Review

Checkpoints in the progression of autoimmune disease: Lessons from diabetes models

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ABSTRACT In the last few years, data from experiments employing transgenic models of autoimmune diseases have strengthened a particular concept of autoimmunity: disease results not so much from cracks in tolerance induction systems, leading to the generation of an anti-self repertoire, as from the breakdown of secondary systems that keep these cells in check. T cells with anti-self specificities are readily found in disease-free individuals but ignore target tissues. This is also the case in some transgenic models, in spite of overwhelming numbers of autoreactive cells. In other instances, local infiltration and inflammation result, but they are well tolerated for long periods of time and do not terminally destroy target tissue. We review the possible molecular and cellular mechanisms that underlie these situations, with a particular emphasis on the destruction of pancreatic β cells in transgenic models of insulin-dependent diabetes.

Organ-specific autoimmune disease is often considered to result from a deficiency in tolerance induction systems, resulting in their failure to eliminate or defuse self-reactive lymphocytes. T cells that slip through the mesh of the tolerance safety nets would attack and destroy peripheral tissues. Diabetes, for example, would result from rogue T cells reactive to pancreatic antigens such as glutamate decarboxylase (1, 2). Yet, it is clear, from recent work with transgenic systems in which most T cells express receptors directed against natural organ-specific antigens, that additional mechanisms allow the immune system to control potentially devastating lymphocytes and avoid pathology. We will review the concept of checkpoints in the progression of autoimmune disease, focusing on insulin-dependent diabetes mellitus (IDDM) in the nonobese diabetic (NOD) mouse and in a T-cell receptor (TCR) transgenic model derived therefrom.

IDDM is a chronic disorder that results from destruction of the insulin-producing β cells of the pancreatic islets (for review, see ref. 3). In its initial phase, which is clinically silent, T lymphocytes and other inflammatory cells invade the islets, eventually destroying them. The disease then becomes clinically overt, with the patho-

logical consequences (hyperglycemia, ketosis, and neuropathy) of the inability to maintain glucose homeostasis. The overall progression of disease, as well as the polygenic and environmental influences that condition it, are well modeled in the NOD mouse (4). Insulitis in this inbred strain appears spontaneously around 3–4 weeks of age and is well established by 10 weeks. Progression to overt diabetes occurs in 80% of female mice between 10 and 30 weeks of age. The major histocompatibility complex (MHC) represents the most important contributor to a complex array of genes that influence susceptibility (5, 6).

There is extensive evidence supporting a crucial role for T cells and MHC-restricted self-antigen recognition for diabetes pathogenesis, as disease can be transferred by T-cell populations or clones. Haskins and colleagues (7, 8), in particular, isolated clones of $CD4^+$ T cells capable, alone, of conferring disease; these clones recognized pancreas-specific antigens presented by NOD MHC class II molecules. To facilitate analysis of the selection and tolerance induction forces operating on such cells, a line of transgenic mice expressing on most T cells the TCR of one such clone (BDC2.5) was generated (9). Detailed evaluation of the lymphoid compartments showed an absence of tolerance induction; there was no sign of negative selection in the thymus and T-cell reactivity was normal in the periphery. However, autoimmune pathogenesis followed a particular course. There was no manifestation whatsoever in the first 3 weeks of life, with pancreatic islets completely free of infiltration; insulitis sets in abruptly at 3 weeks, very quickly becoming massive and involving essentially all islets. Yet, in spite of this overwhelming insulitis, diabetes only appeared much later and onset was widely spread over 10–25 weeks within a cohort of transgenic animals, in a manner not fundamentally different from that of disease in nontransgenic NOD mice.

