a still unknown triggering event such as the local inflammation brought by a putative virus or an antigen mimicry phenomenon. Alternatively but not exclusively, it might involve a defect in protective immunoregulatory circuits. An intriguing possibility is that the onset of diabetes is secondary to the loss of the active tolerance that characterizes the 2-3-month-long period of clinically latent insulitis (prediabetic stage). If this is indeed the case, how does this tolerance operate (Th1/Th2 imbalance)? What are the factors that control this loss of active tolerance?

Another issue is that of the respective role of CD4 and CD8 T cells. Both cell subsets are required to transfer diabetes into neonates or irradiated syngeneic adults (Bendelac *et al.*, 1987; Wicker *et al.*, 1986), but both CD4 and CD8 T-cell clones have been shown to transfer the disease when injected alone in NOD SCID mice (Peterson and Haskins, 1996; Wong *et al.*, 1996). What is the respective role of CD4 and CD8 cells at both the initiation of the autoimmune process and the effector phase? The initial prejudice was that the effector cells were CD8^{+} cytotoxic T lymphocytes whose differentiation required CD4^{+} T-cell help. Recent data from several laboratories suggest a more complex situation.

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# Questions about NOD mouse diabetes

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The participants in this Forum have been asked to discuss the immunology of the non-obese diabetic (NOD) mouse focusing on 4 questions: 1) insulin-dependent diabetes (IDD) genes; 2) β-cell antigens; 3) CD4 and CD8 T cells; and 4) self tolerance.

In essence, the traditional 4 questions can be reduced to one general question: what makes this mouse different from all other mice? In discussing each of the questions, we have been requested to explain the importance of the theme, distinguish hard facts from hypotheses, and draw conclusions

and mark points of disagreement. I will organize my discussion according to this format.

## IDD genes

*Importance*

The spectrum of genes associated with the development of insulin-dependent diabetes mellitus (IDDM) is important because characterization of

these genes and their products could help us to appreciate the genetic basis of autoimmune disease in general, and sort out key factors in the pathogenesis of IDDM in particular.

## Hard genetic facts

Two findings seem to me to be important. The first is that more than a dozen loci critical to the IDDM process have been uncovered by genetic studies (Vyse and Todd, 1996). The second finding is the importance of the MHC class II I-A$^{g7}$ allele of the NOD mouse (Wicker et al., 1995). Expressing the I-A$^{g7}$ molecule is not sufficient to cause IDDM, but the molecule is very important among all the collective of Idd genes. Because MHC II molecules function in the presentation of peptide epitopes to T cells, my colleagues and I have studied the peptide-binding motif of I-A$^{g7}$ (Reizis et al., 1997a). We found evidence for the existence of four anchor positions accommodating 9 amino acids in which the pocket at position (P) 9 is critical, anchors P6 and P4 are important, and, in contrast to the common finding for DR/IE molecules, anchor PI is relatively degenerate. The details of the I-A$^{g7}$ motif and a molecular explanation for the role of residues 56 and 57 in the $\beta$ chain of I-Ag7 can be found in our paper (Reizis et al., 1997a). The important feature of the NOD mouse model for this discussion is that the I-A$^{g7}$ motif seems to be remarkably similar to the motif of the human DQ8 (DQB*0302) IDDM susceptibility molecule independently arrived at by Nepom's group (Kwok et al., 1996). Extending this investigation, we studied biochemical features of the DQ8 molecule compared to those of the I-A$^{g7}$ molecule, including stability and half-life of peptide binding, and compact dimer formation (Reizis et al., 1997b). In these aspects, too, we find a close similarity between the DQ8 and I-A$^{g7}$ molecules, which together differ from the IA/DQ molecules associated with resistance to IDDM. In fact, a particular hsp60 peptide uniquely induces compact dimer formation in both DQ8 and I-A$^{g7}$, but not in other DQ or IA molecules. Hence, a peptide can serve as a probe to define a biochemical feature characteristic of the critical class II molecule in both species.

## Implications and disagreements

To my mind, the genetic facts are both discouraging and encouraging. The bad news is that we will probably not achieve a major breakthrough by characterizing yet more Idd genes. A disease associated with more than a dozen loci is not a genetic disease, in the sense that we will not be able to reduce the disease to genetic components that are both *necessary* and *sufficient* to cause the disease. Indeed, the

concordance rate of probably less than 50% in homozygotic twins (Barnett et al., 1981) tells us that inherited genes are not sufficient factors in causality. Genetics will tell us more faithfully who is exempt from IDDM than who is going to get IDDM. The role of the environment is highlighted in the NOD mouse by the importance of infection and diet in determining the incidence of IDDM (Bowman et al., 1994; Elias, 1994).

