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Autoimmune tolerance and Type 1 (insulin-dependent) diabetes mellitus

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Summary. The autoimmune process that results in Type 1 (insulin-dependent) diabetes mellitus may be viewed as a failure to develop or maintain tolerance to self-antigens expressed in the islets of Langerhans. During T-cell development in the thymus, cells that are reactive with self antigens encountered there may undergo clonal deletion or, as more recently described, clonal anergy which effectively removes these cells from the pool of mature antigen reactive T cells. For antigens not found in the thymus, tolerance to self antigens is more complex and may depend on site of antigen expression, ambient concentrations of lymphokines, and availability of antigen-presenting cells that can deliver co-stimulatory signals. Transgenic mice in which the majority of T cells express T-cell receptors against "self" antigens or in which expression of antigens is targeted to peripheral tissues have proven useful for studies of tolerance in both T- and B-cell compartments. In general, T-cell reactivity against foreign antigen expressed on Beta cells does not occur because of the failure to activate T cells reactive with the antigen, termed clonal ignorance. This may be broken with, for example, viral infection or cytokines. In one transgenic model, dendritic cells that surround the islets of Langerhans have been shown to be responsible for presentation of islet antigens to the immune system. B-cell tolerance can also involve mechanisms of clonal deletion or clonal anergy similar to that occurring with T cells. In addition, a mechanism for changing the affinity of the B-cell antigen receptor termed "receptor editing" has been described, which may play an important role in diversifying the B-cell repertoire while removing self-reactive cells. Tolerance to antigens may also be inducible. For example, monoclonal antibodies against T-cell epitopes may induce antigen-specific tolerance that is transferable to other animals, and MHC blocking peptides which can inhibit T-cell responses that are restricted by disease associated MHC molecules. In conclusion, although several possible triggers and mechanisms of autoimmune diabetes can be envisioned, none can be excluded by existing data. However, advances in understanding mechanisms of tolerance to islet and other self antigens suggest potentially useful therapeutic approaches to arresting the autoimmune response.

Key words: Autoimmunity, tolerance, diabetes mellitus, immunotherapy, lymphocytes, autoantibodies, antigens.

Introduction

By the time of clinical presentation of Type I (insulin-dependent) diabetes mellitus, tolerance of both T & B cells responsible for recognition of islet antigens has failed. A number of developmental events during normal T- and B-cell ontogeny prevent the occurrence of autoreactivity, which may be disrupted in the development of autoimmune disease. In this summary, we shall discuss model systems of central and peripheral T- and B-cell tolerance with special emphasis on mechanisms that are of importance to maintenance of non-responsiveness to antigens expressed on peripheral organs such as islet cells. The work group showed that the property of tolerance could reside in both T- and B-lymphocyte populations. For each population, tolerance could be ascribed, under some circumstances, to an actual deletion of self-reactive T or B cells, and under others, to a functional inactivation, frequently referred to as clonal anergy. There were also some situations in adult animals where tolerance could somehow be imposed by a tolerant T-lymphocyte population on a normal lymphocyte population, though the detailed cellular mechanisms underlying this are by no means clear.

Experimental approaches to understanding T-cell tolerance

Factors involved in thymic education of potentially autoreactive T cells

T-cell receptor (TCR) expression and thymic tolerance (von Boehmer). In the developing thymocyte, one can

distinguish several developmental stages of alpha, Beta ($\alpha\beta$) T cells, such as the early T-cell precursors of immature $\alpha\beta$ T cells which are CD4+CD8+TCR^{low}, and cells which have undergone positive selection which are small in size and are CD4+CD8+TCR^{high}. In the CD4+CD8+TCR^{low} cells, α chain TCR gene rearrangement continues in spite of the surface expression of an $\alpha\beta$ TCR while the rearrangement machinery is inactive only in CD4+CD8+TCR^{high} cells as evident by their very low expression of RAG1 and RAG2 genes. Thus, at this early stage, allelic exclusion appears to have occurred in TCR β chains but is not operative for α chains, and α chain gene rearrangement continues. This raises the question whether the central deletion mechanism is still operative at the level of the CD4+CD8+TCR^{high} cells or is censorship of T cells no longer possible after the completion of TCR gene rearrangement. This question has been addressed with an in vitro assay for apoptosis on various thymocyte subsets (Table 1) expressing anti-HY TCR transgene [1, 2]. CD4+CD8+TCR^{low} as well as CD4+CD8+TCR^{high} cells underwent apoptosis whereas thymic and mature CD4-CD8+ T cells did not.

There is now evidence that not all $\alpha\beta$ T cells follow the above outlined developmental pathway. A distinct lineage of $\alpha\beta$ T cells develops without being censored by positive and negative selection mechanisms in thymus. Some of these cells express receptors specific for self antigens, and one can actually show that these cells are being activated by autoantigens in the periphery. The significance of this lineage of cells is unclear at present as is whether they can be involved in the generation or suppression of autoimmunity.

Expression of alloantigen in the thymic medulla can induce clonal anergy of alloreactive T cells (Haemmerling). Double transgenic mice were prepared in which low levels of alloantigen (K^b into K^k or $K^{d/k}$ mice) were expressed in the thymic medulla without expression in the thymic cortex, and anti-K^b TCR was expressed on a high proportion of the T cells [3]. The mature K^b-reactive T cells from these mice did not undergo thymic deletion, as detected using a clonotypic monoclonal antibody (mAb), Desire (DES), and were tolerant of the alloantigen in vitro. T cells from these transgenic mice were unable to mount an anti-K^b cell-mediated lysis response. Furthermore, activation of T cells from the periphery could not be induced with the anti-clonotypic mAb. These data suggest that clonal anergy is an alternative to clonal deletion of T cells that are reactive with antigens expressed in certain regions of the thymus such as the medulla. In addition, they indicate that thymic deletion may be influenced by factors such as location of antigen expression in the thymus and affinity of the TCR for antigens expressed intrathymically. It is semantic as to whether anergy induction in the thymus medulla is classified as central or peripheral tolerance. On the one hand, it occurs within the thymus and is strictly central. On the other, it involves a fairly mature T cell and thus shares features with peripheral tolerance.

