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Immunology

The phenotype of lymphoid cells and thymic epithelium correlates with development of autoimmune insulitis in NOD ↔ C57BL/6 allophenic chimeras

(NOD mouse/autoimmune diabetes)

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ABSTRACT **The mechanisms contributing to the development of autoimmune insulin-dependent diabetes mellitus have been analyzed in allophenic mouse chimeras of the NOD ↔ C57BL/6 strain combination (where NOD is nonobese diabetic). Occurrence of lymphoid cell infiltration (insulitis) in pancreatic islets was observed in the majority of such chimeras. The development of insulitis was found to correlate with major histocompatibility complex chimerism in lymphoid cells and in thymus cortical regions. Chimeras with more than 50% of C57BL/6 lymphoid cells rarely developed insulitis. Our data suggest that the correlation with the thymic cortical region is absolute. Thus, all individuals displaying NOD or NOD/C57BL/6 thymic cortical regions developed insulitis, whereas we have not observed insulitis in chimeras with only C57BL/6 thymic cortical regions. Thus the positive selection of T cells appears to play a crucial role in the development of insulin-dependent diabetes mellitus.**

One of the best animal models of human insulin-dependent diabetes mellitus is represented by the nonobese diabetic (NOD) mouse (1). In these mice overt diabetes develops spontaneously in a majority of female mice and less frequently in male mice. As in human insulin-dependent diabetes mellitus, development of overt diabetes in NOD mice is preceded by infiltration of lymphoid cells into the pancreatic islets and is observed only after massive destruction of insulin-producing β cells (2).

Predisposition to the disease is under polygenic control, one of the genes having been identified as the major histocompatibility complex (MHC) class II locus both in man and in the NOD mouse strain (3, 4). The involvement of MHC genes in the development of autoimmunity in NOD mice indicates that T cells play a crucial role in this process. Indeed, the disease can be transferred with cells from diabetic animals (5) requiring both $CD4^+$ and $CD8^+$ cells (6, 7).

NOD mice express a unique I-A molecule ($I\text{-}A^{NOD}$) where aspartic acid in position 57 of the β chain is replaced by serine (8). This is observed also in the HLA-DQ β chain of many Caucasians with insulin-dependent diabetes mellitus (9–11). The I-E antigen is absent in the NOD strain due to a defect in I-E α chain expression (3). Recent reports showing that insulitis in NOD mice can be prevented by the introduction of I-E α chain or non-NOD I-A transgenes have supported the hypothesis that I-A and I-E expression affects development of insulitis (12–15).

I-E expression is known to mediate intrathymic clonal deletion of sets of T-cell antigen receptors carrying specific β-chain variable regions (16, 17). Based on these observations, the protective effect of I-E has been suggested to reflect negative selection through intrathymic clonal deletion of potentially hazardous T-cell clones (12, 18). However, the observation that NOD mice expressing promoter-mutated I-E α chain genes mediating intrathymic deletion of I-E-reactive T cells still develop insulitis has cast doubt about this hypothesis (19).

To further investigate the role of MHC class II and cellular mechanisms in the development of insulitis, we have studied allophenic chimeras (20) constructed from NOD and C57BL/6 (B6) mouse strains. Since the cell mixing for construction of allophenic chimeras occurs at the 8-cell stage of development, all cell components of interest (T cells, macrophages, thymic epithelium, β cells, etc.) develop into chimeric structures and will be physiologically functional in adult mice. As the proportions of the two cell populations (chimerism) vary among individual chimeras (21, 22), analysis of MHC chimerism and autoimmune response (measured by degree of insulitis or disease) may elucidate the critical cell components involved in the initiation of the autoimmune response. In this report we have measured MHC chimerism in various immune tissues and pancreas by flow cytometer analysis or immunofluorescence staining of tissue sections. The results show a positive correlation between the MHC chimerism of the lymphoid cells (in the thymus and the periphery) and the degree of insulitis in pancreas. Furthermore, whereas all chimeras developing insulitis were found to display thymic cortical regions (thymic epithelium) of only NOD type or NOD plus B6 type, no insulitis was observed among chimeras displaying thymic cortical regions of only B6 type. These observations strongly indicate that thymic positive selection constitutes a crucial step for development of autoimmunity in NOD mice.

