was examined. mRNA for glucagon, somatostatin, pancreatic polypeptide, trypsin and elastase were found to be expressed intrathymically in both newborn and adult thymus. These findings taken together suggest a role for the thymus in inducing tolerance to 'tissue specific' proteins normal thought of as being peripheral.

To determine the role that thymic T antigen plays in establishing the tolerant phenotype, thymus from newborn embryonic onset RIP-Tag mice was transplanted into nude mice and the immune system allowed to develop in a setting where the only source of T antigen was the newly transplanted thymus. Since the B cells in these chimeras had developed prior to transplantation, they were non-tolerant of T antigen. Therefore, both the in vitro proliferation assay and the capacity to produce anti-T antigen antibodies provided measures of the tolerance of the $CD4^+$ T cells. In these two assays, the chimeric mice looked identical to the intact tolerant RIP-Tag mice. Thus, thymic T antigen expression is sufficient to confer tolerance of the $CD4^+$ T cells. However, when the $CD8^+$ T cell tolerance was assessed using the cytotoxic T cell assay, the majority of the animals generated cytotoxic T cells able to lyse T antigen expressing targets. Since the $CD4^+$ T cells in the chimeric mice are tolerized to the same extent as in intact

RIP-Tag mice, this result suggests that the $CD8^+$ T cells require a signal outside the thymus to become tolerant of T antigen. It seems likely that this signal is provided through an interaction of the $CD8^+$ T lymphocytes with T antigen presented by MHC class I in the periphery, possibly on the pancreatic ß cells.

In the transplantation studies, intact newborn thymus was employed as a source of transgenic thymus. This tissue contained both thymic epithelium and bone marrow derived residents of the thymus. Studies have shown that both medullary epithelium and bone marrow derived cells, most likely dendritic cells, can induce tolerance (Robey and Fowlkes, 1994). Although these studies have demonstrated that both cell types can tolerize developing T lymphocytes in the thymus, they have not addressed the capacity of thymic cells expressing 'tissue specific' genes to induce tolerance. Since T antigen expressed in the thymus of embryonic onset RIP-Tag mice induces tolerance of the $CD4^+$ T cells and recapitulates that of the endogenous insulin genes, the identification of the thymic cell type producing T antigen may provide insight into the way in which tolerance is established to proteins expressed by rare cell types.

To identify T antigen expressing cells in the thymus, two complementary approaches, immunohistochemistry and cellular fractionation, were taken. Both studies provide support for the conclusion that the T antigen expressing cell is a dendritic cell. In the thymus T antigen expressing cells have large, indented nuclei, surrounded by a large pale cytoplasm, and are situated at the cortico-medullary junction, the appropriate morphology and anatomical location for a dendritic cell. T antigen mRNA can be detected in a low density fraction of cells purified

from thymus. These cells are enriched in bone marrow derived cells, and represent only one percent of the total thymic population. Transcripts for several other 'peripheral' pancreatic genes were also found in this fraction. The signal for insulin is depleted from the low density fraction by cell subtraction with antibodies recognizing class II MHC and dendritic cells. These characteristics are all consist with the T antigen expressing cell being a dendritic cell, which can induce tolerance of $CD4^+$ T cells.

Three other studies using transgenically expressed antigens have implicated thymic epithelial cells as inducing tolerance of $CD8^+$ T cells. In all three studies the transgene was found to be expressed in the thymus. In two of the studies, the keratin IV promoter (Schönrich et al., 1992) and a milk protein promoter (Husbands et al., 1992) were used to direct expression of H-$2K^b$ to epithelial cells. In the mice employing the keratin promoter, thymic epithelium was demonstrated to express H-$2K^b$. In the second study, using the milk protein promoter, it was inferred that the H-$2K^b$ expressed in the thymus was produced by the epithelial cells, since all other tissues producing it were epithelial. In the third study, mice expressed the SV40 T antigen under the control of the elastase promoter. Thymi from newborn transgenic mice were heavily irradiated, with 1300 rads, to deplete all the bone marrow derived precursors, and then transplanted into athymic immunodeficient hosts. These irradiated thymi were shown to allow for the reconstitution of an immune system in which $CD8^+$ T cells recognizing T antigen were tolerant. The authors inferred that the tolerance was induced by the thymic epithelium (Antonia et al., 1995). Although the cell inducing the tolerance in these mice may well be an epithelial cell, this has not been formally proven. Notably, I have shown that elastase mRNA is expressed in the low

density fraction of cells, which is enriched for bone marrow derived components of the thymus. It is formally possible that the resident dendritic cells of the thymus resisted the radiation treatment. The resolution of these conflicting results awaits further investigation.

