Pre-diabetes in the spontaneously diabetic BB/E rat: pancreatic infiltration and islet cell proliferation

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Abstract. A cohort of BB/E rats derived from litters with a high and low incidence of IDDM was studied prospectively to examine the relationship between circulating autoantibodies, islet insulin secretion, pancreatic infiltration, and islet cell replication during the pre-diabetic period. Although a higher incidence of islet cell surface (ICSA) and insulin autoantibodies (IAA) was detected in the diabetes-prone than in the low diabetic-incidence BB/E rats, there was no correlation between the two antibodies in individual animals. Moreover, ICSA, but not IAA, were associated with loss of first phase islet insulin release. Between 75 and 105 days of age the number of diabetes-prone rats with ICSA and impaired islet insulin secretory function increased. Over the same period, there was a concomitant increase in the proportion of diabetes-prone animals with pancreatic infiltration, and increased islet endocrine cell proliferation. All these interrelated phenomena were observed in diabetes-prone BB/E rats at a time when the animals were normoglycaemic.

The spontaneously diabetic insulin-dependent BB rat displays a diabetic syndrome closely resembling that of human type 1 (insulin-dependent) diabetes mellitus (IDDM) in several important aspects Nakhooda et al. (1977). In particular, there is a genetic predisposition linked to the major histocompatibility complex RT1 (Colle et al. 1981), a long pre-diabetic period, and a mono-

nuclear cell pancreatic infiltrate associated with selective destruction of islet β-cells (insulitis), which invariably precedes and accompanies the appearance of ‘clinical’ diabetes. The pathogenesis of the disease has been extensively investigated and there is evidence (reviewed by Yale & Marliss 1984) suggesting an involvement of cellular and humoral immunity in the destruction of β-cells. These findings together with the reported prevention of diabetes in this animal model by immunosuppression in the pre-diabetic period (Laupacis et al. 1983; Boitard et al. 1985) confirm its importance as a tool for defining precisely the sequence of events leading to the development of Type 1 diabetes.

Loss of pancreatic β-cell mass leading to overt IDDM results from a negative balance between islet cell proliferation and destruction. Attention has focussed on the latter process and there is no information about β-cell regeneration as spontaneous IDDM develops. We have therefore examined directly the relationship between circulating autoantibodies, islet morphology, and β-cell function/replication in the pre-diabetic period.

Materials and Methods

Animals

The animals used in this study were from the Edinburgh colony (subsequently designated BB/E), the nucleus of which was kindly donated in 1982 by Dr Pierre
Protocol

Table 1.

<table>
<thead>
<tr>
<th>Parental phenotype</th>
<th>No. of litters</th>
<th>Normoglycaemic study rats (age in days)</th>
<th>Rats not included in study</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td>Low diabetic- incidence</td>
<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diabetes-prone</td>
<td></td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>D x D</td>
<td></td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>17</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

Excluded from study: *a 3 rats; *b 1 rat; *c 3 rats (N x D mating) diagnosed diabetic on study day.


Thibert from the colony maintained at the Animal Resources Division of Canada, Ottawa (designated BB). The BB/E colony consists of two sub-lines of animals created by selective outbreeding. The diabetes-prone line, predominantly maintained by crossing diabetic males with non-diabetic females, has a diabetic incidence of approximately 60% with a mean age at onset of diabetes of approximately 96 days. The low diabetes incidence subline has an incidence of diabetes of < 2% at 120 days of age. Details of the cohort of BB/E rats used in the study are shown in Table 1. Groups of 16 normoglycaemic rats were selected (as shown in Table 1) for study every 15 days between 30 and 105 days of age.

Protocol

At each time point, selected animals received an ip injection of tritiated thymidine (0.5 µCi/g body weight) one hour before being killed under light halothane anaesthesia. The pancreata (splenic portion) were then rapidly excised and plasma samples were taken for determination of plasma glucose (glucose analyser—Beckmann Instruments Inc, Fullerton, CA), insulin concentration, and autoantibodies to islet cell surface (ICSA), cytoplasm (ICA), and rat insulin (IAA). A portion of the excised pancreatic tissue was fixed in Bouin’s solution for autoradiography and examination of mononuclear cell infiltration. The remainder was used to prepare isolated islets for insulin secretion studies in a multichannel perfusion system.