Table 1. Susceptibility of developing T cells to negative selection:

	Increasing stage of differentiation →			
	Thymocyte			Peripheral T Lymphocytes
	CD4+CD8+ TCR^{low}	CD4+CD8+ TCR^{high}	CD4+CD8+ (after+ selection)	CD4-CD8+
susceptability to negative selection	+++	+++	+	-

Effect of changes in the affinity of the TCR/antigen interaction on thymic education (Haemmerling). Two experiments were carried out with double transgenic mice to evaluate effects on T-cell development of expression in the thymus of a transgene with altered affinity for the TCR. In the first, a K^b molecule with a modification of the α3 domain was expressed in the thymus, together with the anti-K^b TCR described above. Target cells expressing this modified K^b molecule are not susceptible to lysis by T cells reactive with unmodified K^b molecules. However, in the double transgenic animal, clonal deletion of the anti-K^b reactive cells was found to occur, and skin grafts that express normal K^b were rejected when applied to the double transgenic animals. In a second experiment, the Bm1 mutant K molecule was expressed as a transgene in the thymus of TCR transgenic mice. Similar to the K^b molecule with reduced affinity, targets expressing the Bm1 mutant K molecule are not susceptible to lysis by the anti-K^b CD8+ T cells. In these double transgenic animals, evidence for negative selection in the thymus was also found, but, in addition, the lymph nodes in the periphery were heavily populated with T cells expressing the anti-K^b TCR and CD8, that were tolerant of the K^b molecule.

These experiments indicate that 1) thymic anergy may represent an alternative fate to clonal deletion for autoreactive T cells developing in the thymus, and 2) negative selection appears to still occur even in the presence of relatively low affinity/TCR interactions.

Development of T-cell tolerance for autoantigens that are expressed in the periphery

Antigens that are expressed only in the peripheral tissues and not in the thymus may elude the immune system in a number of ways, such as location in privileged sites that are unavailable to immune surveillance, expression on cells without or with low levels of MHC that are incapable of presenting antigens to the immune system, surface concentrations of antigen at levels that are below a threshold for recognition even in the presence of MHC molecules, or weak avidity of the antigen for TCR and CD4 or CD8 so that T- cell activation requirements are not met. The following experiments explore these possibilities.

Mechanisms of tolerance to antigens expressed on Beta cells in TCR/K^b, anti-K^bTCR double transgenic mice or in triple transgenic mice with interleukin-2 (IL-2) expression in the Beta cells (Miller). The gene encoding the class I molecule, K^b, was linked to the rat insulin promoter (RIP) and microinjected into fertilized eggs derived from mice of non-b haplotypes [4-7]. Immunohistochemical techniques revealed abundant expression of K^b in the Beta cells, but the transgene was not detected in the thymus using these techniques. No lymphocytic infiltration was seen in the islets even if the mice were immunised with K^b antigen. Beta-cell dysfunction and diabetes became evident only after 3 to 4 weeks of age, and resulted not from an autoimmune reaction as seen in autoimmune diabetes but from the toxic effects of overexpression of the transgene and the accumulation of the class I MHC molecules in Beta cells (Table 2). This is supported by the fact that even expression of *syngeneic* class I molecules on the islets leads to diabetes. Nonetheless, these animals exhibited tolerance to K^b, because K^b-bearing skin was not rejected by the young transgenic mice despite the ability of the mice to reject skin from a third-party donor.

In order to determine the fate of the autoreactive T cells, it became necessary to construct double transgenic mice which express the K^b transgene and an anti-K^b TCR gene on a high proportion of the T cells (Table 2). The TCR transgene was specific for K^b and could be detected with the mAb DES. When peripheral CD8+ T cells in single TCR and double transgenic mice were compared by flow cytometry, the cells expressing the highest density of TCR were lacking in the double transgenic animals. Similarly, the high DES-expressing cells were not present in the thymus, and a 50% reduction in the percentage of CD8+ T cells in the thymus of the double transgenic mice was seen compared to the single TCR transgenic animals. This suggested that a few molecules of K^b must have been present in the thymus and were able to cause deletion of T cells with the highest density of TCR and presumably with the highest avidity for K^b. Although K^b expression could not be detected by techniques including immunohistochemical staining, Northern blotting, and S1 nuclease mapping, the K^b gene could be amplified from thymus by the polymerase chain reaction (PCR). Therefore, these and previous data from this model must be reinterpreted in view of this finding indicating that the transgene expression was present to a small extent in the thymus and may have been responsible for the 50% reduction in the CD8+ transgenic TCR+ bright cells that were found in these mice through mechanisms of thymic deletion. If this is the correct explanation, it is of interest that the deletional mechanism affects TCR avidity rather than affinity. Of course, the anti-K^b TCR on the TCR dull thymocytes is identical to that on the putatively deleted cells. It seems probable that the low K^b antigen expression within the thymus allows this subtle affinity difference to play a role.

Studies by other groups have indicated that anergy in T cells may be broken by proliferation induced with IL-2. To determine whether a similar mechanism might explain the failure of the "dull" DES TCR+ cells to respond to the K^b transgene on the islet cells of double transgenic mice, these mice were mated to animals in which IL-2 was expressed in the Beta cells under direction of the RIP (Table 2). Single RIP/IL-2 transgenic mice and double RIP/IL2 + K^b transgenic mice had peri-islet infiltrates composed primarily of T lymphocytes, and in the latter group diabetes developed only after 20 days of life. However, this did not represent K^b-specific autoimmunity because K^b-bearing islet grafts transplanted into the kidney were not destroyed. Mice with all three transgenes developed diabetes within the first few days of life in contrast to the other transgenic mice in which diabetes developed because of toxic effects and only after day 20. Thus, the addition of IL-2 production in the islets in the setting of antigen and antigen-specific T cells overcomes the non-responsiveness that occurs in the absence of the cytokine.