MATERIALS AND METHODS

Animals. B6 and NOD mice were bred in our animal facilities at University of Umeå. The NOD colony was derived from a breeding nucleus kindly provided by C. Carnaud (Hospital Necker, Paris). All NOD mice and the NOD ↔ B6 allophenic chimeras (see below) were screened weekly, from the age of 15 weeks, for glucosuria using test strips and a calorimetric assay. In our NOD colony, the frequency of overt diabetes reaches 64% in females and 10% in males at the age of 30 weeks.

Allophenic Chimeras. Methods of producing allophenic chimeras can be found elsewhere (23). Briefly, two 8-cell-stage mouse embryos were paired in the culture droplets after removal of zonae pellucidae by treatment with 0.5% Pronase. Aggregated embryos were then cultured for 24–48 hr at 37°C in 5% CO_2/95% air. These chimeric blastocysts were surgically transferred into uteri of pseudopregnant foster mothers of $(CBA/J \times B6)F_1$ on the third day (vaginal plug discovery

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Abbreviations: B6, C57BL/6; MHC, major histocompatibility complex; NOD, nonobese diabetic; FITC, fluorescein isothiocyanate.

= day 1). Allophenic chimeras that showed clear coat-color chimerism (NOD, albino coat; B6, black coat) were selected for experiments.

Cytofluorimetry. Approximately 10^5–10^6 cells from the respective organ were double stained by incubating with primary antibodies coupled to fluorescein isothiocyanate (FITC) for 30 min at 4°C, washed, and incubated with a secondary biotinylated antibody followed by avidin-phycoerythrine (Vector Laboratories). Primary antibodies used were goat anti-mouse immunoglobulin-FITC (Becton Dickinson), anti-mouse CD4-FITC (L3T4 Becton Dickinson), and anti-mouse CD8-FITC (anti-Lyt2 Becton Dickinson). Secondary antibodies were Ox6, a mouse anti-rat MHC class II antibody crossreacting with class II^{NOD} but not reacting with class II^b (24), 25.9.17S specific for the $I\text{-}A^b$ antigen (25), K9.18 specific for the K^d MHC class I antigen (26), and K10.56 specific for the K^b MHC class I antigen (27). Incubation and washing were always done in phosphate-buffered saline (PBS)/5% (vol/vol) fetal calf serum. Labeled cells were then analyzed on a FACScan (Becton Dickinson). Dead cells were excluded by virtue of high propidium iodide fluorescence and 10^4 cells were accumulated for histograms using logarithmic amplification of fluorescence intensity.

Histopathology. Insulitis was determined by histological examination of pancreas. Paraffin sections (4 μm) of formalin-fixed pancreata were stained with hematoxylin/eosin. A minimum of 20 islets per individual was examined and only mice in which no affected islets could be detected were scored as negative.

Immunohistology. The monoclonal antibodies used have been listed above. Serial cryostat sections (5 μm) of thymii or pancreata were double stained with the respective combination of antibodies. Sections were fixed in acetone and stored at −70°C until further processed. After rehydration in PBS, slides were incubated with normal rabbit serum [20% (vol/vol)] for 20 min followed by incubating with a biotinylated primary antibody followed by rhodamine-conjugated avidin D (Vector Laboratories). This was followed by incubating with a second FITC-conjugated antibody. In the case of guinea pig anti-insulin (Sigma) staining, FITC-conjugated goat anti-guinea pig IgG (Cappel Laboratories) was used as secondary antibody. All incubations were followed by three washes in PBS at room temperature.

RESULTS

Qualitative and Quantitative Distribution of Chimerism in NOD ↔ B6 Allophenic Chimeras. Embryos from NOD and B6 mice were aggregated at the 8-cell stage for allophenic chimera production. As the result of cell mixing at this early stage of ontogeny, all tissues become chimeric and MHC-incompatible chimeras develop stable tolerance mainly due to clonal deletion (28, 29). By monitoring coat color mosaicism, mice displaying black and albino regions were selected for the experiments. Table 1 shows the distribution of chimerism among 15 individuals of the NOD ↔ B6 chimeras analyzed. In this series of chimeras, coat color distribution was found to range from 50% to 99% albino (NOD).