The good news for those who seek genetic causality is the striking similarity between human and NOD mice in peptide binding (Reizis et al., 1997a) and biochemical (Reizis et al., 1997b) aspects of the DQ8/I-A$^{g7}$ molecules. The implications of the close similarity between the DQ8/I-A$^{g7}$ molecules are: 1) that the NOD MHC class II molecule is likely to model a genetic element important in human IDDM; 2) that the peptide-binding motifs of the DQ8/I-A$^{g7}$ molecule present similar peptides of importance; and 3) that biochemical features of DQ8/I-A$^{g7,}$ in addition to specific peptide binding, may play a role in disease.

The I-A$^{g7}$ molecule is controversial, however. An I-A$^{g7}$ peptide-binding motif has been proposed that differs markedly from ours (Harrison et al., 1997). We believe that the contending I-A$^{g7}$ motif may be explained by an error in interpreting the reading frame of the reference peptide used in those studies. It has also been claimed that the I-A$^{g7}$ molecule has an intrinsic defect in its ability to bind peptides (Carrasco-Marin et al., 1996). We find no such defect (Reizis et al., 1997a). Indeed, the well documented ability of the NOD mouse to make adequate T-cell responses argues against any general defect in MHC II function. Fortunately, any disagreement here can be resolved by experimentation.

## $\beta$-cell antigens

### Importance

Identifying target antigens is the key to early diagnosis of the autoimmune process. Target antigens also promise to be useful tools for specific immune modulation of the autoimmune process.

### Hard facts

Rather than a single, organ-specific antigen, IDDM in NOD mice and humans is marked by many antigen reactivities (Roep, 1996). Except for insulin, none of the many other antigens targeted in IDDM are limited in their expression to $\beta$ cells.

Immunization to only two of the antigens has been reported to induce a form of diabetes. Immunization to the whole hsp60 molecule (Elias et al.,

1990) or its p277 peptide (residues 437-460) conjugated to an immunogenic carrier can induce transient hyperglycaemia and insulitis in prediabetic NOD mice or in some standard strains of mice (Elias et al., 1995). Paradoxically, the induction of transient diabetes in NOD mice prevents the development of spontaneous diabetes. T-cell clones reactive to hsp60 or its p277 peptide can adoptively transfer hyperglycaemia and insulitis (Elias et al., 1991).

The other antigen associated with the induction of IDDM is insulin. T cells reactive to insulin can adoptively transfer disease (Wegmann et al., 1994). Active immunization to insulin has not been reported to induce IDDM.

T-cell reactivity to hsp60 and to p277 peptide appears in C57BL/KsJ mice induced to develop IDDM by an ultra-low dose of the $\beta$-cell toxin streptozotocine (Elias et al., 1994). Antibodies to insulin also appear in this model of IDDM.

Spontaneous IDDM in NOD mice (Elias and Cohen, 1995) and toxin-induced IDDM in C57BL/KsJ mice can be prevented or treated by subcutaneous administration of hsp60 or peptide p277 in oil given once or twice (Elias and Cohen, 1996). Transgenic NOD mice expressing mouse hsp60 controlled by the IE$\alpha$ promoter are protected against the development of IDDM (Birk et al., 1996). Treatment with peptide p277 downregulates immune reactivity to the other IDDM-associated antigens, glutamate decarboxylase (GAD) and insulin (Elias et al., 1997). The hsp60 transgenic NOD mice also manifest downregulation of their spontaneous T-cell reactivities to hsp60 and to GAD (Birk et al., 1996).

Treatment with GAD (Tian et al, 1996) or insulin can also prevent IDDM (Zhang et al., 1991) and downregulate T-cell reactivity to $\beta$-cell antigens including hsp60.

*Implications and disagreements*

Despite expectations for a single, primary, organ-specific antigen targeted at the initiation of the autoimmune process, the experimental evidence points to a collective of autoantigens that can be mutually regulated by any of 3 dominant antigens : hsp60, GAD or insulin (Tisch and McDevitt, 1996). There is as yet no molecular explanation for these findings, and the difficulty cannot be resolved by asserting that only one of the antigens is the true, primary antigen. I will discuss more about target antigens in the section on self tolerance.

## T cells, CD4 and CD8

*Importance*

T cells are important in IDDM because they appear to cause the destruction of $\beta$ cells. The interest of my laboratory in NOD diabetes has grown out of our studies of autoimmune T cells, first initiated in the models of EAE, thyroiditis and adjuvant arthritis (Cohen, 1995a).

