Pancreatic NOD beta cells express MHC class II protein and the frequency of I-A^{g7} mRNA-expressing beta cells strongly increases during progression to autoimmune diabetes

U. Walter^{1, 6}, T. Toepfer¹, K. E. J. Dittmar², K. Kretschmer¹, J. Lauber¹, S. Weiss¹, G. Servos⁴, O. Lechner¹, W. A. Scherbaum⁵, S. R. Bornstein⁵, H. von Boehmer⁷, J. Buer^{1, 8}

¹ Department of Cellbiology and Immunology, German Research Centre for Biotechnology, Braunschweig, Germany

² Department of Molecular Biotechnology, German Research Centre for Biotechnology, Braunschweig, Germany

⁴ Department of Anatomy, University of Duesseldorf, Duesseldorf, Germany

⁵ Department of Endocrinology, University of Duesseldorf, Duesseldorf, Germany

⁶ INSERM U373, Institut Necker, Paris, France

⁷ Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA

⁸ Institute of Medical Microbiology, Hannover Medical School, Hannover, Germany

Abstract

Aims/hypothesis. In the NOD mouse model, attempts to show MHC class II expression by pancreatic beta cells were unsuccessful so far. We readdressed this question by analysing I-A^{g7} expression in single pancreatic beta cells.

Methods. Single-cell multiplex RT PCR and single-cell immunofluorescence were used to study MHC class II expression in NOD and NOD/SCID beta cells.

Results. Pancreatic beta cells from NOD mice express the I-A^{g7} protein as well as the corresponding mRNA. The frequency of MHC class II mRNA-expressing beta cells is drastically increased during the progression

to overt diabetes. MHC class II protein is accumulated intracellularly, and invariant chain is co-expressed. Beta cells from 9- to 10-week-old NOD/SCID mice express MHC class II at the same low frequency as beta cells from 3-week-old NOD mice.

Conclusion/interpretation. NOD beta cells express I-A^{g7} and could be a direct target of autoreactive CD4+ T cells. This MHC class II expression is triggered by infiltrating lymphocytes. [Diabetologia (2003) 46: 1106–1114]

Keywords Beta cell, MHC class II, NOD mice, Type-1-diabetes mellitus, single-cell multiplex RT PCR.

IDDM is an autoimmune disorder characterised by the destruction of the insulin-producing beta cells in the islets of Langerhans of the pancreas. The reference model of the human disease is the NOD mouse that spontaneously develops IDDM in a multistep process [1]. Clearly, T cells are key players in the pathogene-

sis and both T cell subsets seem to be essential for the development of diabetes in this model [2, 3, 4, 5]. Beta cells constitutively express MHC class I molecules; their expression is up-regulated upon exposure to IFN- γ and TNF- α [6]. Therefore, autoreactive CD8+ T cells can directly recognise beta cells.

A series of publications also provided evidence that beta cells can be induced to express MHC class II molecules [6, 7, 8, 9, 10, 11, 12, 13]. However, since it has been reported that MHC class II molecules in NOD islets are expressed exclusively by CD45+ cells [14], that is, cells of haematopoietic origin, it is generally accepted that NOD beta cells are I-A^{g7}-negative and therefore cannot directly be recognised by self-reactive CD4+ T cells [15]. This idea has recently been challenged by a clear-cut demonstration that an I-A^{g7}-derived peptide is an autoantigen in the NOD mouse [16].

Received: 21 January 2003 / Revised: 19 March 2003

Published online: 10 July 2003

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Corresponding author: Dr. U. Walter, German Diabetes Research Institute, University of Duesseldorf, Auf'm Hennekamp 65, 40225 Duesseldorf, Germany

E-mail: Ulrich.Walter@med.uni-duesseldorf.de

Abbreviations: APC, antigen presenting cell; DC, dendritic cell; IDDM, insulin-dependent diabetes mellitus; Ii, invariant chain; NOD, non obese diabetic; PPI, preproinsulin; SCID, severe combined immunodeficiency.