The Thymus, Is That All?

The reconstitution of the immune system of athymic mice with thymus from embryonic onset RIP-Tag mice unequivocally demonstrates that thymic expression of a tissue specific antigen is sufficient to induce tolerance of the $CD4^+$ T cells. The cell type expressing T antigen and insulin is most likely a bone marrow derived dendritic cell. Thymic dendritic cells express exceptionally high levels of class II MHC molecules and may be specialized for the presentation of endogenous synthesized antigens by these class II gene products. However, these same transplantation studies provide evidence that intrathymic expression of tissue specific antigens, such as the insulin promoted T antigen, is insufficient to induce tolerance of T antigen reactive $CD8^+$ T cells, since the majority of the chimeric mice tested can produce T antigen reactive cytotoxic T cells. These findings raise the intruiging possibility that tolerance to a novel tissue specific protein occurs intrathymically for $CD4^+$ T cells and extrathymically for $CD8^+$ T cells.

The finding that many transgenically expressed 'peripheral antigens' are expressed in the thymus has led investigators to ask if extrathymic tolerance exists. Extrathymic mechanisms of tolerance induction have been documented in a number different models, in which high levels of a foreign

antigen, a superantigen, the male specific antigen HY, or the lymphocytic choriomeningitis virus (LCMV), are introduced to a naive mature immune system (Webb et al., 1990; Rocha and von Boehmer, 1991; Moskophidis, et al., 1993). In two of these studies, the mice were athymic to ensure that the thymus could not contribute to the final tolerant state. A unifying theme in these studies is that the naive host first mounts a strong immune response to the newly introduced abundant antigen. This strong immune response is followed by deletion of the majority of the reactive T lymphocytes. Those remaining T cells which bear receptors capable of recognizing the newly introduced abundant antigen are now non-responsive upon further challenge. This mechanism of extrathymic tolerance induction is thought to be caused by terminal differentiation of the reactive lymphocytes, and has been termed 'peripheral exhaustion'.

In a recent study, this mechanism has been further explored. In this work the amount of tolerogen was manipulated, resulting in two different outcomes. When large amounts of antigen were present, the T cells did not first expand, but rather were rendered directly non-responsive, or anergic. However, when less antigen was available, peripheral exhaustion of the transgenic T cells ensued (Rocha et al., 1995). The authors concluded that tolerance was induced extrathymically as long as the antigen is not cleared from the organism, but rather persists. In all of these systems of extrathymic tolerance induction a mature immune system is suddenly flooded with an over abundance of a novel antigen and the T cells react to this foreign agent. While these studies demonstrate that tolerance can be induced extrathymically, they do not address the question of whether these same

mechanisms apply to proteins synthesized by normal cells in peripheral tissues.

The Role of Thymus in Inducing Tolerance to Tissue Specific Peptide Antigens: a Perspective

The thymus is the site of T cell maturation, where developing thymocytes undergo the two processes that shape the final T cell repertoire, positive and negative selection. Recent work has demonstrated a role for peptides presented in the antigen binding groove of MHC molecules in both of these processes (Robey and Fowlkes, 1994). Many investigators have assumed that if a protein is expressed in the thymus, tolerance to it will ensue. Two proteins, myelin basic protein and acetylcholine receptor, are expressed in the thymus, yet T cells recognizing them develop intrathymically, emigrate to the periphery, and, under the appropriate circumstances, can be activated to participate in an autoimmune attack on the hosts own tissues (Steinman, 1995). How then is it that some proteins expressed in the thymus induce tolerance and some do not? One possible explanation is that T cells with high affinity to these self antigens are deleted during intrathymic maturation and only those with low affinity escape to the periphery. These would only be activated under exceptional circumstances. The second possibility is that not all antigens expressed in the thymus are efficiently presented by MHC molecules. The simplest way in which to explain this possibility is to propose the only certain cells in the thymus are capable of inducing tolerance. This mechanism would demand

that those antigens expressed by specialized antigen presenting cells, such as the thymic dendritic cells and also, in this case, medullary epithelium, would induce tolerance, while proteins expressed by thymocytes and other rare cells in the thymus, such as neurons, fail to provide the necessary tolerization signal.

