Ontogeny of islet cell antibodies, insulin autoantibodies and insulinitis in the non-obese diabetic mouse

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Summary. The predictive value of insulinitis, islet cell cytoplasmic antibodies and insulin autoantibodies for insulin-dependent diabetes was studied in young female non-obese diabetic mice. The ontogeny of the three markers was examined cross-sectionally at days 15, 25, 40 and 90 while islet cell antibodies and insulin autoantibodies were studied longitudinally from day 35 or day 144–168 until approximately day 250. Insulinitis was first observed at day 40 (50%) and subsequently at day 90 (70%). Islet cell antibodies and insulin autoantibodies were present at day 15 in 46% and 54% of the animals respectively. The rate of islet cell antibodies was slightly higher at day 25 (60%) than at day 40 (40%) and day 90 (54%) whereas antibodies to insulin were present in all samples from day 25–90. At day 40 and day 90 insulinitis and insulin autoantibodies were present together in 42% and 70% of the animals, respectively, while insulinitis and islet cell antibodies had a lower rate of concordance (17% and 42%, respectively; diabetes rate, 30%). The concordance rates for islet cell antibodies and insulin autoantibodies were 42% at day 40 and 54% at day 90. Concordance for all three markers was first observed at day 40 (17%) which increased to 38% at

day 90. In longitudinal studies, islet cell antibodies and insulin autoantibodies were often present together whether or not diabetes supervened. In the islet cell antibody procedure, immunoreactive cells were shown immunohistochemically to correspond with insulin and/or glucagon cells. However, this staining was not suppressible with insulin- or glucagon- absorbed sera, implying the presence of non-hormonal autoantigens. We conclude that the three markers investigated are expressed early after birth and well before clinical symptoms appear in this animal model. Both islet cell antibodies and insulin autoantibodies preceded insulinitis but the prevalence rate for each marker or their degree of concordance was different from the anticipated rate of diabetes in our colony. Consequently, the early expression of the three markers alone is not predictive of diabetes although concordance for the two, or all three markers may be of some value. However, no animal developed diabetes without the prior appearance of both islet cell antibodies and insulin autoantibodies.

Key words: NOD mice, insulinitis, islet cell antibodies, insulin autoantibodies, ontogeny, immunofluorescence.

The non-obese diabetic (NOD) mouse develops insulin-dependent diabetes spontaneously from approximately 120 days onward. The rate of diabetes depends on the particular colony but is always considerably higher in females than in males [1]. The histopathology of the pancreas includes insulinitis and B-cell loss relatively early after weaning [1, 2]. The severity of insulinitis shows good correlation with the degree of impairment of first phase insulin release in response to glucose perfusion in vitro [3]. Humoral antibody changes, such as the presence of islet cell cytoplasmic antibodies (ICA) and islet cell surface antibodies (ICSA) have also been detected in the sera of prediabetic NOD mice [4, 5]. In humans with Type 1 (insulin-dependent) diabetes it is known that such antibodies (ICA and ICSA) are usually present well before diagnosis and several recent

studies have now shown that antibodies directed against endogenous insulin (insulin autoantibodies (IAA)) also precede the disease [6]. In light of these findings, we were interested in studying possible markers of diabetes proneness (insulinitis, ICA and IAA) cross-sectionally and longitudinally in the NOD mouse to establish their timing of onset and assess their predictive value.

Materials and methods

NOD mice from an in-house strain held by the University of Ehime City, Japan, have a rate of 30% diabetes in females between the ages of 120 and 200 days. This was confirmed in 41 females from this strain housed and fed in New Zealand (13/41 diabetic between 119 and 241 days old). Diabetes was defined as the presence of persistent glycosuria over 3–4 days (Testape 3+).

NOD female mice were studied at day 15 ($n=13$), 25 ($n=8$), 40 ($n=12$) and 90 ($n=13$). None showed glycosuria at any stage. Animals were bled, killed and the pancreas removed and fixed in Bouin's fluid for 2 h. After embedding in paraffin, sections (3 μ m) were stained with haematoxylin and eosin and examined for the presence of lymphocytic infiltrates within the islet or in peri-insular areas. Lymphocytic cells within ducts or blood vessels close to the islet were not included.