In this study, we readdressed the question of I-A^{g7} expression by pancreatic beta cells. We found the frequency of MHC class II expression on NOD beta cells to be drastically increased at the mRNA level during progression to overt diabetes and MHC class II protein was clearly detected intracellularly. Moreover, our data show that MHC class II expression in beta cells is triggered by infiltrating lymphocytes.

Materials and methods

Preparation of islets and islet cells. Individual pancreata from female NOD and NOD/SCID mice (principles of laboratory animal care were followed) were cut with scissors in PBS, 5% FCS, 1% glucose and digested with 5 mg/ml collagenase (Roche Diagnostics, Mannheim, Germany) in PBS, 15% FCS, 1% glucose for 4 to 5 min at 37°C. After three washing steps with ice-cold PBS, 5% FCS, 1% glucose, islets were harvested under a microscope by aspiration into a drawn and siliconised Pasteur pipette, reseeded into an intermediate chamber and aspirated once again. Islets were then preincubated for 10 min at 30°C in buffered Earle's medium without Ca²⁺ (EH, pH 7.3) containing 1 mmol/l EGTA (Sigma, Steinheim, Germany) and digested by incubation with 25 mg/ml trypsin (Roche) for 10 min at 30°C in the same medium. In some experiments, dissociation of islet-cells was achieved using a PBS-based, enzyme-free cell dissociation buffer (Invitrogen, Karlsruhe, Germany) under the same conditions. During preincubation and trypsinisation, the suspension was aspirated twice per min into a siliconised Pasteur pipette. After washing in EH, 1 mmol/l EGTA, single cells were handpicked with a drawn capillary using an intermediate chamber under a microscope. For multiplex RT-PCR, the picked cells were transferred to PCR tubes containing 4 µl DEPC (Sigma)-treated PBS (pH 7.2) and stored at -80°C until use.

Immunofluorescence staining of pancreatic islet cells. Prior to all steps of the staining procedure, the cell surface and intracellular structures were saturated with PBS, 2% human serum and protein blocking reagent (Beckman Coulter, Unterschleissheim-Lohhof, Germany) for 30 min each. Single cells were indirectly stained for 30 min on ice for surface MHC class II expression with the monoclonal antibody 10.2-16 (reported to cross-react with I-A^{g7} [17]) (kindly provided by S. z. Lage) or the control antibody 14.4.4.S (anti-mouse I-E^k, BD Biosciences, Heidelberg, Germany) which does not cross-react with

NOD MHC class II ([3] and own observations). After washing, cells were incubated with biotinylated R2-40 (anti-mouse IgG 2a/2b, BD Biosciences) for 30 min. After another washing step, they were stained with Cy5 labelled streptavidin (Dianova, Hamburg, Germany). For intracellular staining, cells were fixed with PBS, 3% formaldehyde for 30 min and permeabilised with PBS, 0.2% Triton-X100 for 1 min. Intracellular MHC class II molecules were detected with the same specific monoclonal antibodies mentioned above using Texas red-labelled streptavidin (Dianova) as the fluorochrome. Staining for intracellular insulin was carried out by indirect immunofluorescence testing with guinea pig anti-insulin antibody (DAKO, Hamburg, Germany), followed by a fluorescein-labelled anti-guinea pig IgG (Vector Laboratories, Burlingame, Calif., USA). All antibodies and fluorochromes were incubated for 30 min. After washing in PBS for 2×10 min, nuclear DNA was stained with DAPI (Roche) for 5 min in a final step. For confocal microscopy cells were embedded in mounting medium (NeoMount, MERCK, Darmstadt, Germany). Only cells with an intact nucleus displaying insulin-positive granules were considered to be intact pancreatic beta cells and taken into consideration.