Table 2. Development of diabetes in transgenic mice

Transgenes	Day of life for appearance of diabetes (*n*)			
	6-12	15	18	>20
DES/TCR + K^b	-	-	-	1
K^b + IL-2	-	-	-	7
DES/TCR + K^b + IL-2	3	3	1	-

The effect of the IL-2 transgene may have been to overcome anergy in the K^b-reactive T cells or to cause quiescent K^b-reactive (and immunologically "ignorant") T cells to home in on and recognise their targets. To differentiate these possibilities, single transgenic RIP/K^b mice were thymectomised, irradiated, and reconstituted with bone marrow from transgenic TCR mice and thymus grafts from non-transgenic donors. This was to ensure that the K^b transgene was not present in the thymus. In these mice there was no deletion of the CD8+ TCR bright cells that had been seen previously, and K^b-bearing skin grafts were rejected. These results indicate that the K^b-reactive T cells were not rendered anergic in the presence of the K^b transgene and that the effect of the IL-2 production in the Beta cells was to stimulate proliferation of K^b-reactive T cells.

Although the finding of expression of the K^b transgene in the thymus of the transgenic mice calls into question the development of post-thymic tolerance in this model, other studies of similar design have shown that tolerance can develop after a powerful immune response to a persistent antigen. In these studies, syngeneic spleen cells were injected into irradiated transgenic mice expressing the K^b molecule on liver cells (linked to the met-allothionein promoter). Initially, activated lymphocytes infiltrated the liver lobules and mediated piecemeal necrosis of the adjacent liver cells. With increasing time after inoculation, the response became more subdued, and by 12 weeks, the few portal tracts that remained infiltrated showed no accompanying hepatocyte necrosis, and the lymphocytes

were small and apparently inactive. Hence, mature T cells may become non-responsive to a persisting antigen following an initial response.

Influence of tissue-specific promoters on development of tolerance to antigens expressed in the periphery (Haemmerling). The effect of site of expression of a transgene (K^b) on the development of tolerance in DES/TCR transgenic mice [8-10] was examined using different tissue-specific promoters (Table 3, Fig. 1). In all of these animals, no evidence for expression of the K^b transgene in the thymus has been found using conventional techniques. PCR results have not yet been reported.

When K^b was expressed on hepatocytes under the direction of the albumin promoter, skin grafts that expressed K^b were not rejected, and transgenic TCR and $CD8^+$ were expressed at lower density on peripheral T cells. The mechanism of this effect appeared not to involve deletion of the K^b reactive T cells in the thymus, since thymocytes from these mice reactive to K^b could be demonstrated in vitro. In the peripheral lymph nodes, there was a 5-40% decrease in the TCR+ cells, but further studies indicated that the reduction in TCR expression was not due to deletion of T cells. As many as 62% of the cells in the lymph nodes were Thy1+, CD2+, CD3-indicating that many of the T cells did not express TCR. In addition, modulation of the TCR could be shown to be due to a post-translational event, since TCR protein could be detected in the cytoplasm of the cells, and expression of the TCR could be induced onto the surfaces of T cells with CD2 mAb.

In mice in which the K^b transgene was expressed on Schwann cells, brain, and small intestinal cells, slightly different findings were obtained. Skin grafts that were K^b+ were not rejected, and again, modulated expression of the TCR transgene and CD8 were found. However, a parallel loss of responsiveness to K^b antigen could not be demonstrated in mixed lymphocytic reaction (MLR) in vitro. Furthermore, clonotype+, CD8+ cells that were non-responsive in vivo were present in the spleen and lymph nodes, suggesting the induction of a state of clonal anergy.

Finally, when K^b expression was directed to the skin using the keratin promoter, tolerance to K^b+ skin grafts occurred, but the non-reactivity of the clonotypic TCR+, CD8+ T cells could not be overcome in vitro by the means described above.

Table 3. Effect of site of K^b transgene expression on development of tolerance in DES/TCR transgenic mice

Site of Kb expression	Promoter	Tolerance to skin grafts	Responsiveness of TCR/DES+ cells
Hepatocytes	Albumin	yes	reversible with anti-CD2 stimulation
Schwann cells+ brain+small intestine	GFAP	yes	reversible in MLR
Keratinocytes	keratin	yes	non-responsiveness

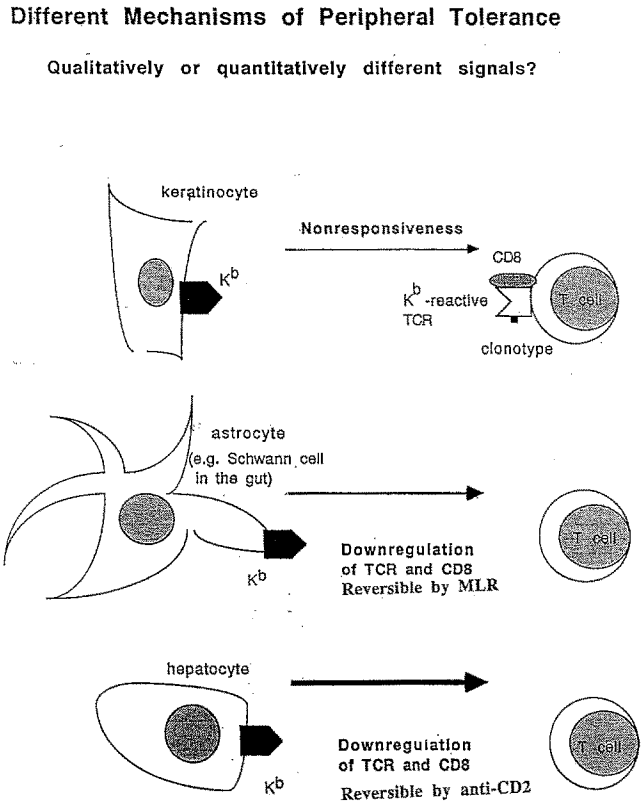

Fig. 1. To determine the effect of the site of antigen expression on the development of tolerance, transgenic mice ($H\text{-}2^{dxk}$) were prepared in which, as a model antigen, the K^b MHC molecule was under the control of tissue specific promoters that are not expressed in the thymus. These mice were crossed with mice transgenic for an anti-K^b T-cell-receptor (TCR), and the fate of the K^b specific T cells was followed by anticlonotypic antibody and FACS analysis. All mice were tolerent as judged by their acceptance of K^b+ skin grafts. No deletion or anergy was observed in the thymus. In the periphery, distinct levels of tolerence were seen: with K^b expressed in skin cells (keratin-IV promoter), all anti-K^b TCR+CD8+ cells were still present and could be activated in vitro by antigen. With K^b expressed on cells of neuroectodermal origin(GFAP promoter), TCR and CD8 were down-regulated (not completely) on peripheral T cells, but could be activated in vitro by K^b antigen. With K^b expressed on hepatocytes only (albumin promoter), the peripheral T cells were completely surface negative for TCR and CD8 but were positive in the cytoplasm. They could not be up-regulated by K^b alloantigen, but by crosslinked anti-CD2 antibodies. The distinct levels of peripheral anergy could be due to qualitatively different signals, but are more likely due to quatitatively different signals depending on intensity of contact, timing,etc.