This transgenic line, overexpressing a TCR that recognizes a natural autoantigen recognized in IDDM, thus allowed us to define two major checkpoints in pathogenesis. Checkpoint 1 controls the onset of insulitis: before 3 weeks of age there is no islet infiltration, although potentially aggressive T cells are circulating in large numbers through the immune system; pro-

gression across this checkpoint leads to a massive influx of T cells into the islet environment. Checkpoint 2 controls the switch to overt diabetes: in spite of extensive and active insulitis, intact β cells persist for long periods of time and no diabetes occurs. Eventually, this balance is lost, and the insulitis becomes terminally aggressive. In retrospect, both checkpoints also exist in regular NOD mice, but they are more clearly delineated in the transgenic mice. The recruitment and expansion of an array of autoreactive cells is obviously not required in the transgenic mouse as it is in the NOD mouse, and transitions are sharper: the onset of insulitis is brutal at 4 weeks, and when hyperglycemia sets in, it is quickly maximal. The molecular and cellular mechanisms that underlie these checkpoints are not just of academic interest, since therapeutic intervention against IDDM may stem from reproducing or influencing such control mechanisms.

Checkpoint 1: The End of Ignorance

BDC2.5 transgenic T cells ignore the islets for 3 weeks and then brutally invade them. This time course is actually quite similar to that of the NOD mouse itself. This delay in NOD animals correlates with the appearance of T cells reactive with a variety of autoantigens (see, for example, refs. 1, 2, 8, 10–13) and had been thought to represent the period necessary to recruit and activate self-reactive cells. This is clearly not the case in TCR transgenic mice with their preformed repertoire.

Clonal ignorance, defined by the pathologically harmless presence of antigen-reactive cells that are not deleted or inactivated, is not without precedent. In the pioneering study of Ohashi *et al.* (14), T cells expressing a transgenic TCR specific for a peptide from the gp protein of lymphocytic choriomeningitis virus ig-

nored their cognate antigen expressed in the pancreatic β cells. In this instance, autoimmune infiltration of the islets could be provoked by priming the T-cell compartment through systemic infection with lymphocytic choriomeningitis virus (14, 15). Similarly, significant numbers of T cells reactive against myelin basic protein or other components of the central nervous system exist in healthy individuals, apparently without harm (16). This situation is exaggerated, with huge numbers of autoreactive cells, in TCR transgenics expressing anti-myelin basic protein reactivity (17, 18); here, also, clonal ignorance reigns, but it can be reversed by peripheral immunization or by nonspecific environmental stimuli.

Several possible interpretations of the lack of islet infiltration before 3 weeks of age in BDC2.5 mice (and, by extension, in NOD) are shown in Fig. 1. Some interpretations, *a priori* tenable, are unlikely. We know, for example, that T cells are not generally incompetent in young mice, as splenic T cells at 10 days of age can be stimulated by mitogen or specific antigen (J.K., unpublished results). It is also unlikely that the absence of antigen is involved: we have found that the antigen recognized by the BDC2.5 receptor is present in islet tissue at 1 week of age (J.K., unpublished results). Antigen is also of course present in the double transgenic system of Ohashi *et al.* (14), as is myelin basic protein in neural tissue. On the other hand, it is conceivable that antigen-presenting cells are absent or deficient in the connective tissue between the blood vessels and the islets and thus that T cells that would venture in the vicinity of the islets would actually not encounter antigen in processed and recognizable form. Indeed, Jansen *et al.* (19) have documented changes in the composition of macrophage/dendritic cell populations around the onset of insulitis.

Yet, the results of transfer experiments in which cells from adult transgenic diabetics home readily to the islets of neonatal mice point more to a defect in the homing ability of the T cells. An important clue to checkpoint 1 may reside in timing. The onset of insulitis coincides (as it does in the NOD mouse) with weaning and the large immunological changes that take place at that time: major shifts in food

[• Inactive T cells]

[• Delayed synthesis of antigen]

• No or inappropriate APCs

• No homing: - Naive T cells
                  - Unattractive endothelium

FIG. 1.   Checkpoint 1: transition from clonal ignorance to organ infiltration. Brackets denote unlikely or disproven possibilities. APCs, antigen-presenting cells.