Confocal microscopy. For the analysis of fluorescence intensities, cells were examined by fluorescence microscopy using a dual-laser scanning confocal imaging system (MRC-1024UV, BioRad, Munich, Germany) equipped with filter set T1/E2 for detection of DAPI/FITC/Texas red/Cy5, an argon ion UV laser (351 nm, 363 nm, 488 nm, and 514 nm) and a krypton/argon laser (488 nm, 568 nm, and 647 nm). Transmission images were generated with blue light (488 nm). The images were collected with a PlanNeofluar 63x (oil)—NA1.25. To avoid spectral overlapping in multicolour staining, each fluorescence dye was selectively excited, and each fluorescence was separately detected in photon-counting mode.

Single-cell multiplex RT-PCR. Single cells were thawed by incubation at 65°C for 2 min. After "chilling-out" on ice, 10 µl of RT mix were added and the tubes incubated at 37°C for 1 h followed by inactivation of the enzyme for 15 min at 70°C. Final concentrations were 1×RT buffer (Thermo Hybaid, Ashford, UK), 1 µmol/l Oligo(dT) primers (Invitrogen, Karlsruhe, Germany), 0.5 µmol/l dNTP (Amersham, Freiburg, Germany), 0.01 mol/l dithiothreitol (Invitrogen), 40 U RNase inhibitor (RNasin, Promega, Mannheim, Germany), 100 U reverse transcriptase (TrueScript, Thermo Hybaid) and distilled DEPC-treated water. Subsequently, cDNA was amplified by a fully nested two-step-PCR. In principle, samples were assayed for preproinsulin (PPI) mRNA expression in order to clearly dis-

Table 1. I-A^{g7} mRNA expression in single beta cells from individual NOD mice

	3 weeks		6 weeks		9 weeks		11 weeks		Diabetic	
	<i>n</i>	(%)	<i>n</i>	(%)	<i>n</i>	(%)	<i>n</i>	(%)	<i>n</i>	(%)
1	0/10*	(0)	9/27	(33)	7/15	(47)	21/26*	(81)	11/15*	(73)
2	0/20*	(0)	5/11	(45)	9/18*	(50)	6/13*	(46)	11/12*	(92)
3	2/13	(15)			13/26	(50)			2/4*	(50)
Total	2/43	(5)	14/38	(37)	29/59	(49)	27/39	(69)	24/31	(77)

MHC class II mRNA expression in single beta cells from individual NOD mice at different stages of diabetogenesis. Single handpicked islet cells were analysed for the expression of preproinsulin (PPI), and PPI+ cells then for the expression of I-A^{g7}.

In the indicated (*) experiments, single cells were first tested for CD45 expression to exclude contamination by haematopoietic cells. CD45- cells were further analysed for the expression of PPI, and CD45-PPI+ cells then tested for MHC class II

Table 2. Primer sequences for first and second RT PCR

Gene		Sequence	Size (bp)
Preproinsulin	1 st PCR	CCA CCA GCC CTA AGT GAT CC TTT ATT CAT TGC AGA GGG GTA GG	434
	2 nd PCR	CAG CAA GCA GGT TAT TGT TTC A GCT GGG TAG TGG TGG GTC TA	374
CD45	1 st PCR	TGC ATC CAT CCT CGT CCA CT GGG CTC GCA GAA CCA TTG	411
	2 nd PCR	GGA TCC CAG CAG ACA GG TGG GCT TTA TTC AGA GGA C	299
MHC class II	1 st PCR	GAT CCC CAG CCT CCT CCT CTC GCC CCC GAT GCC GCT CAA C	681
	2 nd PCR	GCG GGC CGA GCT GGA CAC G CGC ACT TTG ATC TTG GCT GGG TAG	179
Invariant chain	1 st PCR	GTG CGA CGA GAA CGG TAA CTA TTT GGG GGT GGC AGG ATG TGA G	532
	2 nd PCR	CGC GGG CGC CAT AAC TG GAG CCC CAC ATT CAA GAC TAC TAA	350