Evidence for the ability of thymically expressed antigens to tolerize the highest affinity T cells comes from an elegant study by von Herrath and coworkers (1994). They created transgenic mice which express either the glycoprotein (GP) or nucleoprotein (NP) of the lymphocytic choriomeningitis virus (LCMV) under the control of the rat insulin promoter. Some families of mice express the transgene in the pancreatic ß cells and not in the thymus, while other lines express the transgene in both tissues. When the mice which express the transgene solely in the pancreas are infected with LCMV, they develop diabetes within 10 to 14 days due to the destruction of the insulin producing ß cells. This autoimmune destruction depends only on presence of high affinity $CD8^+$ T cells, since depletion of $CD4^+$ T cells in the mice does not alter the kinetics or severity of disease. However, when mice which express the transgene in both thymus and pancreas are infected with LCMV, they too develop diabetes, but with delayed kinetics, only after several months. The authors demonstrate that in these lines of mice, autoimmunity develops only when both $CD4^+$ and $CD8^+$ T cells are present. The $CD8^+$ effectors which develop are not decreased in number, but rather show reduced affinity, and now require help from $CD4^+$ T cells to recognize the self antigen. These findings are supported by Heath and coworkers (1992), who demonstrate that the alloantigen H-$2K^b$, when expressed in the thymus under the control of the rat insulin promoter, only

allows for development of T cells with low affinity to H-2K^b. Thus, thymic expression of a "tissue specific" protein can result in tolerization of high affinity T cells.

In principle, any cell present in the thymus can induce tolerance to its own peptides on MHC class I molecules, as long as the cell is present in the correct anatomical site. The two main candidates are medullary epithelial cells and interdigitating dendritic cells. Bonomo and Matzinger (1992) provide evidence that thymic tolerance induction is cell type specific. These authors demonstrated that when thymic epithelium, which is devoid of hematopoietic cells, from one strain of mouse, C57BL/6 which is H-2^b, is transplanted into athymic recipients which possess a different MHC haplotype, BALB/c which are H-2^d, that the chimeras are tolerant of C57BL/6 epithelial cells, but not C57BL/6 bone marrow derived spleen cells. Other chimeric combinations are also studied which yield similar results. Thus, these authors provide evidence that tolerance is induced to the peptide-MHC complexes expressed by a given cell type. The same argument that tolerance is only induced to the peptides-MHC combinations found in the thymus has been made for bone marrow derived cells, and other thymic constituents (Nossal, 1994).

The majority of the above studies focus on tolerance induction of CD8$^+$ T cells and antigen presentation by MHC class I molecules. The case for CD4$^+$ T cells and MHC class II is less well studied. CD4$^+$ T lymphocytes play an important role in limiting the immune response to self antigens. Without help from antigen specific CD4$^+$ T cells, B cells are unable to produce antibody to protein antigens. Although cytotoxic CD8$^+$ T cell responses can be generated in the absence of help from CD4$^+$ T cells, helper-

independent cytotoxicity, lack of help from CD4+ T cells may result in a less than optimal response (Doherty, 1993; Bodmer et al., 1993; von Herrath et al., 1994). Therefore, it is important to ask: which antigens are presented by these class II expressing thymic cells? Clearly soluble proteins, present in the blood gain access to the antigen processing compartments of these tolerance inducing cells (Mitchison et al., 1984; Nossal, 1994). It has been more difficult to assess tolerance to endogenously synthesized proteins produced by these class II MHC expressing cells.

In embryonic onset RIP-Tag mice, low levels of T antigen are produced by discrete cells in the thymus. The evidence suggests that the cells producing T antigen are most likely dendritic cells, which express high levels of MHC class II. Whether or not this cell is ultimately proven to be a dendritic cell, the data are unequivocal that the thymically expressed T antigen produced by these cells is sufficient to tolerize the CD4+ T cells. This result argues that a thymic cell type is capable of processing a nuclear protein and presenting it on MHC class II molecules, thus inducing tolerance of CD4+ T cells which recognize this endogenously produced protein. It is still possible that T antigen is released by the cell which synthesizes it, and picked up by an adjacent class II MHC bearing cell. This seems unlikely since the amount of T antigen produced is quite low and once released would have to compete with the universe of proteins present in the extracellular environment. Nevertheless, it is intriguing that an extrathymic, 'tissue specific', nuclear protein, produced in the thymus, which should not normally gain access to the class II presentation pathway, enters this pathway and tolerizes the developing CD4+ T lymphocytes. These findings raise the possibility that thymic dendritic cells and possess unique antigen processing

ability. Although much is known about the antigen processing and presentation pathways in macrophages and B cells (Germain and Margulies, 1993), these abilities of dendritic cells are just beginning to be elucidated (Steinman and Swanson, 1995).