Autoantibodies

Plasma samples were applied undiluted to cryostat sections (4 µm) of normal Wistar rat pancreas for detection of ICA using fluoresceinated goat anti-rat immunoglobulins (Nordic, Maidenhead, Berks, UK) as the second layer. ICSA were estimated using a 125I-protein A radioligand assay Dyrberg et al. (1982). Briefly, dispersed islet cell suspensions were prepared from β-cell-rich foetal rat islets and were incubated with plasma samples for 60 min at 4°C. The cells were then washed and incubated for a further 30 min at 4°C with 125I-protein A. After washing and centrifuging, the radioactivity in the cell pellet was determined in a gamma scintillation counter (Nuclear Enterprises, Edinburgh, UK). Cell-bound IgG was expressed as cpm/10^6 islet cells. Intra- and inter-assay variation was 6% and 14%, respectively (N = 8) with non-specific binding (islet cells incubated in absence of plasma) contributing < 0.02% of total counts. Animals were regarded as being ICSA-positive when counts bound - 3 SD exceeded mean binding values (± 3 SD) for normal Wistar (518 ± 159 cpm/10^5 cells; N = 32) and low diabetes incidence BB/E rats (486 ± 138 cpm/10^5 cells; N = 132). IAA were measured by a modified direct immunospecific enzyme-linked immunosorbent assay (Elisa) (Dean et al. 1987), employing purified rat insulin (Novo Industri A/S, Copenhagen, Denmark) and horseradish peroxidase conjugated rabbit anti-rat immunoglobulins (Miles Laboratories, Stoke Poges, Slough, UK). Reference plasma from normal Wistar and diabetic BR/E rats were included in each assay as controls. Intra- and inter-assay variation were 5.6% (N = 5) and 15.2% (N = 3), respectively. Plasma samples were considered IAA-positve when OD values were two times the mean non-specific binding value and mean ± 3 SD of binding for normal Wistar rats (OD = 0.014 ± 0.018; N = 12; aged 90–150 days).
Islet insulin secretion

The numbers of age-matched ICSA-positive and negative rats did not allow a full statistical evaluation of islet insulin release, but we considered it important to confirm that islets from the present study animals showed a pattern of insulin secretion similar to that observed in a previous study of diabetes-prone BB/E rats Bone et al. (1983).

Thus, islets were isolated from pooled pancreatic tissue of ICSA-positive or negative rats sacrificed at 75, 90 and 105 days of age. On each experimental day the islet insulin secretion was determined for up to 8 batches of 30 islets (4 channels each from the ICSA-positive and ICSA-negative groups) and the data combined to compare the pattern of insulin secretory response of ICSA-positive and ICSA-negative rats. The multichannel system and experimental protocol used for islet perfusion have previously been described in detail (Ashby & Shirling 1980). Briefly, the islets were equilibrated by a 40-min perfusion with basal media (2.7 mmol/l glucose) and then subjected to two consecutive stimulations with 16.7 mmol/l glucose applied 20 min apart. Samples of the perfusate were collected throughout and stored at −20°C until assayed for insulin. At the end of the second high glucose stimulation period, the islets were perfused for a further 20 min with basal media containing no BSA and recovered from the perfusion chambers. The islets were ultra-sonically disrupted, and their protein content determined (Bio-Rad protein assay kit, Richmond, CA). Insulin secretion was expressed as µU 1RI per µg islet protein so as to correct for any possible differences in the size of islets isolated from ICSA-positive and negative animals.

Autoradiography

Incorporation of tritiated thymidine into DNA during the S phase of the cell cycle has been widely used as an index of islet cell proliferation (Logothetopoulos 1972). Autoradiography is considered to be the most sensitive method for detecting tritiated thymidine incorporated into islet DNA (Hellerstrom 1977).

Pancreatic paraffin sections (5 µm) of Bouin fixed tissue were mounted on gelatinised slides. The slides were dipped in Kodak NTB2 photographic emulsion and exposed for five weeks at 4°C. After developing for 5 min in Kodak D19 and fixing for 10 min in Kodak F24, the emulsion layer was allowed to dry overnight and the sections were then lightly counterstained with haematoxylin and eosin. The labelling index was determined by counting the number of radioactively labelled islet cell nuclei and expressing this value as a percentage of the total number of islet nuclei scored. Evaluation of sections containing several islets ensured that a minimum of 800–1000 nuclei were examined for each individual pancreatic tissue sample. Pancreatic sections were also examined for the presence of mononuclear cell infiltration. All observations were performed blind on coded samples.

Samples of pancreas from a subsequent biopsy study (Bone et al. 1986) were examined to confirm that a possible proliferative activity of infiltrating cells was not contributing towards the islet cell labelling index recorded in those rats showing pancreatic infiltration. Cryostat sections of pancreas from 2 confirmed pre-diabetic BB/E rats (biopsied 10 and 13 days prior to onset of IDDM) were pre-stained with a monoclonal antibody to rat class II antigens and subjected to autoradiography as described above. The infiltrating mononuclear cells showing class II antigen expression did not possess radiolabelled nuclei whether located either at the periphery of islets or at a distance from islet tissue.