Cytofluorimetric analyses of class I MHC expression in spleen cells, lymph node cells, or thymocytes, derived from the same set of chimeras, revealed a phenotypic distribution ranging from 23% to 97% NOD type. As expected from the ontogenic process, where pigment cells and lymphoid cells develop from different stem cells (21, 22), no direct correlation between coat color chimerism and H-2 chimerism in lymphoid organs was observed. In contrast, the fraction of NOD cells in different lymphocyte subpopulations was found to be very similar. Thus, within the same animal, roughly the same proportion of NOD-derived cells would constitute the B-cell, $CD4^+$, or $CD8^+$ compartments (Fig. 1).

Table 1. Correlation between lymphoid cell MHC chimerism and occurrence of insulitis

Mouse	Sex	Insulitis	% NOD coat color	Fraction of cells with the NOD phenotype		
				Spleen	Lymph node	Thymus
B6			0	<0.02	<0.01	<0.01
NOD			100	>0.97	>0.98	>0.98
NOD + B6			—	0.44	0.36	0.50
Ch 24	M	−	50	0.23	0.27	0.32
Ch 40	M	−	55	0.23	0.28	0.29
Ch 49	F	−	85	0.23	0.33	0.30
Ch 44	M	−	60	0.41	0.46	0.49
Ch 60	F	+	60	0.42	0.44	0.44
Ch 39	M	+	70	0.47	0.47	0.47
Ch 13	M	+	70	0.68	0.71	0.55
Ch 43	M	−	80	0.41	0.44	0.61
Ch 14	M	+	80	0.97	ND	0.96
Ch 22	M	−	90	0.63	ND	0.51
Ch 31	F	+	95	0.63	0.52	0.71
Ch 18	F	+	95	0.69	0.55	0.57
Ch 16	M	+	98	0.93	0.92	0.92
Ch 30	M	+	99	0.96	0.94	0.92
Ch 12	F	+	99	0.80	0.75	0.77

M, male; F, female; −, no; +, yes; ND, not done.

Immunofluorescence studies of thymic tissue sections revealed a distribution of class II chimerism in medullary cells that corresponded to the distribution of thymocytes and other lymphoid cells observed by fluorimetric analyses. As illustrated in Fig. 2, the medullary region of the thymus consisted of a mixed population of class II^{NOD}- and class II^{B6}-expressing cells. In contrast, the thymus cortical region was

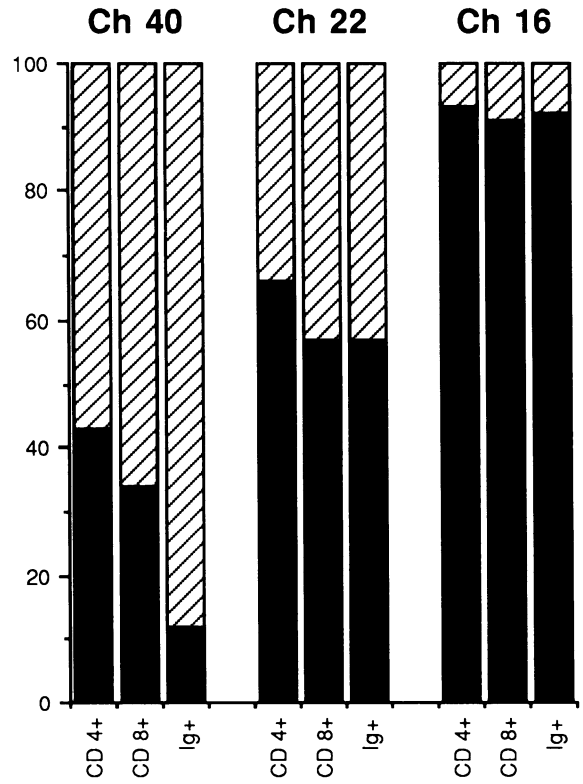

FIG. 1. Distribution of chimerism in spleen cells expressing CD4, CD8, or immunoglobulin (Ig) in three representative chimeras, Ch40, Ch22, and Ch16. Spleen cells were double stained with antibodies specific for MHC class I or class II antigens of NOD (hatched bars) or B6 (solid bars) in combination with anti-CD4, anti-CD8, and anti-immunoglobulin antibodies as indicated.