Although thymic expression of the insulin promoted T antigen results in tolerance induction of $CD4^+$ T cells, it is inadequate to ensure tolerance induction of the $CD8^+$ T cells. Chimeric mice whose only source of T antigen protein is a transgenic thymus are capable of generating T antigen specific cytotoxic T cells, although the extent of this ability varies from mouse to mouse. However, intact embryonic onset RIP-Tag mice, which express T antigen in both thymus and pancreas fail to generate T antigen specific cytotoxic T cells under the same conditions. Thus, it appears that T antigen encountered in the periphery plays a role in inducing tolerance of the T antigen reactive $CD8^+$ T lymphocytes. Several investigators studying tolerance induction of $CD8^+$ T cells have also found that intrathymic antigen encounter is insufficient to completely tolerize $CD8^+$ T cells. They observed that a second antigen encounter in the periphery drives the $CD8^+$ T cells into a more profound state of non-responsiveness (Kosaka and Sprent, 1993; Arnold et al., 1993). Thus, the thymus and the periphery may both play roles in limiting the reactivity of $CD8^+$ T cells.

The two mechanisms of thymic tolerance induction discussed above, namely deletion of high affinity T cells and cell type specific tolerance to epithelial and bone marrow derived cells, are not mutually exclusive. They would each serve to limit the number and affinity of T cells to the antigens which they encounter the most frequently once they emigrate from the thymus. There are many potential autoantigens which are not included in

this set of thymus specific proteins. It has been postulated that fragments of all proteins are expressed in the thymus (Boon and Van Pel, 1989). However, it seems that not all proteins in an organism are expressed and presented at sufficient levels to induce tolerance. For example, some proteins expressed in the periphery are ignored by the immune system (Mitchison, 1984; Ohashi et al., 1991). In the course of my thesis research I provide evidence that the thymus expresses some genes normally thought of as being 'extrathymic tissue specific proteins'. This expression can lead to tolerance of the developing $CD4^+$ T cells which recognize at least one of these antigens. The extrathymic tissue specific genes expressed in the thymus include an insulin promoted transgene T antigen, as well as several endogenous genes, normally produced by different pancreatic cell types, both endocrine and exocrine. I have provided preliminary evidence that at least some of these genes are expressed in a population of cells highly enriched for dendritic cells, which have been implicated in tolerance induction. Thymic expression of organ specific proteins may be yet another component of this complex system, operating in the thymus to limit reactivity to self proteins, which are more rarely encountered.

Final Summary

To conclude let me briefly summarize the lessons I have learned from studying RIP-Tag mice, which express a neo-self antigen that alternatively confers systemic tolerance or organ specific autoimmunity:

1) Functional class I MHC molecules and $CD8^+$ T cells are not required for the development of tolerance in embryonic onset in RIP1-Tag2 mice or the initiation of autoimmunity in delayed onset RIP1-Tag5 mice. However, this deficiency results in an autoimmune response of reduced severity, indicating that $CD8^+$ T cells are important effector in the development of this response.

2) Immune surveillance of tumors by cytotoxic T cells or natural killer cells does not limit tumor development in tolerant RIP1-Tag2 or non-tolerant RIP-Tag5 mice, since a deficiency of these two types of lymphocytes does not result in an increased tumor burden in either line of mice. Rather, the autoimmune response directed at the pancreatic ß cells in RIP1-Tag5 mice results in an increase in the number of tumors which develop, as seen by comparisons of tolerant RIP1-Tag2 or class I deficient RIP1-Tag5 mice. This increase in tumor progression may be due to local release of cytokines, by infiltrating lymphocytes, which promote tumor cell growth.

3) Embryonic onset RIP-Tag mice express T antigen in the thymus. This thymic expression is sufficient to induce tolerance of the $CD4^+$ T cells. These tolerant $CD4^+$ T cells limit the ability of the non-tolerant B cells to produce T antigen specific antibody.

4) Thymic expression of T antigen in embryonic onset RIP-Tag mice is not sufficient to induce complete tolerance of the $CD8^+$ T cells, which may require an additional interaction with T antigen presented by class I MHC molecules extrathymically to become tolerant. This interaction most likely takes place on the pancreatic ß cells, the main source of T antigen protein in these mice.

5) Delayed onset RIP-Tag mice fail to express T antigen intrathymically. Thus, thymic expression of T antigen correlates with tolerance and lack of thymic T antigen expression correlates with non-tolerance.

