Quantification and Three-Dimensional Imaging of the Insulitis-Induced Destruction of β-Cells in Murine Type 1 Diabetes

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OBJECTIVE—The aim of this study was to refine the information regarding the quantitative and spatial dynamics of infiltrating lymphocytes and remaining β-cell volume during the progression of type 1 diabetes in the nonobese diabetic (NOD) mouse model of the disease.

RESEARCH DESIGN AND METHODS—Using an ex vivo technique, optical projection tomography (OPT), we quantified and assessed the three-dimensional spatial development and progression of insulitis and β-cell destruction in pancreata from diabetes-prone NOD and non–diabetes-prone congenic NOD.H-2b mice between 3 and 16 weeks of age.

RESULTS—Together with results showing the spatial dynamics of the insulitis process, we provide data of β-cell volume distributions down to the level of the individual islets and throughout the pancreas during the development and progression of type 1 diabetes. Our data provide evidence for a compensatory growth potential of the larger insulin-producing islets during the later stages of the disease around the time point for development of clinical diabetes. This is in contrast to smaller islets, which appear less resistant to the autoimmune attack. We also provide new information on the spatial dynamics of the insulitis process itself, including its apparently random distribution at onset, the local variations during its further development, and the formation of structures resembling tertiary lymphoid organs at later phases of insulitis progression.

CONCLUSIONS—Our data provide a powerful tool for phenotypic analysis of genetic and environmental effects on type 1 diabetes etiology as well as for evaluating the potential effect of therapeutic regimes.

Diabetes 59:1756–1764, 2010

Type 1 diabetes is an autoimmune disorder resulting from the destruction of insulin-producing β-cells by an autoreactive immune response involving CD4+ and CD8+ T-cells as well as other leukocyte subsets. Our present understanding of the natural history of type 1 diabetes (1) depends, to a large extent, on analysis of rodent models of the disease, like the nonobese diabetic (NOD) mouse (2). In NOD mice, the development of clinical diabetes is preceded by an inflammation of the pancreatic islets. It is generally thought that an initial event in triggering the development of insulitis and β-cell destruction is the processing of β-cell antigens by macrophages and dendritic cells residing in the pancreatic islets (3). The antigen-presenting cells are then drained to the pancreatic lymph nodes where they will present the antigen to autoreactive T-cells. In the absence of proper peripheral tolerance mechanisms, this leads to activation and insufficiently controlled expansion of these T-cell clones and eventually to their migration back to the pancreatic islets, where they mediate β-cell destruction (4). While it is well established that the insulitis progresses over an extended time period, detailed information on the quantification of spatial dynamics is largely lacking, in part, due to limitations in existing technology.

Recently, we have developed an optical projection tomography (OPT)-based approach (5), allowing for ex vivo, global evaluation of molecularly labeled pancreatic constituents (e.g., insulin-producing islet cells or infiltrating CD3+ T-cells) (6,7). This provides a method for direct quantification and three-dimensional–spatial assessment of both infiltrating lymphocytes and remaining β-cell mass during the progression of type 1 diabetes in NOD mice. Using this approach to extract information of islet number, β-cell distribution, and volume down to the level of individual islets throughout the pancreas, we provide a detailed account of the kinetics and spatial extension of the insulitis and β-cell mass destruction process during the development and progression type 1 diabetes.

RESEARCH DESIGN AND METHODS

NOD. Both mice were originally obtained from Bomholtgaard (Ry, Denmark), while NOD.B10.H-2b (NOD.H-2b) mice were kindly provided by Dr. Linda Wicker, (Cambridge University, Cambridge, U.K.). Mice were bred and maintained in the animal facility at Umeå University. All animals were kept on normal diet, and the NOD mice were screened for diabetes by urine analysis for significant glucosuria once a week (BM-test Glucose; Boehringer Mannheim, Mannheim, Germany). Positive results were thereafter verified by daily urine analyses for a week. In our NOD colony, the frequency of diabetes reached 70% in females and 20% in males by 30 weeks of age. All experiments were performed in compliance with the relevant Swedish and institutional laws and guidelines.
**Organ preparations.** Pancreata from female NOD and NOD.H-2b mice were isolated, stained for insulin, and prepared for OPT scanning, as described previously (6). To minimize variation in the staining procedure, groups consisting of one pancreas at each time point were stained simultaneously. For practical reasons, the gastric and duodenal lobe of the pancreas was scanned as one body (referred to as duodenal) and the splenic lobe as the other. For studies of insulitis, the same protocols were applied with the addition of Rb anti-CD3 (C7930; Sigma) primary and Alexa 488 anti-Rb (Molecular Probes) secondary antibodies.