These data indicate that a range of non-responsiveness may be induced in T cells depending on the site of expression of the transgene (Fig. 1). The reasons for these differences are still unclear and may include factors such as the K^b-bound peptide, or variation in the density of the alloantigen expressed on the surfaces of the cells. Taken as a whole, the results suggest that a gradient of tolerance induction, from very profound to less complete, may be induced by encounter of T cells with antigen in the periphery and depends on factors including the site of transgene expression. If further research substantiates different degrees of anergy, reversible by differing levels of intensity of stimulation, this would indicate a degree of fine-tuning of lymphocyte responsiveness more subtle than the simple apoptotic death of a cell.

Table 4. Possible antigen-presenting cells associated with the islets of Langerhans

Dendritic cells in the islet
Dendritic cells outside of the islet
B cells in the lymph nodes draining the islet
Recuited macrophages
Islet cells with induced MHC antigens

Pathogenesis of diabetes in RIP-haemagglutinin transgenic mice (Lo). The immune response to PR8 haemagglutinin antigen (HA) has been well characterised, making this a good model antigen for study of immunologic responses to antigens expressed only in the periphery [11, 12]. Transgenic mice were prepared in which HA was expressed on Beta cells under direction of RIP. A B-cell (specific antibody) response was found in these transgenic mice when the mice were immunised with HA peptide, but diabetes did not develop and both CD4 and CD8 cells from the same mice appeared tolerant to the transgene. There was no evidence of cytotoxic T lymphocyte (CTL) reactivity against HA+ targets in vitro. In further studies, bone marrow chimeras were prepared in thymectomised, irradiated, HA-transgenic animals that were grafted with neonatal thymus, non-transgenic bone marrow. These chimeras similarly showed no response to HA, indicating that tolerance was dependent on the islet-specific expression of the HA and eliminating the possibility that thymic mechanisms were responsible for generation of tolerance.

Since islet cells express low levels of class I MHC molecules under basal conditions, do not express class II MHC antigens, and are therefore unlikely to be responsible for antigen presentation, studies were undertaken to determine which cells might be responsible for presentation of HA antigen to the immune system (Table 4).

To determine the contribution of these antigen-presenting cells (APC) to the development of diabetes in virus-infected mice, naive or primed B10.D2 lymphocytes were transferred with virus into sub-lethally irradiated HA transgenic animals. The islet histopathology was studied over the 3-week period during which diabetes develops. In the first week after transfer and infection, an infiltrate of CD4+ cells was seen outside the islet, and I-E expression was increased on dendritic cells (mAb F480+) surrounding the islet. With time, the number of CD4+ cells increased and some CD8+ cells were found. The number of macrophages present in the islet and class II MHC expression increased further, but all of the class II MHC expression in the islet was on the infiltrating macrophages. A cycle of recruitment, further increase in expression of MHC and other accessory molecules such as selectins (ICAM-1) and cellular infiltration then developed. Eventually, the recruited macrophages entered the parenchyma of the islet and the islet cells were destroyed.

Thus, the initial presentation of shed antigen in HA-transgenic mice appears to be carried out by the dendritic cells that reside on the outside of the islet. As the inflammatory response accelerates, APCs and T cells enter the islet and the islet cells are destroyed.

Pathogenesis of diabetes in lymphocytic chorio-meningitis virus (LCMV) transgenic mice (Zinkernagel). Non-cytopathic viruses may not cause disease directly, but may induce T cells or B cells or both, that may then recognise antigens derived from these viruses on target cells. An example of this is infection of Beta cells with LCMV or presentation of LCMV antigens on Beta cells as a transgene under control of RIP [13, 14].

It should be noted that there are important limitations with these model systems and the methodologies as illustrated by the following two examples. First, CTLs specific for L^d+NP118-126 of LCMV cross-react in vitro by proliferation when stimulated with L^q+NP118. CTL activity is "specific" for virus-infected targets, but cross-reactive to some extent with peptide coated L^d vs L^q targets. However, when tested in vivo for anti-viral protective capacity there was no cross-reactivity seen. Thus, in vitro methods, particularly proliferative responses, may reveal reactivities of little or no relevance to in vivo studies. Second, there is evidence that T-B cell co-operation is contact dependent and MHC restricted, whereas T- cell help, if necessary for CTLs, is neither.

T-cell mediated pathology to a foreign antigen (i.e. LCMV antigen on Beta cells) is not fundamentally different from immunopathology to an "ignored" self antigen such as those postulated to be important in Type 1 diabetes (Table 5). Relevant examples of tolerance to self are shown using transgenic animals with differing LCMV-carrier status. For example, expression of LCMV on the haematopoietic stem cel ls using the K^b promoter results in deletion of the LCMV reactive T cells and failure of T cells to respond to islet LCMV in double transgenic mice, whereas islet-directed responses do occur in virus-infected mice expressing LCMV/RIP transgene only. Other examples of immunopathology caused by T cells are found in the LCMV model in the form of T help-dependent autoantibodies to LCMV in carriers, autoaggressive hepatitis mediated by CD8+ T cells after infection with a hepatotropic LCMV, and in LCMV-induced immuno-

Table 5. Immunologic "ignorance" as a mechanism of non-responsiveness of T cells

A viral glycoprotein (GP) from the lymphocytic choriomeningitis virus (LCMV) is targeted to Beta cells by RIP
RIP/GP transgenic mice are healthy, express only low levels of GP
RIP/GP transgenics challenged with live LCMV get insulitis and sometimes Type 1 diabetes
Study double transgenics – cross to T-cell receptor/anti-GP transgenics
No immunity, no disease, no deletion but live LCMV – Type 1 diabetes
Conclusion: No tolerance in T or B compartment. LCMV triggers anti-viral responses against GP. Islets suffer cytotoxicity and other damage

Table 6. Differences in T- and B-cell compartments in tolerance induction to viral glycoproteins

Render mice transgenic for a cell membrane-associated glycoprotein (GP) of vesicular stomatitis virus (VSV), targeted to kidney, heart, and brain, but not lymphoid tissue.
Immunise with GP in CFC or expressed in vaccinia virus – little or no antibody formed
Infect intravenously with living VSV – Good antibody formation, excellent secondary response
Conclusion: "Self" GP induces T cells, but no B-cell tolerance. "Autoreactive" anti-GP B cells take up VSV particles, present non-tolerated T-cell epitopes to T cells and thereby get triggered

suppression leading to an adult carrier status.