6) T antigen expression recapitulates that of the endogenous insulin genes, which are expressed intrathymically throughout development. In addition, several other pancreatic genes, normally produced by both endocrine and exocrine cell types, are expressed by the thymus.

7) The thymic cell type expressing T antigen is most likely a thymic dendritic cell, which has previously been implicated in tolerance induction.

8) Several endogenous pancreatic genes are also expressed by the same population of thymic cells which expresses T antigen. This population is greatly enriched for dendritic cells. Thus, the thymus may play a role in inducing tolerance to proteins which are normally thought of as being extrathymic 'tissue apecific' proteins.

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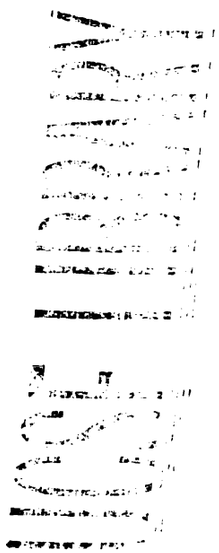

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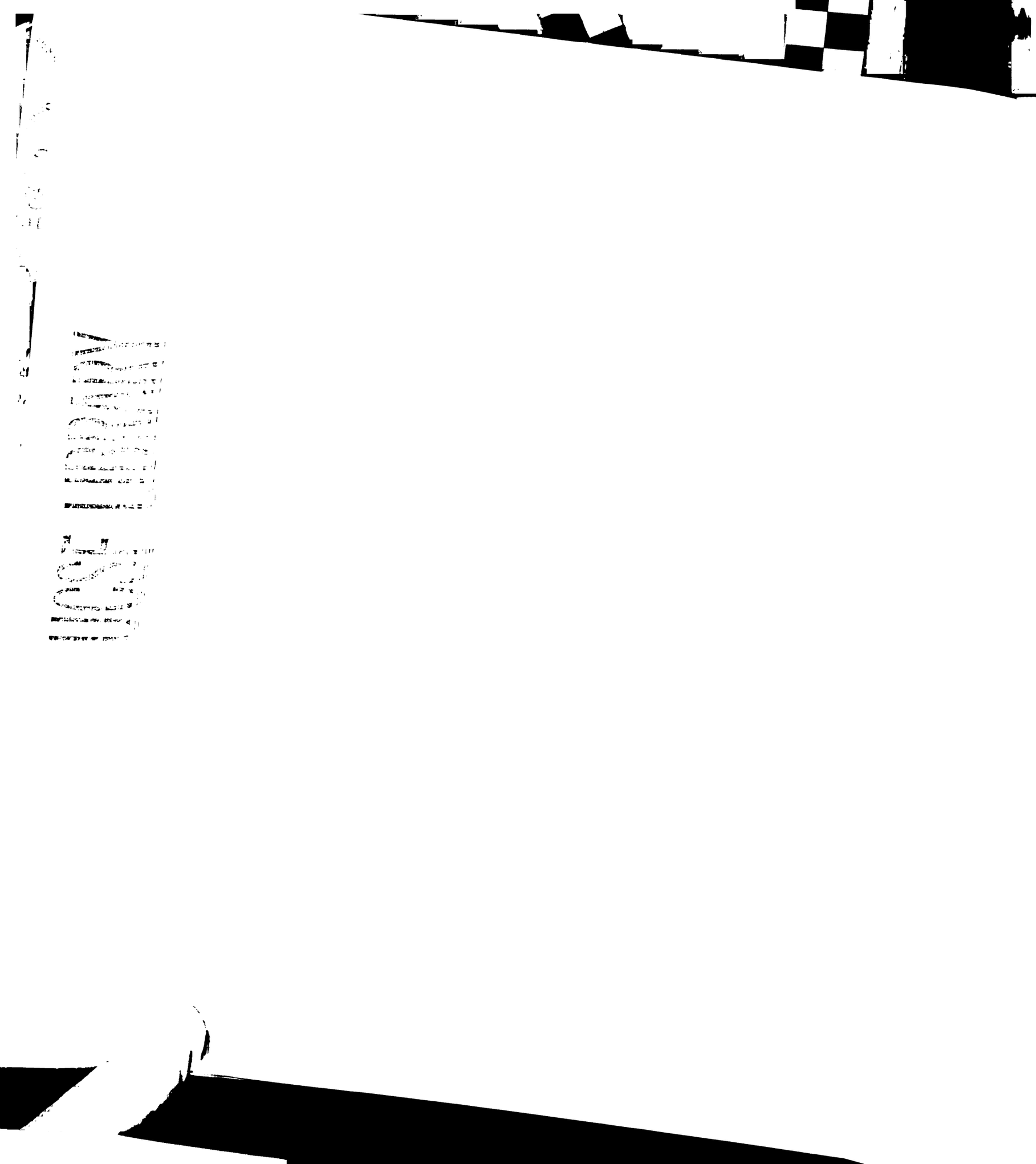