intake and in intestinal flora, resulting in a novel array of antigens, which confront the immune system in large quantities. Since adhesion molecules, in particular $\alpha 4$ integrin, have been shown to be important in the insulitic process (20–22), it seems reasonable to hypothesize that the broad T-cell stimulation that takes place at weaning modifies the homing potential of T cells (or at least of a fraction thereof) and endows them with the ability to migrate into pancreatic connective spaces (much as lymphocytic choriomeningitis virus infection does in the Ohashi model). Whether such alterations in the capacity of T cells to home to the islets will prove sufficient to explain all the phenomenology remains an open question. There are also documented changes in the expression of addressins (MAdCAM and PNAd) on the pancreatic blood vessel endothelium around the onset of insulitis (23–25). These changes in the expression of adhesion molecules by the endothelium could of course be merely secondary to the local inflammatory process (e.g., in response to local interferon γ production), but they may also play an integral role in the process. Checkpoint 1 would correspond, then, to increased homing potential of T cells coupled with increased attractiveness of the endothelium.

How do these phenomena relate to what happens in the NOD mouse? Some form of "checkpoint 0" must exist there as well—namely, the generation of the anti-pancreas repertoire, which preexists in BDC2.5 transgenics (under the reasonable assumption that the NOD thymus does not spontaneously select for anti-pancreas reactivity, which must thus be somehow expanded by autoimmunization). The appearance of measurable reactivity to pancreatic antigens (1, 2, 8, 10–13) has been reported to take place also around the 3-week period, concomitant with insulitis onset. The NOD system thus appears to progress simultaneously through checkpoints 0 and 1. One might speculate that a local insult around 3 weeks of age renders the endothelium attractive and releases sequestered pancreatic antigens, attracting T cells whose homing potential had been honed, not necessarily specifically, in the gut.

Checkpoint 2: From Controlled Violence to Chaos

The notion of controlled insulitis, in which T cells with potentially devastating capabilities and in direct contact with their source of antigen are nevertheless kept in check, is of clear importance in our understanding of diabetogenesis.

The uncoupling of insulitis and diabetes has been observed in several other experimental settings. In the NOD mouse itself, long-term insulitis does not always progress to disease; for example, in male mice, insu-

litis is as prevalent as in females, but males rarely show clinical diabetes (4). This state of tolerated insulitis in NOD mice can be stabilized by nonspecific immunostimulation (immunization with a variety of antigens, bacterial infection, or adjuvant administration) as shown in numerous studies (for review and references, see ref. 26). T cells attracted to pancreatic islets by the ectopic expression of tumor necrosis factor do not provoke disease in spite of massive insulitis (27, 28). They do, however, provoke disease in double transgenics where pancreatic tumor necrosis factor expression is coupled to an anti-self-TCR or to expression of costimulatory B7 molecules on the β cells (29, 30). Focal expression of interleukin (IL) 2 also leads to a long-tolerated insulitis (except when secreted at very high levels; refs. 31 and 32). Finally, tolerated insulitis has been observed in other TCR transgenic models, such as that of Scott *et al.* (33). These double-transgenic mice express influenza hemagglutinin (HA) on islet β cells (Ins-HA) and a TCR transgene specific for an HA peptide presented by MHC class II molecules. In some genetic backgrounds (see below), this potentially explosive situation only resulted in moderate insulitis without progression to diabetes. Transfer experiments indicate that the transition between tolerated insulitis and full-blown disease involves a switch in the pathogenic potential of the lymphoid populations. As with NOD mice, splenocytes from BDC2.5 mice that have insulitis but not diabetes do not transfer diabetes to neonatal recipients, whereas those from overtly diabetic mice do. New pathogenic capabilities, or a new regulatory balance, have thus been reached by the lymphoid compartment.

What affects this equilibrium? Why and how is it broken in some animals? Genetic influences on the progression through checkpoint 2 can readily be demonstrated, although their nature still needs to be defined. In early studies on the genetics of IDDM in NOD mice, Todd and colleagues (5) showed that some *idd* loci affect only the progression to diabetes and not the incidence of insulitis. In the HA transgenic system, the genetic background controls the outcome—very mild and tolerated insulitis on a BALB/c background vs. aggressive disease on C57BL/10 (33). Note, however, that the affinity of the TCR is also of importance, since the very same Ins-HA target provokes aggressive diabetes when crossed with another TCR transgenic, in this case regardless of genetic background (34). With BDC2.5 TCR transgenics, we have found a profound influence of non-MHC genes on the frequency and timing of progression to diabetes: hyperglycemia appears much earlier on the C57BL/6 than on the NOD background (J.K. and A.G., unpublished results). This balance can also be broken