Analysis of autoantibody induction was done using transgenic mice expressing glycoprotein (GP) of vesicular stomatitis virus (VSV-GP) (Table 6). Autoantibodies were induced by infecting these mice with wild-type VSV, but were not induced with purified GP-antigen of the vaccinia recombinant virus expressing GP. This result is interpreted to reflect induction of a non-tolerant state with the transgene that is linked to T-cell help in the first case but not in the second and third cases. This conclusion is based on the observation that T-cell help induced a switch in IgM to IgG production and a neutralising (i.e. high affinity) antibody response.

CD8 T cell-mediated immunopathology to a transgenic neo-self LCMV-GP antigen expressed in the pancreatic Beta cells has illustrated how "ignored" self antigen may induce a rampant immunopathology following appropriate antigen presentation. There is a dose requirement (vaccinia-recombinants expressing the GP cannot induce diabetes in RIP/GP single transgenic mice, but do in TCR-RIP/GP double transgenic mice) for both antigen and T-cell precursor frequency. Attempts at vaccination against diabetes have proven difficult, and results vary with the different MHC haplotypes.

Induction of tolerance to class I restricted antigens in the periphery after thymic education (von Boehmer). In vitro, it is not possible to induce deletion of mature CD4-CD8+ cells even by high doses of antigen. In vivo, however, anergy can be induced in CD8+ anti-HY T cells with high doses of antigen if T-cell help (provided by CD4+ peripheral lymphocytes) is bypassed [15]. In these experiments, prior exposure of anti-HY T cells to HY antigen (on a nu/nu background) resulted in non-responsiveness of T cells to restimulation with the HY antigen compared to cells that had not previously seen the antigen. Such anergic CD4-CD8+ T cells are refractory to antigenic stimulation or stimulation by antibodies even in the presence of exogenous growth factors, and non-responsiveness could not be overcome by stimulating the cells either with CD3 or clonotypic monoclonal antibodies. However, anergic cells "parked" in vivo in the absence of antigen for several days recover responsiveness.

Experimental approaches to understanding B-cell tolerance

B-cell tolerance to alloantigens expressed as transgenes (Nemazee)

Studies have been carried out to understand the mechanism of clonal deletion in transgenic mice [16-19] carrying rearranged antibody genes (Table 7). In these mice, the majority of B lymphocytes which develop within the bone marrow express a single antibody specificity to the MHC molecules, H-2K^k or H-2K^b, the former with high and the latter with 100-fold lower affinity. If the transgenic mice carry one of these MHC alleles, the majority of B lymphocytes are triggered to die within the bone marrow as a result of binding self antigens early in their maturation. Not all B cells are eliminated in these mice, however, and other experiments indicate that, before they die, the autoreactive B cells may be triggered to undergo further antibody gene rearrangements and generate more useful antibody specificities (receptor editing). Flow cytometric analysis of the bone marrow revealed a pool of undeleted immature B cells which expressed low densities of antigen receptor, and quantitative PCR indicated that these B cells expressed elevated mRNA for the recombinase products, RAG1 and RAG2, enzymes which are normally at low levels in non-deleting transgenics. Moreover, an increase in the excision products of light chain gene rearrangements could also be detected by PCR, and there was a concomitant increase in the number of B cells expressing lambda light chains, indicating the possibility of gene translocation in this light chain gene as a "second try" where kappa gene translocation had led to an anti-self receptor. This "receptor editing" could play an important role in diversifying the B-cell repertoire.

Autoreactive B cells can also be shown to undergo clonal deletion when they bind to self antigens which are not present in the bone marrow but whose expression is limited to the liver. This was shown in transgenic mice in which the H-2K^b gene was controlled by the metallothionein promoter, which is expressed primarily in the liver. Receptor editing appears not to accompany B-cell deletion in this case.

B-cell tolerance in the double transgenic HEL-anti-HEL tolerance model (Goodnow)

Studies of clonal deletion in immunoglobulin gene transgenic mice in which most of the B cells express a high-affinity antibody to hen egg lysozyme have been carried out to understand the mechanism of clonal deletion of B cells [20-23]. These cells were triggered to die if they encountered lysozyme, expressed from an appropriate transgene construct, displayed on cell surfaces within the bone marrow. FACS sorting and cell culture experiments indicated that the death of the autoreactive B cells appeared

Table 7. Cell tolerance: the anti-H-2k^b transgenic model

If mice express H-2k^b (antibody transgene product 100-fold lower affinity), no transgenic B cells leave the bone marrow. The phenotype of these cells resembles that of "central deletion" Mating of anti-H-2k^b B cells to MET-k^b transgenic mice or adoptive transfer leads to a peripheral deletion

Central but not peripherally deleting mice:

1. Have more peripheral B cells
2. Displayed lambda light chains more often than expected
3. Upregulated RAG1 and RAG2 genes

This mechanism has been termed "receptor editing"

to be secondary to a reversible arrest of the B cells' development at an intrinsically short-lived stage. Moreover, the life span and accumulation of these cells could be extended by constitutive expression of the Beta2 oncogene, but this did not override the arrested maturation of the autoreactive B cells.