**OPT.** OPT scanning using the Bioptons 3001 OPT scanner (Bioptons), with exciter D560/40 and emitter D480/30 filter (Chroma) when visualizing Alexa 594 and 488, respectively, was performed as described previously (7). Generation of tomographic reconstructions and quantitations were performed as described previously (7). Iso-surface reconstructions were generated using the visualization software module for Volocity version 4.3.2 (Improvision). To correct for minute-zoom factor aberrations detected in the Bioptons 3001 OPT scanner, the OPT alignment pin (Bioptons) was measured with a digital caliper. This value was compared with digital measurements of the (same) scanned alignment pin using Skyscan Software (Skyscan). All measurements were made in triplicate. A correction factor for each magnification was calculated by dividing the value obtained with the caliper measurement by the

| Time  | Splenic  | Duodenal  |
|-------|----------|------------|
| 3w    | A–E      | A’–E’      |
| 6w    | F–J      | F’–J’      |
| 8w    | A–E      | A’–E’      |
| 12w   | F–J      | F’–J’      |
| 16w   | A–E      | A’–E’      |

**FIG. 1.** Islet β-cell distribution over time in NOD.H-2b and NOD mice. A–J: Isosurface rendered OPT images of representative NOD.H-2b splenic (A–E), duodenal (A’–E’), and NOD splenic (F–J) and duodenal (F’–J’) pancreata labeled for insulin (red). The pancreas outline (gray) is based on the signal from tissue autoflourescence. In contrast to NOD.H-2b mice (A–E’), the expected progressive destruction in islet β-cell volume is clearly observed in NOD mice (F–J’). Scale bar corresponds to 2.3 mm in F; 2.1 mm in C; 2 mm in D, D’, J, J’, and G; 1.9 mm in B and H; 1.8 mm in I and H’; 1.7 mm in C’ and E’; 1.6 mm in A, E, and F’; 1.5 mm in G; 1.4 mm in A’ and B’; and 1.2 mm in F. (A high-quality color representation of this figure is available in the online issue.)

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value obtained using the SkyScan software. Volume measurements were subsequently adjusted according to the calculated correction factors.

Statistical analyses. Different zoom factors were used during the OPT scanning to enable imaging of various-sized pancreata (a pancreas at 16 weeks is approximately four times larger than at 3 weeks). Hence, the smallest detectable object (highest resolution) varies between the largest and smallest pancreas analyzed. To normalize the smallest islet β-cell volume analyzed, between the time series, islet β-cell volumes smaller than the smallest detectable object using the lowest zoom factor were excluded from the statistical analyses. Therefore, only islet β-cell volumes >5,000 μm³ were analyzed. This volume corresponds to a spherical object with a diameter of ~21 μm. The excluded islets, even at the highest zoom factor, contributed only marginally to the overall islet number (~5% at 3 weeks) and volume (~9% at 3 weeks). Subsequent statistical analyses were performed using Excel 2007 software (Microsoft). Mann-Whitney U nonparametric (two-tailed) test in SPSS 16.0 (SPSS, Chicago, IL) was used to compare islet numbers and β-cell volumes, corresponding spatial coordinates, and tomographic scan data files can be accessed via Web link.