All primers are given in 5'-3' direction

tinguish beta cells from non-beta-cells. In most cases (Table 1), an additional RT PCR for CD45 was performed and PPI, I-A^{g7} and eventually Ii mRNA expression was only assayed by single-cell multiplex RT PCR after the sample was tested negative for amplification with CD45 primers. Thus, haematopoietic cells were excluded from further studies. To this end, 13 µl of RT product were used as template for a first round of PCR in a final volume of 85 µl. In this PCR, the external primers for all the genes of interest (CD 45, MHC II, Ii) were included. Remaining aliquots of 2 µl of RT product were used for detection of PPI mRNA in a first round of PCR with a total volume of 40 µl. Final concentrations were as follows: 1×PCR buffer (Perkin Elmer, Brauchburg, N.J., USA), 0.8 mmol/l dNTP (Perkin Elmer), 25 pmol of each primer (MWG, Ebersberg, Germany), 3 U AmpliTaqDNA polymerase (Perkin Elmer) and purified water. PCR was carried out as a touchdown PCR: after an initial denaturation step (1 min at 94°C), during five cycles the annealing temperature was lowered by 1°C per cycle from 61°C to 56°C, followed by 28 cycles with stable annealing temperature of 56°C. Each cycle consisted of 45 s at 94°C, 45 s at annealing temperature and 1 min at 72°C. PCR was finished by a final elongation of 10 min at 72°C. For each gene of interest, 2 µl aliquots of the product of this first round of PCR were used as template in a second, nested PCR containing the internal primers of only one of the genes of interest in a final volume of 20 µl reaction mix (1×PCR buffer, 0.8 mmol/l dNTP, 25 pmol each of sense and antisense primer, 1 U AmpliTaqDNA polymerase). The cycling conditions were the same as above. All primer pairs (Table 2) span at least one intron. PCR products were separated on a 2% agarose gel stained with 0.5 mg/ml ethidium bromide and visualised on an UV screen. Nested PCR is a method which is prone to contamination problems. Therefore, all experiments included RT minus negative controls done in parallel.

Results

Efficiency of single-cell multiplex RT PCR. As the significance of data obtained with single-cell multiplex

RT PCR is dependent on the efficiency of this method, we determined the efficiency by analysing expression of MHC class II, CD45 and Ii in A20 B cell lymphoma cells. From 22 cells tested, 100% were triple positive (Fig. 1).

Increased frequency of I-A^{g7} mRNA-expressing beta cells during progression to overt diabetes. I-A^{g7} mRNA expression by pancreatic beta cells was tested using single-cell multiplex RT PCR. This approach is suited to assess I-A^{g7} mRNA expression quantitatively, that is, as a percentage of I-A^{g7}-positive beta cells. Female NOD mice of different ages were analysed in order to get an impression about the kinetics of MHC class II mRNA expression during the progression of the disease. The frequency of I-A^{g7} mRNA expression by pancreatic beta cells was strongly increased during the course of the disease (Table 1). At 3 weeks of age (no insulinitis), beta cells expressed I-A^{g7} mRNA at very low frequency (5%). At 6 weeks of age (periinsulinitis), expression of MHC class II was upregulated (37% I-A^{g7}+). In mice of 9 and 11 weeks of age (invasive insulinitis) 49% and 69% of analysed beta cells were MHC class II-positive, respectively. Finally, in mice with overt diabetes, as many as 77% of beta cells scored positive for I-A^{g7} mRNA.

Low frequency of MHC class II mRNA-expressing beta cells in NOD/SCID mice. The increased frequency of MHC class II mRNA could be a developmental event due to ageing. Alternatively, it could be a consequence of lymphocytic infiltration and thus correlate with disease progression. To discriminate between these possibilities, we tested beta cells from 9- to 10-week-old NOD/SCID mice for the expression of I-A^{g7} mRNA at the single-cell level. Islets of Langerhans from

Fig. 1. Determination of single-cell multiplex RT PCR efficiency with handpicked A20 murine lymphoma B cell line cells. Single cells were analysed for co-expression of MHC class II, CD45, and Ii. 1: negative control—H₂O only; 2: negative control—RT minus; 3: Positive control from bulk cDNA; 4–25: single cells

Table 3. I-A^{g7} mRNA expression in single beta cells from individual NOD/SCID mice