by activating the T-cell populations: ConA activation *in vitro* endows acute diabetogenic potential on nonpathogenic splenocytes from BDC2.5 mice (ref. 35 and unpublished data). Finally, diabetes is also induced dramatically in BDC2.5 mice a few days after a single injection of cyclophosphamide (I.A., unpublished results). The mechanism of this induction is still under investigation, but it is unlikely to involve "suppressor" lymphocytes, because it also occurs in BDC2.5 transgenics carrying a single receptor because of the introduction of the TCRα° mutation.

Possible events that may underlie the transition through checkpoint 2 are summarized in Fig. 2. They may be cell intrinsic (i.e., reflect modifications taking place within the pathogenic CD4 T cell itself) or cell extrinsic (i.e., correspond to changes in other cells or signals that affect or complement the activity of the autoimmune T cell).

Among the cell-intrinsic changes could be the acquisition of new effector capabilities. The Th1/Th2 paradigm may provide important clues. There has been much interest in, and some convincing data concerning, the idea that recognition of self-antigens by Th1 cells leads to organ destruction and disease, whereas self-reactive Th2 cells are less damaging or even protective (for review, see refs. 26 and 36). Islet infiltration by Th2 cells would thus be harmless. The effects of systemic administration of several cytokines or anti-cytokines (for review and references, see refs. 26 and 37) are consistent with this idea, as is the ratio of IL-4 or interferon γ-producing cells in nondestructive insulitis after adjuvant or insulin therapy (38, 39). In addition, a strong argument for the functional difference between Th1 and Th2 cells in diabetogenesis came from the transfer of pancreas-specific T-cell cultures or clones (35, 40, 41): CD4 cells with Th1-like cytokine patterns provoked disease, whereas cells synthesizing the Th2 array of cytokines were

invasive but did not induce diabetes (but neither were they protective). Yet, even if the Th1/Th2 dichotomy proves to be the whole story, this only sets the question back one step; it is still necessary to determine what provokes this switch and how the switch elicits the final stages of pathogenesis. It is possible that other effector functions are acquired by pathogenic autoimmune T cells: a yet to be discovered cytokine (IL-*x*?) or the ability to kill through the *fas* pathway (42)—the latter is perhaps tied to Th subclass, as Th1 cells are known to express more fas ligand (43, 44). Conversely, the phase of controlled insulitis may result from dampening of the autoimmune T cell through particular surface receptors, recently shown to transmit inhibitory signals: (*i*) CTLA-4, whose absence provokes runaway T-cell responses (45, 46); (*ii*) the high-affinity IL-2 receptor required for the balance between activation-induced T-cell proliferation and death (47, 48); or (*iii*) the inhibitory MHC receptors of natural killer cells, also expressed on T cells (49, 50). A transition to diabetes could then represent an uncoupling of the signaling pathways that mediate negative control through these surface molecules. That CTLA-4 is involved in preventing a runaway insulitis would be consistent with data pointing to an influence of the B7/CTLA4-CD28 system in the control of diabetes (51, 52).

Initially, it seemed possible that the delayed diabetes in BDC2.5 mice reflected a need to recruit other cells, perhaps those expressing non-transgene-encoded specificities because of leaky allelic exclusion by the TCR transgene (9). The recruitment of CD8$^+$ cells appeared a real possibility, given that CD8$^+$ cells are necessary, together with CD4$^+$ cells, to transfer disease in the NOD system (53–55). Yet recent experiments have not supported this possibility; transfer of CD8$^+$ populations did not accelerate disease. Even more convincing was the observa-

tion that elimination of T cells displaying non-transgene-encoded specificities, by crossing the TCRα knockout mutation into the BDC2.5 line, not only failed to prevent diabetes but actually accelerated it (ref. 35; and J.K. and I.A., unpublished data).