RESULTS

Pancreata from diabetes-prone NOD mice and non–diabetes-prone NOD.H-2b congenic mice were isolated at differ-
ent ages, ranging from 3 to 16 weeks; stained with anti-insulin antibodies; and subjected to OPT scanning. In this manner, the volume of insulin-producing β-cells in each individual islet could be directly quantified as well as spatially assessed. As illustrated in Fig. 1, the pancreata from NOD.H-2b mice displayed islets containing insulin-producing β-cells scattered over the whole organ, with a similar pattern observed at all ages studied (Fig. 1A–E). The global assessment of the three dimensional–spatial distribution also confirmed the previous notion (6,8) of an uneven distribution of differently sized islets, with a predominance of small islets in the periphery of the organ and larger islets mainly located centrally in proximity to the large blood vessels and pancreatic ducts (Figs. 1 and 2A–E). The total pancreas volume (Fig. 3A and D) increased over the whole period of observation, with the largest increase occurring between 3 and 8 weeks of age. A similar development was seen in both NOD and NOD.H-2b congenic mice, as illustrated by both strains displaying a similar total pancreas volume at 16 weeks of age (compare Fig. 3A and D). The total β-cell volume increased with similar kinetics (Fig. 3B and E). In contrast, the total number of islets (Fig. 3C and F) had reached adult levels of ~2,000 islets at only 3 weeks, agreeing with previous estimates using stereological techniques (9). These data suggest that the observed increase in β-cell volume between 3 and 8 weeks of age is mainly due to the expansion of already-existing islets rather than through de novo establishment.

In contrast to NOD.H-2b mice, the diabetes-prone NOD mice displayed an expected loss of insulin+ islets due to the autoimmune process, which became evident at ~8 weeks of age. Between 8 and 12 weeks of age, the average number of islets decreased by ~35%, and between 8 and 16 weeks of age the number of islets was reduced by >75% (Fig. 3C and F). Interestingly, the drop in islet number was not accompanied by a corresponding drop in total β-cell volume, which was only reduced by <20% between 8 and 12 weeks and to ~50% between 8 and 16 weeks of age (Fig. 3E). This discrepancy could, in part, be explained by the fact that smaller islets were preferentially lost in the earlier stages of the process (Fig. 1F–J). To confirm this notion, we classified all individual islets into size categories arbitrarily chosen to represent “large” (>5 × 10⁶ μm³), “intermediate” (1–5 × 10⁶ μm³), and “small” (<1 × 10⁶ μm³) islets (Fig. 2) and determined at each time point, the number of islets in each size category and the mean islet β-cell volume for each of these categories. As illustrated in Fig. 2F, the maximum number of islets in each size category was established at 3 weeks for the smallest category and by 6

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**FIG. 3.** A and D: Graphs showing the average whole-pancreas volume (duodenal and splenic) at 3, 8, and 16 weeks and in NOD.H-2b mice (A) and in NOD mice (D) at 3, 6, 8, 12, and 16 weeks. B and E: Average total pancreatic β-cell volume in NOD.H2-b (B) and NOD (E) mice. C and F: Average islet number in NOD.H2-b (C) and NOD (F) mice. n = 5 for NOD at 3, 6, 8, and 16 weeks, n = 4 for NOD at 12 weeks, and n = 3 for NOD.H-2b at 3, 8, and 16 weeks. Values are given ± SE. Significance levels for the decay of β-cell volume and number of islets are indicated. *P < 0.05; **P < 0.01.
weeks for the larger categories. Between 8 and 12 weeks, the number of small islets significantly decreased (39%, \(P < 0.05\)), and between 8 and 16 weeks of age, this reduction was even more prominent (82%, \(P < 0.01\)). In contrast, the number of intermediate and large islets remained comparatively constant between 8 and 12 weeks. After this point, a clear reduction in the number of intermediate-sized islets was observed at 16 weeks (77% reduction when compared with 8 weeks, \(P < 0.01\)), while the number of the largest islets, even at this point, only decreased by 28% when compared with those at 8 weeks. Together, these data reveal that the smallest, peripherally located islets are the first to be destroyed during insulitis.