	<i>n</i>	(%)	
1	2/29	(7)	9 weeks of age
2	1/13	(8)	10 weeks of age
Total	3/42	(7)	

MHC class II mRNA expression in single beta cells from individual NOD/SCID mice. Single handpicked islet cells were first tested for CD45 expression to exclude contamination by haematopoietic cells. CD45- cells were further analysed for the expression of PPI, and CD45-PPI+ cells then tested for MHC class II. In addition, none of the MHC class II+ cells expressed the invariant chain

NOD/SCID mice are characterised by the absence of any lymphocytic infiltration. Beta cells from these mice express MHC class II mRNA at very low frequency (7%), comparable to that observed for beta cells from NOD mice of 3 weeks of age (5%) (Table 3).

Expression of I-A^{g7} protein. To study MHC class II protein expression by beta cells, single pancreatic cells from 11-week-old pre-diabetic NOD mice were analysed by immunofluorescence. Beta cells (green: insulin-containing secretory granules) from 11-week-old NOD mice express MHC class II molecules within the cell (red: intracellular I-A^{g7}) (Fig. 2). Cell surface

expression could not be detected under these conditions (blue: surface I-A^{g7}). Clearly, the failure to detect surface I-A^{g7} was not due to digestion of these molecules, as experiments replacing the trypsin-containing buffer by a PBS-based, enzyme-free cell dissociation buffer had the same outcome (not shown). Staining with the isotype control antibody 14.4.4.S was always negative on insulin-positive cells (not shown), showing that the 10.2–16 antibody-positive staining of beta cells from NOD mice was indeed specific.

In order to exclude the possibility that PPI+I-A^{g7}+ cells could have been macrophages that ingested parts of or an entire apoptotic beta cell or insulin secretory granules released thereof, single islet cells from 11-week-old NOD mice were double-stained with an insulin- and a macrophage-specific antibody (F4/80, kindly provided by S. z. Lage). We found positive staining for these molecules to be mutually exclusive (not shown). In concert with the lack of CD45 expression determined by RT PCR, it can be excluded that the suggested expression of I-A^{g7} by pancreatic beta cells of NOD mice is due to contaminating haematopoietic cells.

Expression of invariant chain by beta cells. We were surprised to find I-A^{g7} to be expressed intracellularly, but not measurably on the surface. This scenario resembles the phenotype of antigen-presenting cells (APC) in Ii-knock out mice in which MHC class II molecules were shown to be retained within the cell, resulting in strongly reduced surface expression. To exclude an impaired Ii expression in beta cells of NOD mice, we tested co-expression of PPI, I-A^{g7}, CD45 and Ii mRNA in single pancreatic beta cells from 11-week-old animals. Of MHC class II mRNA-

Fig. 2a–h. Protein expression of MHC class II by pancreatic beta cells of NOD mice. Confocal microscopy of insulin and MHC class II co-expression by a single pancreatic beta cell (a–d) and by a beta cell and an infiltrating MHC class II-positive APC (e–h) with single-cell immunofluorescence of cells

of two different NOD mice (11 weeks of age). (a,e) composite picture; (b,f) staining of intracellular insulin (green) and surface I-A^{g7}(blue); (c,g) staining of intracellular and surface MHC class II (red); (d,h) transmission picture

Fig. 3. Co-expression of MHC class II and invariant chain in CD45-, PPI+ handpicked single cells derived from pancreatic Langerhans islets from an 11-week-old NOD mouse. Data of a second experiment are given in parenthesis. The bands in lanes 1, 6, 8 and 13, last photograph, represent the 978 bp-amplicon of genomic Ii DNA. 1: RT minus; 2: Positive control from bulk cDNA; 4–15(20): Single cells

positive beta cells (PPI+I-A^{g7}+CD45-) 67% co-expressed Ii (Fig. 3). Thus, the failure to detect surface expression of I-A^{g7} was unlikely to be due to a lack of Ii expression in pancreatic beta cells of NOD mice.