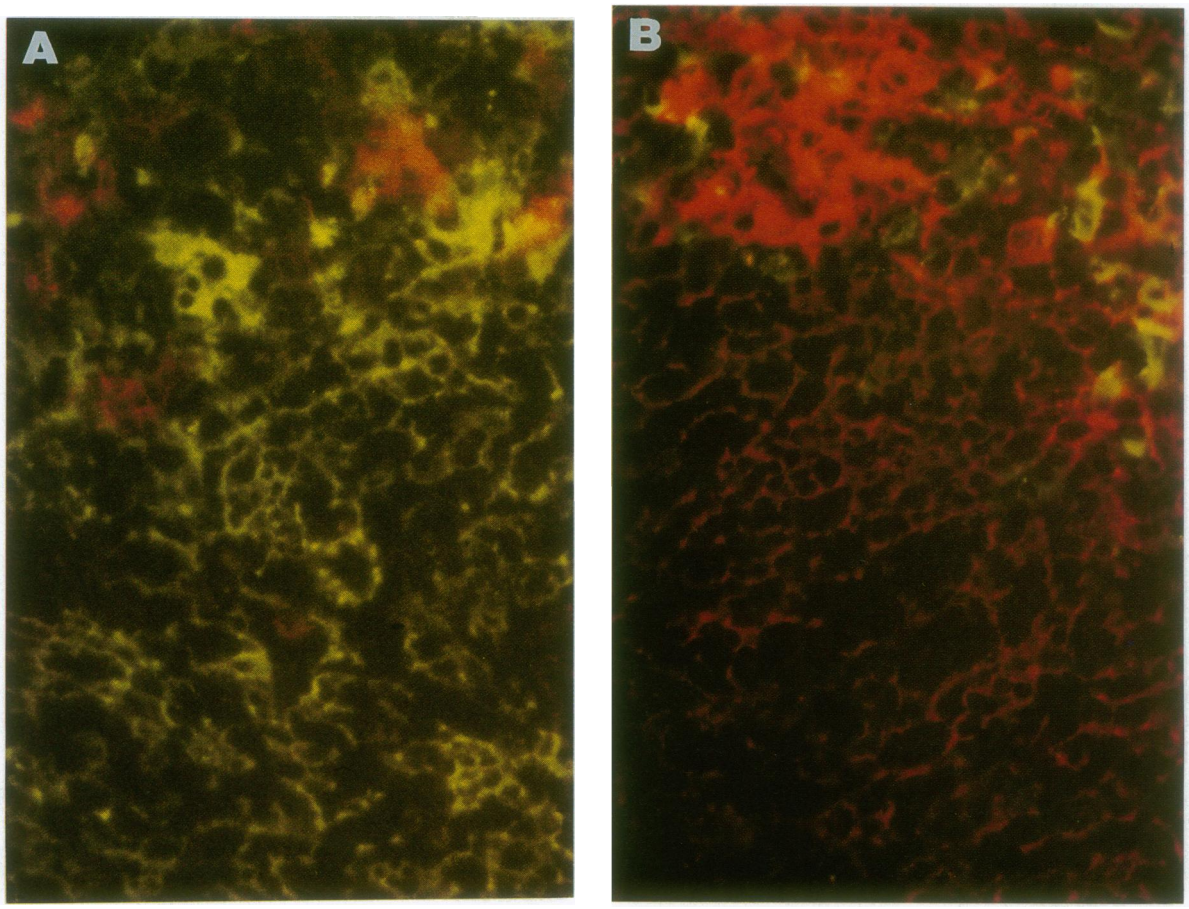

FIG. 2. Immunohistochemical analysis of thymic tissue from EA chimeras double stained with anti-MHC class II^{NOD}-FITC and anti-MHC class II^{B6}-rhodamine. (*A*) A chimera with a cortex region solely expressing MHC class II^{NOD} (lower right corner) and a medullary region containing a mixture of cells expressing either MHC class II^{NOD} or class II^{B6} (upper left corner). (*B*) A chimera with a cortex area solely expressing MHC class II^{B6} (lower left corner) and a medullary region containing a mixture of cells expressing either MHC class II^{NOD} or class II^{B6} (upper right corner).

found to be constituted by homogeneous areas of cells expressing either class II^{NOD} or class II^{B6} only.

Our observation of the H-2 chimerism in thymus is compatible with thymic epithelium being essentially immobile cells and may reflect a small number of precursor cells at the ontogenic time point at which cell type commitment occurs for thymic epithelial cells. On the other hand, most MHC class II-expressing cells in the medulla are mobile lymphocytes and macrophages, leading to extensive cell mixing. Further analyses of thymic sections obtained from younger animals confirmed the observed pattern of chimerism.

Development of Insulitis Correlates with the Proportion of Lymphoid Cells Expressing Class II^{NOD}. Chimeric mice were tested weekly for glucosuria using test strips. Although 64% of the females and 10% of the males in our NOD colony develop glucosuria before the age of 30 weeks, none of the chimeras up to 1 year or more of age did. However, more than half of the chimeras showed various degrees of insulitis when sacrificed at 1 year of age. In the majority of mice developing insulitis, at least some of the affected islets showed heavy intraislet infiltrates and a decrease in insulin production (Fig. 3). In comparison normal NOD mice at this age show a 100% incidence of insulitis. No obvious correlation was found between development of insulitis and sex. Thus, of a total of 32 chimeras analyzed (17 males and 15 females), 19 (9 males and 10 females) had developed insulitis.