*Hard facts*

Adoptive transfer experiments indicate that CD4$^+$ and CD8$^+$ T cells combine to cause IDDM in NOD mice (Bendelac et al., 1987).

CD4$^+$ T cells, however, can by themselves transfer disease (Elias et al., 1991, 1995).

Pathogenic CD4$^+$ T cells seem to be of the Th1 class (Liblau et al., 1995; Elias et al., 1997). Th2 cells were not found to cause IDDM, nor could they induce protection in some studies (Katz et al., 1995). However, we have found that administration to NOD mice of a Th2 line of T cells specific for hsp60 can induce resistance to IDDM (Elias and Cohen, personal communication).

The treatment of NOD diabetes by GAD (Tian et al., 1996) or by hsp60 peptide p277 (Elias et al., 1997) seems to involve a shift of the T cells reactive to the administered antigen, from a Th1 to a Th2 phenotype.

*Implications and disagreements*

Despite the importance of CD8$^+$ T cells in adoptive transfer of NOD IDDM (Bendelac et al., 1987), little is known about the antigen-specificities of cytotoxic T cells involved in the disease. Most workers have focused on the CD4$^+$ population and the Th1/Th2 dichotomy (Cohen, 1997). The major implication of the Th1/Th2 findings is that IDDM is not the result of autoimmunity per se, but rather the result of the biological phenotype of the autoimmune process. If the T cells targeted to the $\beta$ cells produce proinflammatory cytokines, notably IFN$\gamma$ (Elias et al., 1997), the $\beta$ cells are damaged and IDDM can result. If, in contrast, the T cells targeted to the $\beta$ cells produce antiinflammatory Th2 cytokines, then the $\beta$ cells are spared and the disease-causing Th1 cells are suppressed. Although a simple Th1/Th2 dichotomy is probably an unreal oversimplification (Cohen, 1997), the empirical observation that autoimmunity can exist in a protective mode heralds a new awareness; some forms of autoimmunity may be a good thing (Cohen, 1992). It is disturbing that islet hyperexpression of IL10, a Th2

cytokine, can in itself exacerbate insulitis (Wogensen *et al.*, 1994). It may be important that the activation of a specific Th2 shift by administration of the hsp60 peptide p277 is transient; the expression of IL10 and IL4 spontaneously regresses to very low levels after the Th1 response is aborted by treatment (Elias *et al.*, 1997). Thus, the autoimmune response does not remain in an activated state, neither Th1 nor Th2. The cytokine profile seems to be reset to a "harmless" baseline. Indeed, following p277 treatment, islet T cells fail to produce IFN$\gamma$ even when globally activated by mitogenic anti-CD3 antibodies (Ablamunits, A., Elias, D., Reshef, T. & Cohen, I.R., Islet T cells secreting IFN$\gamma$ in NOD mouse diabetes: arrest by p277 peptide treatment, submitted for publication).

Controversy centres on which antigens should be used to induce a therapeutic change in the autoimmune T-cell phenotype, and which route of administration is preferable. The populations of IDDM patients suitable for the early-phase clinical trial is also an open question; one insulin trial is aimed at treating persons at risk, while trials using insulin or hsp60 peptide p277 have begun with newly diagnosed IDDM patients. The impact of the NOD mouse on all of these developments is great. Study of autoimmune T cells in the NOD mouse has been essential to the progress in the field. It will take time and resources, but the contending therapies based on the NOD mouse will be resolved in the clinic.

## Self tolerance

### Importance

The participants have been asked to discuss the "mechanism of the rupture of self tolerance", or what it is that triggers the disease in the NOD mouse. The pathogenic trigger is the key, at least conceptually, to rational arrest of the destructive process.

### Hard facts

Despite the development of autoimmunity to insulin, GAD, hsp60 and other antigens in the IDDM collective, not all NOD mice, females or males, develop IDDM.

Development of IDDM can be prevented by exposure to infection, and modified by diet and by male sex hormones (Elias, 1994).

Active induction of autoimmunity to most of the antigens targeted in NOD IDDM does not appear to cause disease. As mentioned above, active immunization to hsp60 (Elias *et al.*, 1990) or its p277 pep-

tide (Elias *et al.*, 1995) may induce a transient bout of disease that seems to prevent the development of chronic, progressive damage and permanent IDDM.

### Implications and disagreements

Self tolerance and the mechanisms by which it may be "ruptured" are subjects of disagreement, and any implications that one may find reasonable can be thought to be quite unreasonable by others. Each of the following points is open to disagreement.