Discussion

Beta cells are destroyed selectively during the development of diabetes and self-reactive CD4+ T cells are

essential for IDDM. Moreover, the disease can be transferred to NOD/SCID mice by CD4⁺ T-cell clones in the total absence of CD8⁺ cells [18, 19, 20]. These important observations strongly suggest that CD4⁺ T cells are capable of destroying beta cells in an antigen-specific manner. Indeed, several reports propose that the beta cell itself could be capable of presenting antigens in the context of MHC class II molecules. MHC class II expression on beta cells has been shown to be inducible in vitro by IFN- γ and TNF- α in normal mouse islet beta cells [6, 11], on cultured human islets [7] and by IFN- γ alone on beta cells from diabetes-prone BB rats [12]. Moreover, beta cells from the pancreas of patients with recent-onset Type-1-diabetes [9, 13] as well as from diabetes-prone BB rats [10] express MHC class II molecules. It has also been shown that I-A^{g7} itself is an autoantigen in IDDM in the NOD mouse [16]. Hence, MHC class II molecules should be expressed by beta cells. However, these findings notwithstanding, the failure to detect surface MHC class II molecules on NOD beta cells [14, 21] had led to the generally accepted notion that NOD beta cells do not express MHC class II and, therefore, could not be a direct target of CD4⁺ T cells.

The study revisited this question by analysing pancreatic beta cells by single-cell multiplex RT PCR and single-cell immunofluorescence. This is the first report to show that NOD beta cells express I-A^{g7} mRNA and the corresponding protein. On the mRNA level, the frequency of MHC class II-expressing beta cells was found to be drastically increased during progression to overt diabetes. While beta cells from 3-week-old NOD mice expressed negligible levels of MHC class II mRNA, 37% of beta cells were already I-A^{g7} mRNA-positive in mice displaying periinsulinitis. When invasive insulinitis became manifest, 49% and 69% of beta cells expressed MHC class II mRNA in NOD mice aged 9 and 11 weeks, respectively. Finally, in diabetic animals, as many as 77% of beta cells were I-A^{g7} mRNA-positive. This up-regulation of kinetics during diabetogenesis resembled that of kinetics of Fas and TNFR2 mRNA up-regulation [22] inasmuch as the most important transition is from 3 to 6 weeks of age—the transition from the absence of any infiltration to periinsulinitis.

It is likely that the expression of I-A^{g7} mRNA in beta cells is induced by TNF- α and IFN- γ released by infiltrating cells. This is based on the following observations: (i) induction of MHC class II expression (as well as expression of Fas and TNFR2) comes along with the occurrence of insulinitis; (ii) in 9- to 10-week-old NOD/SCID mice, the islets of which are free of any lymphocytic infiltration, beta cells express MHC class II mRNA at the same low frequency as beta cells from insulinitis-free islets of NOD mice of 3 weeks of age (excluding the possibility that MHC class II expression is a developmental event); (iii) TNF- α and IFN- γ have been shown to be expressed by infiltrating

cells [23]; (iv) these cytokines have been shown to be able to trigger expression of MHC class II on beta cells in vitro [6, 11]. In the same way TNFR2 and Fas could be triggered by these cytokines, possibly in concert with IL-1 (also known to be expressed by islet-infiltrating cells early in pathogenesis [23]) in the case of Fas.

It has been reported that NOD/SCID islets, although free of infiltrating lymphocytes, are indeed infiltrated by macrophages and dendritic cells (DC) [24]. Therefore, our observation that MHC class II mRNA is expressed in 9- to 10-week-old NOD/SCID mice at the same low frequency as in 3-week-old NOD mice strongly suggests that induction of I-A^{g7} is caused by the lymphocytic fraction of infiltrating cells, but not by infiltrating macrophages or DCs.

These findings underline the impact the lymphocytic infiltrate already has on pancreatic beta cells at early stages of the disease. Although still free of invasive infiltration, pancreatic beta cells undergo important changes in receptor expression that is presumably triggered “at distance” by cytokines released by the infiltrating cells surrounding the beta-cell mass.