As shown in Table 1, the vast majority of chimeras scoring positive for insulitis were found to have 50% or more lymphoid cells of NOD type. Two of the chimeras (Ch 22 and Ch 43) that showed 50% or more lymphoid cells of NOD type did not develop insulitis (further discussed below). The observed correlation between insulitis and the proportion of NOD type spleen cells was similar when cells of other lymphoid organs, such as peripheral lymph nodes and spleen, were analyzed. Essentially the same results were obtained when the MHC class I antigen was substituted for class II antigens as markers for cellular phenotype (data not shown).

We also analyzed the phenotype of the infiltrating cells in animals scoring positive for insulitis. The distribution of class II-expressing cells at this location was found to be representative of the overall phenotypic distribution in peripheral cells.

Absence of Class II-Expressing NOD Type Cells in Thymic Cortical Regions Coincides with a Lack of Insulitis. As discussed above, the thymic medullary and cortical regions display distinctly different patterns of chimerism. Whereas the former shows a pattern of mixed NOD and B6 cells proportional to the distribution in other lymphoid organs, the latter is constituted by homogeneous areas of class II-expressing cells of only one of the phenotypes. In only a small number of chimeras sacrificed at 1 year of age could distinct cortical and medullary regions be unambiguously identified due to thymic involution. Comparing the class II expression in the various regions of thymus of these animals with the incidence of insulitis revealed a strikingly strong positive correlation between H-2 chimerism in cortical regions and development of insulitis. Thus, when five chimeric animals scoring positive for insulitis were analyzed, all were found to display cortex regions of either NOD plus B6 type or only NOD type (Table 2). In contrast, in four chimeras with no