These findings differ from those made in transgenic mice expressing lysozyme as a soluble protein, where the autoreactive B cells continued to mature and leave the bone marrow, but appear to become blocked in their developmental programme at the stage of long-lived B cells and inhabit the follicular mantle zone (Table 8). In this case, while the B cells were not eliminated, they were profoundly compromised in their ability to respond to stimuli such as helper T cells and antigen. These "anergic" B cells could nevertheless recover their function when "parked" in a HEL-free environment and strongly stimulated with antigen plus T-cell help. This indicates that the functional silencing of the cells is potentially reversible.

Induction of tolerance in normal adult animals

Immunologic tolerance within the secondary B-lymphocyte repertoire (Nossal)

The B-cell response to continued or repeated antigenic stimulation is characterised by affinity maturation due to hypermutation of VH and VL genes and selection of high affinity variant cells. To determine what happens when a B cell, proliferating appropriately to a foreign antigen fortuitously mutates towards high affinity anti-self specificity, soluble freshly deaggregated antigens were used as surrogate self antigens [24, 25]. The B-cell repertoire was cross-examined in a special way to focus on B cells with reasonably high affinity for antigen. The system used LPS stimulation aided by 3T3 filler cells, a mixture of IL-2, IL-4, and IL-5, and an ELISA readout which detected only bivalent IgG1 antibody and ignored decavalent IgM antibody. Under these circumstances, non-immunised mouse spleens contained only a few cells capable of making antibody of requisite affinity, but 2 weeks after immunisation with alum-precipitated antigen plus pertussis adjuvant, about 50,000 antibody-forming cell precursors could be detected in the spleen.

In the first series of studies, the antigen was human serum albumin (HSA). Injection of soluble HSA before or even up to 6 days after challenge immunisation virtually completely abrogated the appearance of HSA IgG1 antibody-forming cell precursors. While in vitro studies did not reveal a profound susceptibility of recently activated pre-memory cells to tolerance induction by soluble antigen, adoptive transfer studies showed a profound functional defect in CD4+ anti-HSA helper T cells and a lesser but still definite defect in anti-HSA B cells harvested from soluble antigen-injected mice. In vitro studies of CD4+ T-cell proliferation to antigen recall also showed functional silencing of HSA-reactive CD4+ T cells.

In a second study, nitrophenol (NP)-specific responses were studied in C57BL mice. NP18-HSA on alum with pertussis was the immunogen. Injection of soluble NP2-HSA up to 6 days after challenge immunisation nearly completely ablated the appearance of high-affinity anti-NP B cells. However, soluble NP8-cytochrome C did not have this effect. Soluble carrier HSA did achieve tolerance. Thus, the frustration of memory cell generation seemed to be due largely to a T-cell lesion in these mice. It appears that carrier-specific T cells are required for the germinal centre reaction to produce affinity-matured B cells (Fig. 2).

Single cell studies using PCR of the anti-NP heavy chain showed very few mutations by day 7 of the challenge response, but already by day 12 the majority of T cells of control animals showed a critical tryptophan to leucine mutation in complementarity determining region 1 (CDR1). There are so few strongly NP-binding B cells in tolerant mice that PCR results are not available yet, but it seems likely that the failure of affinity maturation in the tolerant mice is due to a frustration of the mutation and selection process.

Induction of tolerance with MHC binding peptides (Adorini)

The most desirable immuno-intervention strategy in autoimmune diseases, including Type 1 diabetes, would be reinduction of tolerance to the autoantigen. This is not yet feasible because the autoantigen in most cases is not known, although candidates (e.g. glutamic acid decarboxylase in Type 1 diabetes) are being evaluated. In the meantime, other attack points for selective

Table 8. Beta-cell tolerance: the double transgenic soluble HEL-anti-HEL tolerance model

HEL cause T-cell tolerance at a concentration of less than 10^{10} mol/l but Beta cell tolerance at only greater than 10^9mol/l

Beta-cell tolerance is due to anergy not deletion

The tolerant Beta-cell phenotype is sIgM^{low}, sIgD^{high}, follicular mantle seeking

Anergy is partial (since mitogenesis still occurs) and reversible with time and strong T-cell-dependent stimulation

Anergy and the anergic phenotype can be imposed on mature Beta cells within a few days