The terms "self tolerance" and "rupture (breaking) of self tolerance" should probably be replaced by more precise terms. Tolerance means different things to different immunologists. To some, tolerance implies a total lack of the capacity to respond in any way to an immunogenic stimulus, based on the deletion of all lymphocytes bearing receptors that might be able to recognize the given antigen. Specific deletion might be called "strong" tolerance. The "breaking" of strong tolerance would require the emergence of new receptor-bearing lymphocytes specific for the antigen, and the induction of these lymphocytes into action.

Others might use the term tolerance to indicate an immune response phenotype marked by no apparent immune attack on a specific antigen presented in what should be an immunogenic form. This use of the term tolerance is "weak", compared to classical strong tolerance.

In between the strong and weak concepts of tolerance are individual variations, whether the tolerance is "central" or "peripheral", whether both antibodies and T-cell proliferation are not allowed, whether "suppression" is included, and so forth. Breaking of weak tolerance could mean anything from a loss of regulation to an activation of intrinsically anergic clones of lymphocytes. Any term that means different things to different members of a profession is not the most efficient way to transmit information.

The uniformity of the immunologic expression of IDDM and the stereotypic collective of target antigens in NOD mice and human patients suggests that the disease is not due to the chance emergence of a forbidden clone; strong tolerance is unlikely. The mutual cross-regulation of the disease by GAD, hsp60 and probably insulin also suggests that the autoimmune expression of IDDM is orderly, and therefore likely to be structured within the immune system itself (Cohen, 1992).

If the potential to develop disease is a characteristic of the immune system of the NOD mouse (and of the susceptible human population, too), then the lack of disease (weak tolerance) probably rests on some form of regulation.

Thus, the "rupture of tolerance" is dysregulation, a dynamic imbalance in the relationship between the autoimmune T cells naturally present and the factors (cells, cytokines) that hold them in check. "Non-specific" immune experience obtained, for example, by living in a contaminated environment can contribute to "positive regulation" and prevent disease. In contrast, a variety of "non-specific" factors or toxic insults could "weaken" regulation and trigger disease. In other words, the specificity of the potential disease is programmed into the NOD immune system. Various and different triggers might therefore unleash the same disease program. The lack of a one-to-one relationship between the disease and a uniquely specific trigger is intellectually frustrating, but that's the way the disease acts. Fortunately, intellectual frustration can stimulate new thinking.

The good news about an intrinsically programmed disease is that its regulation is also naturally programmed. Regulation, indeed, can be reinstated using several different self antigens to push the system into a healthier mode of expressing its autoimmunity (Cohen, 1995b).

## Acknowledgements

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# Insights into the chemistry and biology of the I-A$^{g7}$ class II molecule

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During antigen presentation, the protein antigen must be processed intracellularly by antigen-presenting cells (APC) (Ziegler and Unanue, 1981, 1982) in order to generate peptides that, bound to major histocompatibility complex (MHC) class II molecules, are then presented to CD4 T cells (Babbitt *et al.*, 1985; Allen *et al.*, 1985). A number of studies have analysed the molecular and structural basis of the interaction between class II molecules and diverse peptides (for examples, Hunt *et al.*, 1992; Chicz *et al.* 1992; Nelson *et al.*, 1992; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Nelson *et al.*, 1996; Fremont *et al.*, 1996).

We are interested in analysing the interactions of class II molecules with peptides involved in the diabetic autoimmune response. The non-obese diabetic mice (NOD) spontaneously develop a T-cell-dependent autoimmune diabetes, similar to human insulin-dependent diabetes mellitus (IDDM). The main genetic risk factor for developing diabetes in both human and animal models (i.e. NOD mice, BB rats) is the genotype of the MHC class II molecule (Hattori *et al.*, 1986; Castaño and Eisenbarth, 1990; Todd *et al.*, 1987). In the NOD mice, the class II molecule, I-A$^{g7}$, the homolog to human DQ molecules, is composed of an $\alpha^d$ chain plus a unique $\beta^{g7}$ chain that lacks an aspartic acid at the 57 position. All the other murine haplotypes have an aspartic acid residue (Acha-Orbea and McDevitt, 1987). This linkage between the lack of an aspartic acid at the 57 position of the β chain and susceptibility to diabetes also applies to human DQ molecules (but see Todd *et al.*, 1987; Nepom, 1993).

In spite of the undoubted importance of class II molecule in this autoimmune disease, few studies have been conducted to examine in detail the biochemical properties of the I-A$^{g7}$ class II molecule (Reich *et al.*, 1994). The purpose of this research was, first, to study biochemical properties, not previously analysed, of the I-A$^{g7}$ class II molecule, such