The time of onset for diabetes in the NOD mouse is known to display a large individual variation. This is also reflected in the variation in \(\beta\)-cell volume of individual animals. As illustrated in Fig. 2G, the total \(\beta\)-cell volume is kept relatively stable in all animals between 6 and 12 weeks of age, with the exception of one 12-week-old mouse in which a clear reduction was evident. In contrast, four of five 16-week-old mice displayed a similar reduction in \(\beta\)-cell volume, while one mouse appeared relatively unaffected. Diabetes has been suggested to induce \(\beta\)-cell regeneration either through recruitment of progenitor cells from the ductal epithelium or through proliferation of mature insulin-producing \(\beta\)-cells (10–12). It is therefore possible that in addition to a preferential loss of smaller islets, the delay in \(\beta\)-cell volume reduction when compared with the reduction of islet numbers could be a result of \(\beta\)-cell regeneration.

We next estimated the threshold of total \(\beta\)-cell volume required to withstand the development of overt diabetes (Fig. 5). Five sibling pairs of NOD origin were analyzed at the point when one of the individuals (in each litter) developed overt diabetes, while the other retained normal glucose levels. The diabetic mice displayed consistently lower total \(\beta\)-cell volume when compared with their nondiabetic littermates and confirms our previously reported estimates (6) of a threshold at \(\sim 2.7 \times 10^7 \mu m^3\), below which the mice developed clinical diabetes (Fig. 5C). When compared with 16-week-old NOD.H-2b mice, this corresponds to an \(\sim 80\%\) reduction in \(\beta\)-cell volume. These data suggest that the diabetic phenotype of NOD mice
develops at a threshold significantly lower than what has been previously reported by point-counting morphometry (13). It is also worth noting that the approximate threshold for development of clinical diabetes established in our study falls well within the estimated interval of a 80–95% reduction in $\beta$-cell mass for human type 1 diabetes (14).

We next compared the fluctuations in $\beta$-cell volume and islet numbers with the progression of autoimmunity in the NOD mice. Pancreata isolated at the same ages as analyzed above were stained with antibodies against insulin and CD3 to detect $\beta$-cells and infiltrating T-cells and were submitted to OPT analysis (Fig. 6). Using this method, we could assess the progression of infiltrating CD3$^+$ T-cells paralleling the progressive loss of $\beta$-cell mass. In agreement with previous observations (15), the first detectable CD3$^+$-infiltrating cells were already present in NOD pancreata at 3 weeks of age, progressively increasing in abundance over time. Surprisingly, the preferential deple- tion of peripherally located islets was not found to coincide with a preferential insulitis developing in these areas of the organ. Instead, the appearance of the first CD3$^+$ foci do not appear to occur in any preferred region of the organ; however, to what extent it follows different blood vessels or ducts has not been addressed here. A plausible explanation for this observation is that the destruction of the small islets in the periphery of the organ occurs more rapidly because they contain relatively few $\beta$-cells. As a consequence of this, the $\beta$-cell antigen disappears more rapidly and the infiltrating mononuclear cells are no longer retained at the location of the islet. Like unaffected islets, the islets displaying severe insulitis were surrounded by $\alpha$-cells. Thus, while the $\beta$-cells eventually were destroyed also in the larger islets affected by insulitis, no corresponding reduction in $\alpha$-cells was observed (Fig. 7).

At later stages, as the individual foci grew in size, the distribution was less evenly spread over the organ, with some areas of large accumulations of infiltrating cells present at the same time as other areas of the organ appearing to be totally unaffected by the inflammatory process. From ~8 to 12 weeks, larger accumulations of CD3$^+$ cells in direct association with islets became evident. These structures resembled tertiary lymphoid organs (TLOs), with formation of T- and B-cell areas and of high endothelial venules (Fig. 6F and G), previously reported to be associated with chronic inflammatory diseases (16) including in the NOD mouse (17).

**DISCUSSION**

Using a novel tomographic imaging approach to analyze the postweaning development of the pancreas in mice, we have provided detailed three-dimensional-spatial and quantitative data regarding the progression of autoimmune-induced insulitis and $\beta$-cell destruction in isolated pancre-
ata from the diabetes-prone NOD model three times daily.

While this model has been intensively studied using conventional immunohistological methodology, our approach, by enabling directly quantitative and three-dimensional–spatial analyses of complete intact organs, gives a more comprehensive picture including a set of novel insights that will aid in understanding the molecular and cellular basis of disease progression in this system.