Other cell-extrinsic explanations for checkpoint 2 remain distinct possibilities. The transition may coincide with the loss or inactivation of inhibitory cell populations, which would control insulitis by nonspecific interactions such as the secretion of inhibitory cytokines. Indeed, evidence for some form of suppressor or regulatory populations has been observed in the NOD system (e.g., refs. 39 and 56–59). One could draw an analogy with the development of inflammatory bowel disease in several immunocompromised mutant lines or after transfer of particular CD4$^+$ subpopulations (reviewed in ref. 60); inflammatory bowel disease in mutant mice seems to result from the abolition of a T-cell-dependent regulatory system that normally prevents a runaway response and inflammation. The same control system may operate during tolerated insulitis. In the same vein, inhibitory cytokines may dampen insulitis before checkpoint 2: transforming growth factor β, whose broad anti-inflammatory role has been well established (61–63); and IL-10, although experimental approaches to the role of IL-10 in diabetes have painted a confusing picture—while recombinant IL-10 given systemically to adult NOD mice prevents diabetes, local expression accelerates diabetes (64–66). The production of these inhibitory cytokines may of course be a conduit for the regulatory CD4$^+$ populations mentioned above. One could also envision that checkpoint 2 corresponds to the perturbation of an idiotype/anti-idiotype regulatory network, based on TCR–peptide recognition (see ref. 67 for a review). However, this is not a very palatable idea, as it seems difficult to envision a finely tuned network operating in the context of a TCR transgenic mouse expressing an essentially unique TCRβ chain. Finally, one should consider the possibility that the transition from controlled insulitis correlates with a modification of the population of accessory cells, such as macrophages, which would trigger a more aggressive lesion (19).

Conclusion

Autoreactive TCR transgenics simplify, to a degree, the analysis of the autoimmune process, but many questions need to be answered before we reach a good understanding of what these checkpoints represent. It is also likely that the checkpoints are not as rigorously distinct as we have presented them here, for clarity. As an example, the homing potential of Th1 cells seems superior to that of Th2 cells (35). Yet we believe that they correspond to
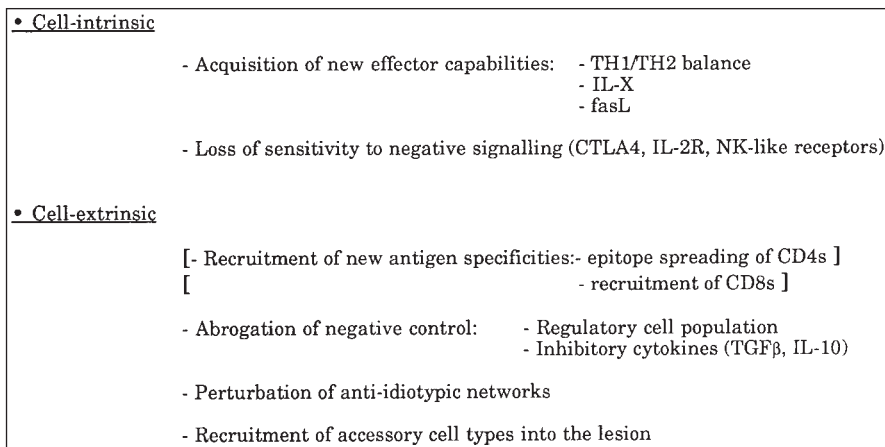

- Cell-intrinsic

       - Acquisition of new effector capabilities: - TH1/TH2 balance
                                               - IL-X
                                             - fasL

       - Loss of sensitivity to negative signalling (CTLA4, IL-2R, NK-like receptors)

- Cell-extrinsic

       [- Recruitment of new antigen specificities:- epitope spreading of CD4s]
       [- recruitment of CD8s]

       - Abrogation of negative control: - Regulatory cell population
                                        - Inhibitory cytokines (TGFβ, IL-10)

       - Perturbation of anti-idiotypic networks

       - Recruitment of accessory cell types into the lesion

FIG. 2. Checkpoint 2: transition from tolerated insulitis to diabetes. NK, natural killer; TGFβ, transforming growth factor β; IL-2R, IL-2 receptor.

mechanistically distinct phases in the progression of autoimmune pathology, which it will be important to understand in order to successfully tackle disease prevention.

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