Blood was also sampled at fortnightly intervals from NOD mice from either day 35 ($n=5$) or from day 144–168 ($n=9$) until the diagnosis of diabetes, or continued until approximately 250 days of age in non-diabetic animals. Pancreata were removed and stained as above.

All sera were stored at -20°C until analysis. Islet cell cytoplasmic antibodies (ICA) were detected by a previously reported indirect immunofluorescence procedure with minor modifications [4]. As substrate for ICA staining, normal pancreata obtained from 45–90 day outbred albino Swiss mice (Animal Resources Centre, Perth, Australia) were fixed in Bouin's fluid, processed and sectioned as described above. Sera from NOD and normal mice after diluting 1:1 with phosphate-buffered saline (PBS) were applied to 3 μ m sections and incubated at 37°C for 1 h. After washing in PBS for 20 min, sections were incubated with goat anti-mouse immunoglobulin (Ig) coupled to fluorescein isothiocyanate (FITC, South Pacific Immunological Laboratories (SPI), Palmerston North, New Zealand) at a dilution of 1:10 in PBS for 30 min at 37°C . Sections were then washed for 20 min, mounted with glycerol-PBS and viewed with a Nikon fluorescence microscope. Test sera with a fluorescence intensity of pancreatic islet cells greater than control sera were assigned positive. All positive sera were re-tested in the procedure described, before and after neutralisation with either rat and bovine insulin or porcine glucagon (Novo, Bagsvaerd, Denmark; 1 μ g hormone per μ l neat serum, 18 h at 4°C). The identity of the islet cell types showing positive staining in the ICA procedure was studied immunohistochemically by staining serial normal mouse pancreatic sections with NOD mouse serum (1:1) and with either guinea pig anti-insulin serum (1:80) or rabbit antiserum to glucagon (1:80), bovine pancreatic polypeptide (1:80) or somatostatin (1:40) as described previously [7]. The immunofluorescence was observed after incubating sections with the appropriate FITC-second antibody (goat anti-mouse Ig, SPI, sheep anti-guinea pig or anti-rabbit gamma globulin, Wellcome, Beckenham, GB).

In addition, sections were stained simultaneously with a mixture of NOD mouse serum and one of the hormone antisera. The resulting immunoreaction was studied by adding two of the corresponding fluorochrome-labelled second antibody to each section (goat anti-mouse Ig-FITC, SPI, goat anti-mouse Ig-rhodamine, Tago, Burlingame, Calif, USA, swine anti-rabbit gamma globulin-rhodamine-Dako, Glostrup, DK, sheep anti-rabbit or anti-guinea pig gamma globulin-FITC, Wellcome) at a dilution of 1:20.

Insulin autoantibodies in NOD mouse sera were determined by minor modifications of previously reported procedures [8, 9] in 96-well, flat-bottom micro-titre plates (Nunc, Kamstrup, DK). Wells were coated with 5 μ g porcine insulin (Novo) in 100 μ l PBS for 3 days at 4°C and then either used immediately or stored at -20°C . Prior to assay insulin-coated plates were washed ($3 \times$ with washing buffer: 0.05% v/v Tween, Sigma, St. Louis, Missouri, USA, in PBS) and blocked with 2% w/v gelatin (Biorad Laboratories, Richmond, Calif, USA) in PBS for 2 h at room temperature. After washing, wells were incubated with 50 μ l of NOD mouse sera (1:50) or normal mouse sera (1:50) for 2.5 h at room temperature. The diluent for serum and in the subsequent step was 0.1% w/v BSA in PBS. Plates were washed and then 50 μ l of goat anti-mouse IgG-peroxidase (heavy and light chains, 1:250, SPI or 1:8000 Vector Laboratories, Burlingame) was added to all wells and incubated for 1 h. After further washing 100 μ l of substrate consisting of 2.2 mmol/l 0-phenylene-diamine (Sigma) and 0.012% v/v H_2O_2 in 0.05 mol/l citric acid and 0.1 mol/l phosphate buffer, pH 5 was added to each well. After 10–20 min at room temperature the reaction was stopped by addition of 50 μ l 10% v/v HCl and the optical density at 486 nm was

read in an ELISA plate reader. In cross-sectional studies IAA values for test sera were expressed after subtraction of mean optical densities of age-matched normal sera (5 normal sera per age group) while in longitudinal studies they were expressed after subtracting the mean values for normal sera of day 40–90 mice (3 samples). Test sera were considered positive if this difference exceeded 2 standard deviations of the means for normal sera.