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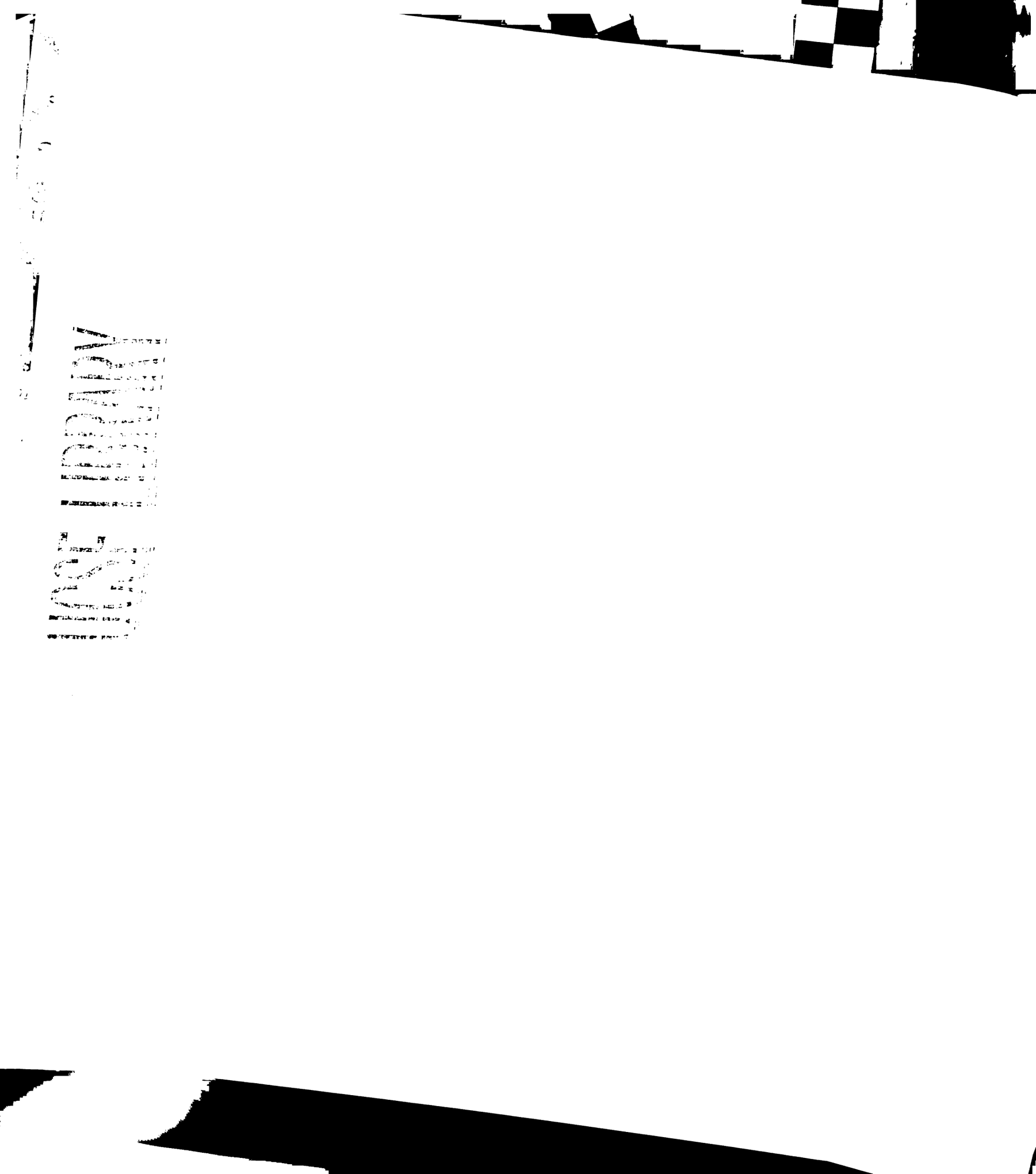

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