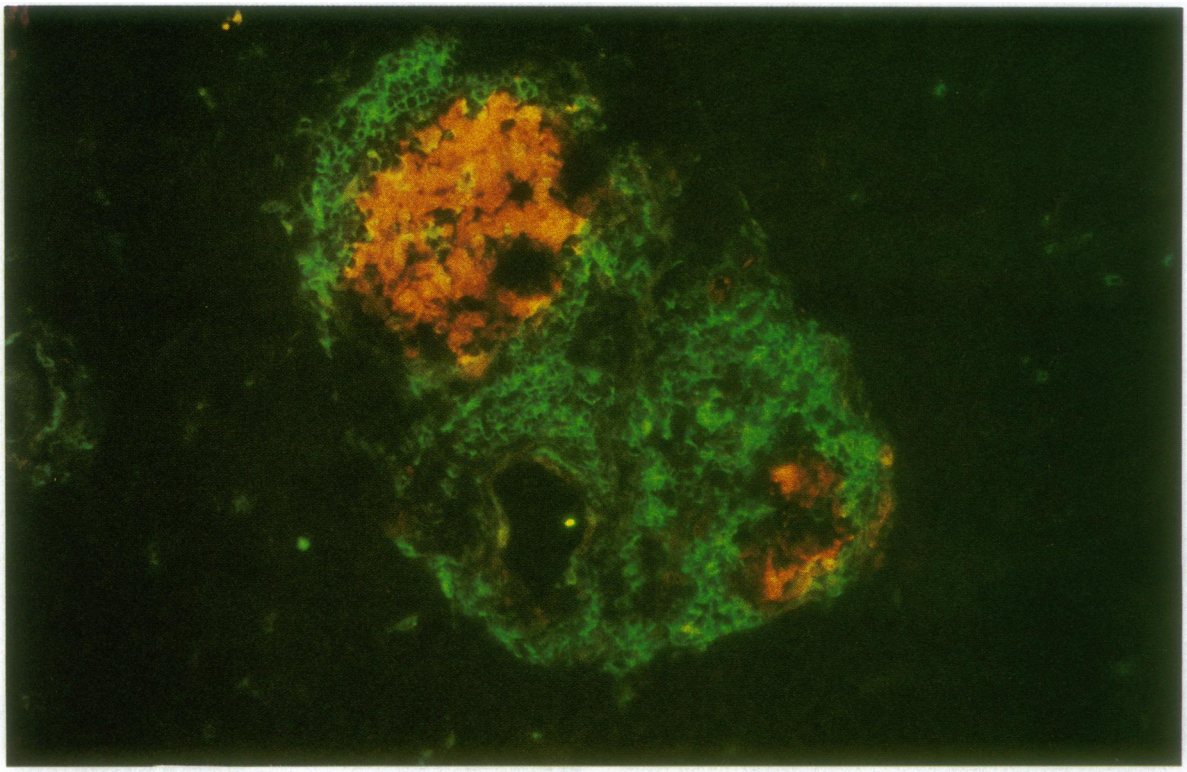

FIG. 3. Immunohistochemical analysis of pancreatic tissue from an allophenic chimera showing insulitis. Consecutive cryosections of the pancreas were double stained with anti-insulin-rhodamine and anti-class II^{NOD}-FITC.

signs of insulitis, the thymic cortex regions were identified as solely expressing B6 class II antigen. In fact, the two chimeras Ch 22 and 43 that did not develop insulitis in spite of displaying more than 50% NOD type lymphocytes were found to exhibit B6 cortex only.

DISCUSSION

Among the various ways the immune system has to cope with the problem of self–nonself discrimination, the selection of T cells by MHC and self antigens at various levels plays the major role. During the process of establishment of T-cell repertoires, the cortical region is known to be crucial for positive selection of T cells (30, 31), whereas, according to the classically held view, tolerance induction mainly occurs through clonal deletion (17, 32) through interactions with cells of hematopoetic origin in the thymic medulla (33, 34).

Interpreted within this frame, our results could be explained in terms of clonal deletion of potentially autoreactive T cells, induced by $I\text{-}A^b$ molecules provided by cells of B6 origin. Thus in chimeras in which B6 lymphoid cells comprise more than 50% and, consequently, $I\text{-}A^b$ molecules are abundant, clonal deletion is complete and insulitis does not develop. In line with this, I-E and $I\text{-}A^b$ molecules in transgenic NOD mice have been shown to prevent insulitis (12–15, 19).