Recently, a peptide derived from the I-A^{g7} beta-chain has been shown to be an autoantigen in NOD IDDM. T cells from diabetic but not from pre-diabetic young NOD mice proliferated in response to this peptide. Moreover, immunisation with this peptide protects NOD mice from diabetes (as immunisation with other autoantigens does [25, 26]) by inducing regulatory T cells of the TH2 phenotype which are capable of preventing adoptively transferred diabetes [16]. These findings conflict with the idea that NOD beta cells are MHC class II-negative. By showing intracellular expression of MHC class II molecules we have solved this contradiction.

However, we could not prove a significant surface expression of I-A^{g7} in non-diabetic NOD mice. The staining pattern of beta cells resembled those of APC from Ii-knockout mice. APC of these animals have been reported to show drastically reduced levels of surface MHC class II molecules that accumulated intracellularly [27]. The rather surprising expression pattern of I-A^{g7} prompted us to determine if beta cells co-express Ii. As 67% of PPI and I-A^{g7}-double-positive cells from 11-week-old NOD mice co-expressed Ii, it is unlikely that the failure to detect I-A^{g7} at the surface is caused by a general lack of expression of Ii. Retention of MHC class II molecules is also a naturally occurring phenomenon that can be observed in immature DCs, in which de novo synthesised MHC class II molecules are retained in lysosomal compartments [28]. Probably, this retention has to be ascribed to the expression of cystatin C, a protease inhibitor which prevents the degradation of the Ii by cathepsin S and thereby the elimination of the lysosomal retention signal present in the cytosolic domain of Ii [29]. Whatever the reason for the retention of I-A^{g7} in the cyto-

plasm of a NOD beta cell is, we would tend to speculate that this retention is leaky. In both, the Ii- and the cathepsin S-deficient mouse, surface expression of MHC class II is reduced but still present. Residual I-A^{g7} surface expression on NOD beta cells might be indistinguishable from isotype control owing to the relatively high background, which is a result of using a primary antibody produced from cells of the same species as the corresponding antigen on the one hand, and a probable Fcγ receptor expression by beta cells on the other (MHC class II and Fcγ receptors are mostly co-regulated). Clearly, the failure to detect surface I-A^{g7} was not due to digestion of these molecules, as experiments replacing the trypsin-containing buffer by a PBS-based, enzyme-free cell dissociation buffer had the same outcome.

Our findings agree with the observation that MHC class II molecules could not be detected on NOD-derived insulinoma NIT-1 cells after stimulation with IFN-γ, despite demonstration of IFN-γ-induced Aα, Aβ, and Ii RNA transcripts [30]. Interestingly, on MIN6N8 cells, another NOD-derived insulinoma, MHC class II surface expression has been shown to be inducible under the same conditions, although to a low extent [31]. Taken together, these findings suggest that NOD beta cells might be generally competent to express I-A^{g7} on the surface, but that the level of expression upon stimulation is very low (perhaps in MIN6N8 cells just passing the detection threshold, in contrast to NIT-1 and NOD beta cells).

If beta cells really do express I-A^{g7} on the cell surface, this expression is likely to have important consequences for the development of diabetes. As T cells are likely to be more sensitive as a read-out system than immunofluorescence suffering from high background, beta cells would then be a direct target of self-reactive CD4+ T cells, which are known to be essential in the development of IDDM. This direct interaction between autoaggressive CD4+ T cells and beta cells might be a critical event in the multistep progression to overt diabetes.