sIgD only transgenics can be anergised

Membrane anchored HEL causes deletion

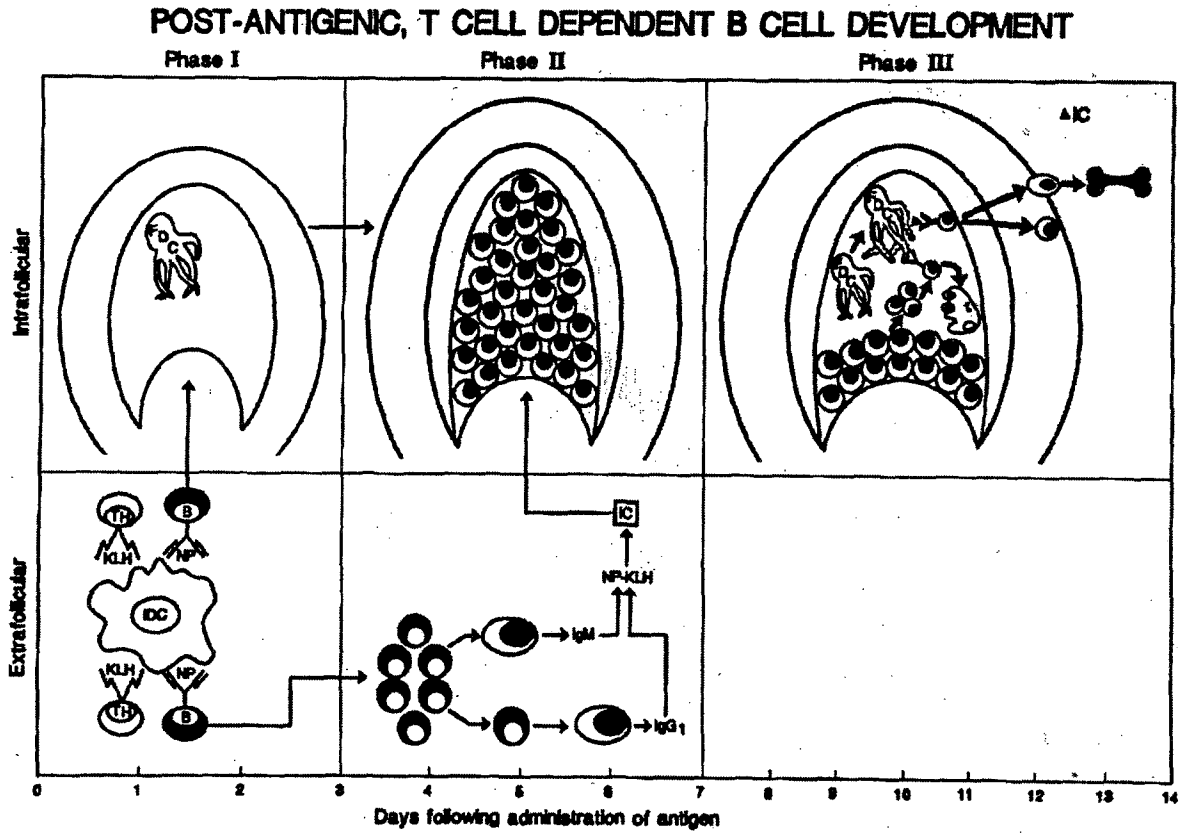

Fig. 2. The germinal centre reaction (after ICM MaClennan et al. [31], adapted by Dr. M.G. McHeyzer-Williams, Walter and Elizah Hall Institute): Phase I shows a primary lymphoid follicle, containing antigen-capturing follicular dendritic cells (FDC) about to be "invaded" by activated anti-NP B cells. In the extrafollicular aprt of the spleen or lymphnode, an interdigiting dendritic cell (IDC or IC) has captured the antigen, NP-KLH. Carrier (KLH)-reactive T cells help NP-specific B cells to become activated; some of these B cells migrate to the follicle.

Phase II shows extrafollicular IgM and, slightly later IgG 1 (and other downstream isotype) antibody production, while within the follicle B blasts, oligoclonally invading the follicle, proliferate very extensively.

Phase III shows the germinal centre in the second week. The proliferating centroblasts lie deep in the centre, away from antigen. They are postulated to be mutating their Ig V genes very rapidly. Centrocytes, smaller and no longer dividing cells, move to where the antigen is on the surface of the FDC. If not positively selected, they die by apoptosis and are rapidly taken up by "tingible body" macrophages within the germinal centre. If effectively able to engage antigen because of an affinity-raising mutation, they are positively selected for either memory cell generation or antibody formation, and are promptly exported from the germinal centre

immunotherapy may be explored. Since autoimmune diseases are frequently associated with particular MHC class II alleles, these molecules represent an interesting target for selective immuno-intervention [26-28]. If the association between class II molecules and autoimmune disease is due to their capacity to present autoantigenic peptides to autoreactive T cells, blocking antigen presentation by these class II molecules should interfere with disease induction or progression or both. It has been shown that in vivo administration of class II-binding peptides selectively prevents, in mice, presentation of antigens by the class II molecule binding the competitor peptide (Tables 9 and 10). Synthetic peptides able to block antigen presentation by the HLA-class II alleles associated with rheumatoid arthritis are currently being developed. If this immuno-intervention strategy proves to be clinically effective, it could be applied to other autoimmune diseases. In the case of Type 1 diabetes, this would require blocking antigen presentation by DQB1*0602/DQA1*0201.

Induction of tolerance with T-cell monoclonal antibodies (Waldman)

For most autoimmune diseases, the antigens are not known. Although tolerance might be possible with judicious application of such antigens once found, or fragments thereof, an alternative approach is to develop ways of "reprogramming" the immune system such that tolerance can be induced to unknown antigens, the same antigens that drive autoimmunity. The antigens show themselves, however, by virtue of interacting with autoimmune T cells. The goal, therefore, of this approach is to turn the recognition event for antigen into one of tolerogenesis [29, 30].

Studies of induction of tolerance with non-depleting CD4 mAb have revealed the following characteristics: 1) Once tolerance is established, it can be maintained throughout life by continuous exposure to antigen. 2) Tolerance cannot be broken with T cells from a normal animal. 3) Tolerant T cells can transfer tolerance to other T cells. Both of these latter two characteristics are strain

Table 9. Direct evidence for MHC blockage in vivo

Deletion of antigenic complexes found in vivo between peptide derived from HEL processing and class II molecules
Inhibition of complex formation in vivo by MHC blockers non-homologous to the antigenic peptide
Absence of inhibitory T cells in the APC population
Absence of evidence for clonal dominance induced by the competitor

combination dependent.

In studies carried out with administration of human gamma globulin to adult mice treated with non-depleting CD4 mAb, the non-responsive state was shown to be independent of thymic mechanisms and could be induced in thymectomised animals. (Although maintenance of tolerance required continual exposure of antigen in euthymic mice, this was not necessary in thymectomised animals.) Other T cell mAb (anti LFA-1, CD11) could also induce tolerance to the antigen, but there was no evidence for involvement of CD8+ T cells either in induction or maintenance of the tolerant state. The CD4+ T cells in the tolerant mice resisted breakage of tolerance by naive CD4+ T cells, and tolerance could be transferred to lethally irradiated recipients that were given normal B cells.

Studies of skin graft transplantation of B10.BR into CBA mice have shed light on mechanisms of tolerance induction. When this allotransplantation was performed together with (non-depleting) CD4 and CD8 mAb treatment, a second B10.BR graft, implanted after 4 weeks, was not rejected despite rejection of a third-party allograft. Adoptive transfer of cells from the animal in which tolerance has been induced have shown that induction of tolerance is not complete until 4 weeks after the initial mAb treatment. In addition, transfer of normal lympho-cytes into these animals was not able to break tolerance, indicating that mechanisms other than clonal deletion or non-responsiveness of the alloreactive T cells were operative. However, this "suppression" of allore-sponsiveness can be eliminated with a second CD4 mAb treatment.

In order to understand the fate of the normal cells given to the tolerant animal, a similar allograft was placed on an animal with a human CD2+ TCR transgene, and tolerance was induced with the T cell mAbs. Adoptive transfer of normal cells into the tolerant mouse was then carried out, and the transgene (CD2+) T cells were depleted from the animal with anti-CD2 mAb. Mice treated in this manner remained tolerant of the original, and a second allograft, indicating that the tolerant state had been acquired by the newly transferred cells. Thus, normal cells co-existing with tolerant cells become tolerant themselves and acquire the capacity of "resisting" normal cells. These studies also highlight the point that tolerance may be a property of a population of cells rather than exclusively occurring at the single cell level.