Statistical analysis

The difference between the rates of the three markers examined in cross-sectional studies were tested using Fisher's exact test while the Student's t-test was used for the IAA values.

Results

The pattern of ICA staining in sections of normal mouse pancreas by sera from NOD or normal mouse is shown in Figure 1a and b. Of the 141 serum samples tested in the ICA procedure, 81 showed staining of the majority of islet cells while 15 sera stained cells which were predominantly located in the periphery of the islets (Fig. 1a, 2a, 3a). In Figures 1c and d and 2b and c adjacent sections have been stained by the indirect immunofluorescence procedure for either insulin or glucagon. Sera showing specific peripheral staining when applied simultaneously with either antiserum to glucagon, somatostatin or pancreatic polypeptide hormone in the immunofluorescence procedure revealed that most of the ICA immunoreactive cells corresponded to glucagon cells (Fig. 3a and b).

Normal pancreatic islets still showed staining when ICA positive sera were re-stained following pre-absorption with insulin or glucagon.

The presence of lymphocytic infiltrations within and in close apposition to pancreatic islets of NOD mice of various ages studied cross-sectionally and the corresponding ICA and IAA results are shown in Table 1. Insulinitis was absent in all mice at day 15 and day 25 but was present from day 40 (50%) and was significantly higher at day 90 than at day 15 ($p=0.05$). Islet cell antibodies showing weak staining were present in 6/13 mice at day 15. At day 25, the ICA rate had increased to almost 60%. Concordance for both markers was seen in 2 mice at day 40 (17%) and 6 mice at day 90 (46%). In contrast, the presence of IAA was seen in 54% of mice studied at day 15 and in all mice at the subsequent ages. However, the mean values for IAA were significantly higher at day 25 (0.38 ± 0.08), 40 (0.27 ± 0.16) and 90 (0.38 ± 0.10) than at day 15 (0.10 ± 0.04 , $p=0.05$). Concordance rates for IAA and insulinitis were 42% at day 40 and 69% at day 90 whereas for ICA and IAA this rate was relatively low at day 15 (15%) but considerably higher at day 25–90 (almost 50%). Animals concordant for all three markers were first observed at day 40 (17%) which increased to 38% at day 90.

The presence of ICA studied longitudinally and the

Fig. 1. Photomicrographs of normal mouse pancreatic sections stained by ICA procedure or by indirect immunofluorescence for insulin or glucagon. **a** Serum from NOD mouse reacted by the ICA procedure; note staining of almost all islet cells. **b** Serum from normal mouse reacted by the ICA procedure; note absence of staining of islet cells. **c** and **d** a single section adjacent to (**a**) stained by indirect immunofluorescence for insulin (**c**) and glucagon (**d**). $\times 550$

corresponding IAA values (for 7 mice) are shown in Table 2. Four out of the 5 mice studied from day 35 (mice 1–4) developed diabetes at the ages indicated. In this group ICA were detected in 4 mice by day 53 and by day 97 in the remaining mouse (mouse 1). Most of the ICA-positive sera from mice 2–5 also had varying levels of IAA whether or not diabetes supervened. The earliness of appearance of either ICA or IAA did not predict the time of onset of diabetes.

Table 2 also includes ICA results for mice studied from day 144–220 (mice 6–9) and day 168–245 (mice 10–14). The corresponding serum values for IAA from mice 7, 8 and 9 are also shown. Islet cell antibodies were detected in all 9 mice but only 4 developed diabetes. Most ICA-positive sera were also positive for IAA. However, 1 mouse which initially showed ICA and IAA (mouse 8) became negative for both antibodies shortly before developing diabetes at 192 days of age.