The observed growth kinetics of the endocrine pancreas in normal mice is not surprising, nor is the progressive loss of β-cell volume observed in the diabetes-prone NOD mice after massive insulitis has been established. However, the kinetics of the decrease in islet numbers and loss of β-cell volume, together with the finding that the mean β-cell volume of the largest and most resistant islet actually increases, is noteworthy. This observation provides evidence of a regenerative process simultaneously underway with the autoimmune-induced destruction of β-cells. This has been previously suggested based on the detection of markers associated with β-cell development reoccurring during the autoimmune destruction of β-cells in type 1 diabetes (13). This is also in line with recently reported findings that abolishment of mature β-cells and insulin expression triggers the regeneration of β-cells mainly due to the expansion of already existing mature β-cells (11). Thus, while we cannot rule out the possibility that the observed expansion of the large islets in the late stage of insulitis could, in part, be the result of β-cell swelling, these findings support the hypothesis that two competing processes are simultaneously ongoing in the autoimmune type 1 diabetes pancreas, one of autoimmune destruction and the other of regeneration of β-cells. Understanding the basic mechanisms controlling these processes remains an important challenge, with potential implications for interfering and halting the destructive arm of autoimmune diabetes. The observed formation of TLOs in stages of advanced insulitis may provide a clue for understanding how this is controlled. The role of these structures, which have been reported to appear in various conditions of chronic inflammation (16), could be to sustain autoaggressive insulitis, tipping the balance in favor of degeneration rather than regeneration of the β-cells. Further insights into when and how these structures are formed in association with insulitis, together with a better understanding

FIG. 6. Spatial assessment of the progression of autoimmune insulitis in the NOD mouse. A–E: Isosurface rendered OPT images of representative pancreata (duodenal) from NOD mice at 3, 6, 8, 12, and 16 weeks. Ins+ islets (red) are reconstructed based on the signal from insulin-specific antibodies and infiltrating T-cells (green) based on the signal from CD3-specific antibodies. A’–E’, insets: High-magnification views corresponding to the enclosed boxes in A, D, and E, respectively. n = 3 for 3, 6, 8, and 12 weeks; n = 5 for 16 weeks. Ins, insulin. F and G: Sections of a pancreata from a 14-week-old female NOD mouse stained with DAPI, anti-CD3 (green), and anti-CD19 (red) (F) or anti-CD3 (green) and anti-MAdCAM-1 (red) (G). F: B-cell areas (arrow head) and T-cell areas (arrow) are indicated by arrow head. G: High endothelial venules (arrow head). The scale bar in E corresponds to 2 mm in E, 1.5 mm in C, 1.4 mm in B, 1.3 mm in D, and 1.0 mm in A. The scale bar in E’ corresponds to 1 mm in E’, E’, D’, D’, and 0.76 mm in A, A’. Scale bar in G corresponds to 100 μm in F and G. Scale bar in I corresponds to 100 μm in H and I. (A high-quality color representation of this figure is available in the online issue.)
of their functional contribution to the chronic inflammation, is much warranted and should shed light on how to interfere with the progression of insulitis and β-cell destruction. Another interesting observation is the unsynchronized spreading of insulitis into different parts of the organ. In view of the prevailing model for the onset and progression of insulitis, the observed coexistence of areas with full-blown insulitis adjacent to areas apparently unaffected by insulitis is remarkable and suggests that local factors may provide cues for the homing of activated lymphocytes back to the pancreas.

The quantitative and three dimensional–spatial characterization of the autoimmune progression, from the earliest stages of leukocyte infiltration into the endocrine pancreas to the complete destruction of the β-cell volume and development of diabetes, provides a novel and more detailed frame of the process that could be used for elucidating the effect of specific genetic factors (e.g., by comparing NOD mice with transgenic or congenic strains) and for efficient evaluation of various treatment protocols.

ACKNOWLEDGMENTS

This work was funded by grants from the Novo Nordisk Fonden, Swedish Diabetes Foundation (to D.H.), the Juvenile Diabetes Foundation, the Swedish Research Council, and the Kempe Foundation (to D.H. and U.A.).

No potential conflicts of interest relevant to this article were reported.

Dr. T. Edlund and I. Jones are acknowledged for helpful comments on the manuscript.

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