Table 10. Characteristics of MHC blockade in vivo in mice

Blockers applied in soluble form at a site distant from antigen injection are able to inhibit T-cell priming.
Peptide delivery by microspheres a.c. is also effective
Blocker delivery in soluble form by osmotic mini-pump is effective (at 1 μg/kg body weight)
T-cell proliferation and T-cell dependent antibody responses can be inhibited by blockers bound to MHC molecules
Inhibition is rapidly reversible
No proliferative responses are seen to the soluble blocker as long as 10 days after continuous s. c. administration

Recommendations

Possible triggers of autoimmune disease

Several mechanisms have been postulated to play a role in the initiation of autoimmune disease (Table 11). Although direct evidence for molecular mimicry is currently not available, this may be because the effector specificity of the cells that mediate the destruction of islet cells may differ from the initiating antigen specificity, so that identification of the initial stimulatory antigen may be difficult. Although direct immunisation with islet cells even with adjuvant is not capable of inducing diabetes, cross-reactivity of islet antigens with antigens from other sources remains a possibility. The number of amino acids presented by class I or class II MHC antigens is small, so that only a limited match between the cross-reactive epitope and islet antigen theoretically needs to be present. The possibility of reversion of normal mechanisms of non-responsiveness to islet antigens is supported by the co-association of lymphopoenia with diabetes in the BB/W rat and the dependence of diabetes in this model on the depletion of RT6+ T cells. However, this mechanism alone would not explain the tissue specificity of the disease.

An example of cytokine-induced induction of autoimmunity is the results with RIP/interferon-gamma transgenic mice previously reported by Sarvetnick [32]. In these animals, a brisk inflammatory response against islet cells is seen, which also develops in MHC-matched intraportal islet grafts. On the other hand, the induction of, or aberrant expression of, MHC molecules on islet cells alone would appear to be insufficient to cause autoimmunity. This mechanism also does not account for the tissue specificity of the autoimmune response, and is refuted by the failure to develop autoimmunity against islet cells expressing class I or class II MHC transgenes on their surfaces.

Table 11. Possible triggers of autoimmune disease

Molecular mimicry or other forms of antigen cross-reactivity
Viral or toxic release of sequestered antigens
Immune response gene-dependent failure of T-cell repertoire purging or pathogen elimination
Cytokine induced hyper-inducibility of B or T cells
Self cellular constituents enter an immunogenic processing pathway
Reversal of anergy

The involvement of antigen-specific "suppressor" mechanisms gains support from studies including inhibitory responses against T cells in models of autoimmune thyroid disease, the need for irradiation of the recipient in adoptive transfer experiments with NOD mice, and studies discussed above concerning effects of T-cell mAb treatment. However, the failure to demonstrate antigen-specific T-cell suppressor mechanisms or antigen-specific suppressor T cells leaves this issue unresolved. Moreover, in none of the recent illuminating transgenic models of tolerance does suppression appear to play a role. Nonetheless, suppression of immune responses, possibly through elaboration of soluble factors or as a property of a population of cells, appears to be an important cause of tolerance induction when antigen first confronts the immune system. The work group drew attention to an important body of work on thymectomy performed in mice not at birth, but after a delay of a few days. Such mice are subject to a variety of organ-specific autoimmune diseases, the nature of which depends on the genetic constitution of the mouse. This suggests the possibility that thymic negative selection is not perfect very early in life, or that slightly later the thymus exports cells capable of exerting a controlling suppressor function on potentially auto-aggressive T cells or both.

Effectors in the pathogenesis of Type 1 diabetes and directions for development of immunotherapies

The data available thus far suggest important roles for several effector pathways including CD4+ and CD8+ T cells, peri-islet dendritic cells, cytokines, and possibly free radical formation. Thus, a clear-cut focus for immunotherapy cannot be identified by the existing data. However, the experience with CD4 mAb treatment in rodents would suggest the use of this reagent, as a human chimeric mAb, may have advantages compared to existing reagents. In the meantime, further studies regarding the pathogenesis of the disease in animal models are most likely to reveal insights into mechanisms of human disease, and would represent a logical testing ground for new immunotherapies which may pose a risk that might otherwise be acceptable if prevention or treatment of disease were a likely outcome.

Conclusions

The observations described above concerning tolerance to islet antigens are taken from animal models which may or may not reflect mechanisms operative in humans. The data presented in this work group, including the results from the expression of transgenes on the islet cells, support the possibility of the release and presentation of sequestered antigens following an insult to the Beta cells as a initiating event in the development of autoimmune diabetes. These antigens may consist of self cellular constituents (such as insulin or glutamic acid decarboxylase) or other antigens, even peptides from foreign proteins, which, after shedding by islet cells, enter an immunogenic processing pathway. Dendritic cells surrounding the islets of Langerhans may play a critical role in presenting islet-derived antigens to the immune system. However, the accessory molecules or cytokines involved in initiating the response are not known, and the earliest affector and effector cells have not been identified.

The islet antigens which are ultimately the targets of the T-cell response do not necessarily reflect the initial insult that leads to their release and presentation. Of note, the recognition of a higher incidence of diabetes with certain occupational exposures (in Australia) and the recently observed increase in the incidence of diabetes in Northern Europe might be explained by this concept of pathogenesis.

Despite our fragmented understanding of the early events in initiation of autoimmune diabetes, potentially fruitful approaches to immunotherapy have been identified. For example, anti-T cell mAbs may be able to induce antigen-specific T-cell tolerance. MHC blocking peptides, which inhibit T-cell responses restricted by disease associated MHC antigens, may also be valuable since Type 1 diabetes is closely associated with particular MHC alleles.

Thus, studies of the development and nature of tolerance to self antigens have provided many insights into the pathogenesis of autoimmune diseases. Further research into this area and application to animal models of Type 1 diabetes are likely to provide answers about the cause of human Type 1 diabetes, and suggest new and effective forms of immunotherapy.

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