Fig. 2. Photomicrographs of normal mouse pancreatic sections stained by ICA procedure or by indirect immunofluorescence for insulin or glucagon. **a** Serum from NOD mouse reacted by the ICA procedure showing staining of some islet cells located either in the periphery or scattered more centrally. **b** and **c** a single section adjacent to (**a**) stained by indirect immunofluorescence for glucagon (**b**) and insulin (**c**). $\times 250$

Fig. 3. A normal mouse pancreatic section stained simultaneously with serum from NOD mouse by ICA (**a**) and with rabbit anti-glucagon serum by indirect immunofluorescence (**b**). Note staining of identical cells by both procedures. $\times 550$

Discussion

There are now several studies which suggest that insulin-dependent diabetes is preceded at least in part by disturbances in the immune system. In humans at risk and in the NOD mouse and the BB rat this phenomenon is characterised by the presence of islet cell and insulin autoantibodies, lymphocytic infiltrations and T-cell aberrations [10–14].

The present cross-sectional and longitudinal studies were undertaken in the NOD mouse to see if

the rates of three likely markers of diabetes (insulinitis, ICA and IAA), their ontogenic expression and/or degree of concordance could confer predisposition to insulin-dependent diabetes. We have shown that all three markers appear relatively early after birth and well before clinical diabetes. Serum ICA and IAA were first detected at day 15 and preceded insulinitis which appeared at day 40. However, the rates for the three markers in our cross-sectional studies were much higher than the expected 30% rate for diabetes in the present colony. These results suggest that the presence of

Table 1. Ontogenic relationship of ICA, IAA and insulinitis in female NOD mice studied cross-sectionally from day 15-90

Age (days)	Number of animals	Number with insulinitis	Number with ICA	Number with IAA	Number with insulinitis and ICA	Number with insulinitis and IAA	Number with ICA and IAA	Number with insulinitis ICA and IAA
15	13	0	6	7	0	0	2	0
25	8	0	5	8	0	0	4	0
40	12	6	5	12	2	5	5	2
90	13	9	7	13	6	9	7	5

In the IAA assay, the mean optical densities $\pm$ SD for age-matched normal sera (5 sera per age group) were: day 15 = 0.442 ± 0.035 ; day 25 = 0.441 ± 0.035 ; day 40 = 0.275 ± 0.046 ; day 90 = 0.285 ± 0.065 . All NOD mouse sera were assayed in duplicate and assigned 'positive' if their mean optical densities were greater than the corresponding mean optical densities of age-matched normal sera by at least 2 SD

Table 2. Presence (+) or absence (-) of ICA in NOD mouse sera sampled longitudinally from day 35-236 (mice 1-5), day 144-220 (mice 6-9) and day 168-245 (mice 10-14). The corresponding serum IAA values are indicated in brackets for mice 2-9 and have been expressed after subtraction of optical densities of test sera from the mean values for normal sera

NOD Mice	Age (days)																				
	35	53	67	82	97	117	141	144	158-159	168-169	179	182	187	192	202-203	206	215-216	220	229-230	236	245
1	-	ND	-	-	+	+	+		+ ↓												
2	+ (ND)	+ (0.16)	- (0.17)	+ (0.18)	- (0.19)	+ (ND)	+ (0.62)		+ ↓ (ND)												
3	+ (0.06)	+ (0.11)	+ (0.14)	+ (0.12)	+ (0.21)	+ (0.20)	+ (0.23)		+ (0.22)	+ (0.24)			+ (0.30)		+ (0.17)		+ (0.19)		+ (0.25)	+ ↓ (0.25)	
4	- (0.09)	+ (0.18)	+ (0.57)	+ (0.20)	+ (0.29)	+ (ND)	+ (0.62)		ND	+ (0.38)			+ ↓ (0.32)								
5	ND	+ (0.11)	- (0.10)	+ (0.10)	+ (0.21)	+ (0.42)	+ (0.60)		+ (0.37)	+ (0.28)			+ (0.23)		+ (0.20)		+ (0.19)		+ (0.25)	+ (0.25)	
6								+	+		+ ↓										
7								+ (0.14)	+ (0.09)		- (0.19)			+ (0.16)		+ (0.14)		+ (ND)			
8								+ (0.17)	+ (0.15)		+ (0.12)			- ↓ (0.00)							
9								+ (0.12)	+ (0.11)		+ (0.08)			+ ↓ (0.07)							
10										+		+			+		+		+		+
11										+		+			+		+		+ ↓		
12										+		+			+		ND		+		ND
13										-		-			-		-		+		+
14										+		+			+		+		+		+

ND: Not determined; ↓: Age at onset of diabetes; All animals were females except mouse 5

either marker on its own cannot be used as a predictor of diabetes, although it is possible that concordance for the two or the three markers may be of some predictive value. Studies in the NOD mouse by others indicate that ICSA also precede the onset of diabetes but may be present in both diabetic and non-diabetic groups perhaps with diminished levels around the time of clinical onset [5].