Table 2. Correlation between thymus MHC chimerism and occurrence of insulitis

Chimera	Insulitis	Phenotype of thymic medulla, % of total		Phenotype of thymic cortex
		NOD	B6	
Ch 43	−	50	50	B6
Ch 24	−	20	80	B6
Ch 22	−	50	50	B6
Ch 20	−	5	95	B6
Ch 39	+	60	40	B6/NOD
Ch 31	+	80	20	NOD
Ch 60	+	40	60	B6/NOD
Ch 16	+	50	50	B6/NOD
Ch 32	+	95	5	NOD

−, no; +, yes.

However, the recent observation (19) that NOD mice with promoter-mutated I-E genes expressed on various subsets of immunocompetent cells still developed insulitis casts some doubt on the simplistic explanation based on clonal deletion. It is possible that the protection from insulitis by I-E or I-A β chain molecules might require cell interaction and active suppression of autoreactive T cells. On the other hand the mechanisms leading to protection from insulitis may be different in these two cases. In neither case do these data contradict our chimera results since such processes are also susceptible to threshold effects restricted by the amount of $I\text{-}A^b$ molecules.

The observation here of an absolute correlation between the NOD-type thymic cortical region and occurrence of insulitis suggests that positive selection of T cells plays a decisive role in the development of insulitis in the NOD mice. When there is not sufficient $I\text{-}A^b$ expression in chimeras, T cells would inevitably escape tolerance. Thus, autoreactive $CD4^+$ T cells, positively selected by MHC class II^{NOD} antigens in thymus epithelium, may escape self tolerance and migrate to the periphery.

Why then do such positively selected $CD4^+$ T cells infiltrate the pancreas and become activated? It has been speculated that putative pancreas-specific peptides might bind more effectively to the $I\text{-}A^{NOD}$ molecules due to the conformational changes caused by mutations around position 57 of the I-A β chain (8, 35). In normal mice, various tolerance mechanisms operating in the thymus and in the periphery would function as fail-safe mechanisms avoiding autoaggression. Since most body tissues express neither the class II MHC antigens nor putative costimulators for T-cell activa-

tion (for review, e.g., see ref. 36), these potentially self-reactive T cells in the periphery become prone to clonal anergy, suppression, or even clonal deletion (37), and autoimmunity is avoided.

Thus, the mutations around position 57 of the I-A β chain may be necessary but not sufficient to cause autoimmunity in NOD mice. Because development of diabetes in NOD mice is under polygenic control, perhaps other genetic loci may disturb normal self-tolerance mechanisms. One such possibility could be disturbances caused by the T-cell hyperplasia observed in NOD mice (38). Excess T-cell proliferation would favor escape of clonal deletion but might not hamper positive selection.

It is likely that the activation of helper T cells would require the presence of macrophages or antigen-presenting cells in pancreas (39, 40) presenting peptides from pancreatic cells. According to the model presently discussed, the diabetes-susceptible mutations at position 57 of I-A β chain should behave as dominant alleles and be expressed at least in thymic epithelium and macrophages. This seems compatible with the observation that insulitis occurred in NOD → (NOD × B10)F_1 bone marrow chimeras (41). In addition, the activation of macrophages and helper T cells in the pancreatic regions may secondarily induce increased expression of class I MHC (42) in pancreatic β cells by cytokines. Cytokines may also induce infiltration and activation of cytotoxic T cells or B cells in pancreatic islets and ultimately cause destruction of β cells. The hypothesis that aberrant expression of class II MHC antigen is the primary cause for the initiation of autoimmune responses (43) can be contradicted by the recent result of transgenic mice where optimally expressed I-A transgenes in pancreatic β cells did not result in insulitis (44).

A puzzling aspect of our chimera experiments was that we have not so far observed diabetic mice over a period of 1.5 years despite the fact that some chimeras had extensive insulitis accompanied by destruction of β cells. The report that cytotoxic T cells in NOD mice are K^d-restricted (45) predicts that only NOD pancreatic cells would be destroyed without touching B6 cells. On the other hand, diabetes in NOD mice could be inhibited under various subtle conditions.

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