With the onset of periinsulinitis, beta-cell-antigen-specific T cells become detectable [25, 32,33]. These islet-specific T cells are of TH1 phenotype and show signs of activation [33, 34]. Therefore, it seems that these T cells encounter their cognate antigen, get primed, expand clonally and acquire a TH1-phenotype as soon as periinsulinitis develops. During this early period of periinsulinitis, it is likely that these T cells encounter their cognate antigen(s) via cross-presentation [35]. This cross-presentation, however, does not trigger destruction of beta cells in NOD mice. Indeed, insulinitis and beta-cell destruction are clearly uncoupled in the sense that periinsulinitis is a necessary, but by no means sufficient prerequisite of autoimmune beta-cell destruction. Male NOD mice, for example, show a stable periinsulinitis that rarely progresses to IDDM. This fact is in line with reports showing that cross-

presentation of peripheral antigens in general, and of antigens expressed as a transgene on beta cells in particular [36, 37, 38, 39], could preferentially lead to the induction of tolerance rather than to autoimmunity. The parameters driving the decision between tolerance and immunity, that is, the transition from harmless insulinitis to beta-cell killing, are still poorly understood. However, it seems likely that an I-A^{g7}-mediated antigen presentation on beta cells provides a context (such as expression of adhesion and co-stimulatory molecules, density of MHC class II / peptide complexes, local concentration of lymphokines etc.) substantially different from that provided by a professional APC in the pancreatic lymph node. This difference could account for the bias towards autoimmunity—self-reactive T cells kept in a tolerant state by APC that cross-present the corresponding peripheral autoantigen in the pancreatic lymph nodes encounter their cognate antigen presented by a beta cell in the islet of Langerhans, and switch from a tolerant to a “violent” state.

Different groups have provided evidence that beta-cell destruction can occur in the complete absence of antigen-specific contact between T cells and pancreatic beta cells [40, 41, 42]. Nevertheless, the experimental approaches and models used in those reports do not rule out a direct presentation of autoantigens to self-reactive CD4+ T cells by the beta cells themselves as an essential step in the pathogenesis of diabetes in humans and NOD mice. For example, the observation of the Mathis group that transferred diabetogenic CD4+ T cells proliferated and got activated in the pancreatic lymph nodes before infiltrating the islets [35] only rules out an essential contribution of islet-resident APC to the presentation of autoantigens within the islets. As Cα⁰/NOD recipients do not have insulinitis before the transfer, beta cells are not triggered to express MHC class II, and are therefore incapable of presenting self-antigens to CD4+ T cells.

The direct presentation to and subsequent activation of self-reactive CD4+ T cells could induce these cells to release chemokines, thus attracting other infiltrating leukocytes to the islet. Additionally, self-reactive CD4+ T cells might also be triggered to release cytokines *in situ*, thus changing the local environment with all its possible consequences. These changes might also affect the compartment of self-reactive CD8+ T cells. Several reports show that CD 4+ T cell help is necessary to avoid CD8+ T cell tolerance induction [43, 44, 45]. Interestingly, this is also true in a transgenic model of IDDM where co-transfer of auto-antigen-specific CD4+ T cells was necessary to inhibit tolerance induction to an antigen expressed by beta cells by deletion of autoreactive CD8+ T cells, thereby favouring autoimmunity [46].

Direct interaction between self-reactive CD4+ T cells and MHC class II-expressing beta cells could also allow these T cells to kill their target. This primary insult might lead to the release of larger amounts of

autoantigen(s). Another study was able to show that the level of antigen expressed by peripheral tissues must be relatively high to facilitate cross-presentation to naive CD8+ T cells. Below this threshold, peripheral antigens did not stimulate by cross-presentation and were ignored by naive CD8+ T cells [47]. Thus, the release of greater amounts of beta cell antigens could allow for an efficient cross-presentation of this antigen to self-reactive CD8+ T cells. Subsequently, these cells become fully competent "beta-cell killers".

Acknowledgements. The authors are indebted to J.-F. Bach for generously providing NOD mice, P. Gatzlaff and S. z. Lage for excellent technical assistance, A. Sechi for advice on cell-staining, S. Amigorena for helpful discussions and U. Brunk for kindly providing the NOD insulinoma NIT-1. We kindly thank B. Rocha, A. Lehen, and H. Kolb for critical review of the manuscript. This work was supported by grants from the Juvenile Diabetes Foundation, the Koerber Foundation, the Deutsche Forschungsgemeinschaft and by the Deutsche Krebs-hilfe.

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