Some of our ontogenic studies are similar to those in the BB rat where insulinitis was observed after day 40 but serum ICSA appeared earlier, at day 28-30 [15]. The age of onset of insulinitis in the NOD mouse report-

ed by others is slightly different from our present observations. For example, Fujita et al. [2] observed insulinitis at 4-5 weeks and Miyazaki et al. [13] at 3 weeks of age by light microscopy whereas Fujino-Kurihara et al. [16] observed these infiltrations at 2 weeks with the electron microscope. Likely reasons for these results may be due to differences in defining insulinitis, ages of mice selected for cross-sectional studies and observation methods used. In our present study there was some evidence of much weaker ICA and IAA levels prior to weaning. Whether a change from a rodent milk diet to one consisting of foreign antigens is re-

sponsible for enhancing the expression of these immune abnormalities is not known. Non-obese diabetic mice fed from weaning on nutritionally adequate diet not containing intact protein are protected against the development of diabetes [17]. It is interesting that ethnic groups who traditionally introduce cow's milk at an early stage have a high childhood diabetes rate, whereas those groups who suckle their infants for relatively long periods of time and do not use cow's milk as a weaning diet, have a very low rate. (R.B.Elliott, unpublished data). Alternatively, our observations may have been due to the passage of ICA-positive maternal immunoglobins in the newborn.

Sera from NOD mice examined longitudinally suggest that autoantibodies to insulin, like ICA, can be detected from an early age whether or not diabetes supervenes. Others have shown that in the high incidence BB rat a majority of the animals were also IAA positive from an early age, none of which were ICSA positive [18]. In the present study, although a formal proof for the presence of insulin autoantibodies by insulin inhibition was not performed, our test system, which utilised porcine insulin as the solid phase ligand would not have detected autoantibodies monospecific for mouse insulin. In our ICA procedure, the failure to suppress immunofluorescence after neutralisation of immune sera with insulin or glucagon, the staining of selective islet cell-types and the demonstration of IAA imply the presence of islet autoantigens of different chemical identities. In addition, autoantibodies with unique epitopes may be present. Further studies are in progress to define more precisely the autoantigenic determinants which show positive staining in our present ICA procedure and their immunochemical relationship to islet hormones. In the NOD mouse others have recently reported that monoclonal antibodies to islet cell surface antigens recognise two major polypeptides of molecular weights 64K and 28K [19]. The 64K value has also been reported earlier by others using serum from Type 1 human diabetic patients and BB rats [15, 20]. In addition, a rabbit antiserum obtained after immunisation with dispersed rat islet cells could immunoprecipitate a 40K glycoprotein [21]. Recently, others have also reported the presence of autoantigens of different biochemical properties [22, 23]. These observations and our present results on the pattern of cellular staining in the ICA procedure, the presence of IAA in the NOD mouse and in humans before diabetes [14] suggest that it is unlikely that a single autoantigen is solely involved in the pathogenesis of insulin-dependent diabetes in this animal model.

Although the quantitation of IAA and ICA as predictors has not been addressed in this study, the present report does demonstrate the expression of ICA, IAA and insulinitis relatively early after birth but at a rate higher than the anticipated rate for diabetes in our NOD mouse colony. These findings are in agreement with recent studies in the BB rat where potential

markers of the disease such as an aberrant first phase insulin release, T-cell lymphopenia and serum ICSA can be a feature of diabetic and non-diabetic animals [24-26]. Evidence for disease susceptibility can therefore be present in a larger group than previously thought but other factors may be necessary for clinical onset. The single mouse which at the time of development of diabetes was ICA and IAA negative, but was previously positive for both, is of interest if this animal model has parallels with the human disease.

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