Results

Table 2 shows the plasma glucose and plasma insulin concentrations of the study animals, details of which are given in Table 1. Plasma glucose concentration increased significantly in the groups of animals from 30–45 days of age but thereafter remained constant at 8 mmol/l. There was a five-fold increase in the mean plasma insulin concentration between 30 and 75 days of age, the values remained steady at 90 days, and declined at 105 days of age. There were no significant differences in either plasma glucose or insulin values between age-matched low diabetic incidence and diabetes-prone rats.

Cytoplasmic ICA were not detected in any ani-

<table>
<thead>
<tr>
<th>Plasma glucose (mmol/l)</th>
<th>Plasma insulin (mU/l)</th>
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<tr>
<td>BB/E rats (age in days)</td>
<td></td>
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<tr>
<td>30 (N = 16)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>45 (N = 16)</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>60 (N = 16)</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>75 (N = 13)</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>90 (N = 15)</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>105 (N = 13)</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>8.2 ± 0.2</td>
</tr>
</tbody>
</table>

Results expressed as mean values ± SEM for the numbers of animals given in parentheses. P values indicate significant differences between mean values for the groups of animals shown (Student’s unpaired t-test).
Percentage of BB/E rats with circulating islet cell surface and rat insulin antibodies in the different age groups studied. The numbers of low diabetic-incidence and diabetes-prone animals per group are detailed in Table 1.

Fig. 1.

The numbers of study animals with ICSA and IAA at the ages studied are shown in Fig. 1. ICSA were not detected in diabetes-prone animals prior to 60 days of age, but were demonstrated with increased frequency thereafter (60 days = 4/12; 75 days = 2/9; 90 days = 6/11; 105 days = 9/11 animals). IAA were found in diabetes-prone rats at 30 (3/12), 45 (2/12), 60 (6/12), 90 (8/11) and 105 (3/11) days of age. There was no correlation (r = 0.104) between IAA and ICSA in individual study animals.

Circulating ICSA and IAA were detected in only a small number of the low diabetic-incidence rats.

The insulin secretory response to glucose was compared between pooled batches of islets isolated from groups of ICSA-positive and ICSA-negative rats aged 75, 90 and 105 days. Findings from three experiments indicated a diminished total secretion of insulin in islets from ICSA-positive rats (414 ± 74 µU IRI/µg islet protein) compared with islets from animals without ICSA (577 ± 72 µU IRI/µg islet protein) with first phase release (98 ± 24 vs 151 ± 16 µU IRI/µg islet protein) being particularly affected.

Fig. 2 shows pancreatic autoradiographs from a low diabetic-incidence (A) and a diabetes-prone (B, C) rat both aged 75 days. The section from the low diabetic-incidence animal shows no mononuclear cell infiltration and no labelled islet cell nuclei. Mild peripheral infiltration and many centrally located, radiolabelled islet cell nuclei are present in the pancreas of the diabetes-prone rat.

Fig. 3 shows labelling index values and the presence of pancreatic infiltration in all individual study animals. The labelling index was remarkably constant at or below 1% in the low diabetic-incidence rats throughout the age range. The diabetes-prone rats showed a similar 1% labelling index up to 75 days of age, but at 90 and 105 days 4/11 and 6/10 rats, respectively, showed a labelling index of greater than 4%. These animals with a high labelling index also showed some degree of pancreatic infiltration. However, mononuclear cell infiltration was also observed in 7 diabetes-prone rats not showing an increase in labelling index values.

Discussion

In this study we have investigated the regulation of islet growth during development of IDDM in the spontaneously diabetic BB/E rat. We have therefore assessed in parallel, 1) metabolic status; 2) occurrence of circulating islet cell and insulin autoantibodies; 3) islet cell function (both insulin...
Fig. 2.

Autoradiographs of paraffin sections of pancreas from normoglycaemic, low diabetic-incidence and diabetes-prone BB/E rats; prepared as described in Materials and Methods.

A. Normal islet showing no mononuclear cell infiltration and no labelled islet cell nuclei (low diabetic-incidence rat aged 75 days; haematoxylin and eosin $\times 300$).

B. Islet showing only mild, peripheral infiltration with no obvious accumulation of non-endocrine cells within the islet core (diabetes-prone rat aged 75 days; haematoxylin and eosin $\times 300$).

C. High power view of B showing numerous islet cell nuclei containing a mass of electron-dense grains ($\times 475$).
secretion and cell replication; and 4) pancreatic morphology in individual, potentially-diabetic BB/E rats prior to the development of 'clinical' diabetes. All findings being recorded at a time when the animals were normoglycaemic and had normal insulin levels.

In agreement with previous findings (Dyrberg et al. 1982, 1984), we were unable to detect ICA in any animal. ICSA and IAA were detected predominantly in diabetes-prone animals but there was no correlation between the presence of these two autoantibodies in individual rats. In addition, of the 19 animals showing pancreatic infiltration, 11 possessed circulating ICSA whilst only 5 had IAA. Our findings confirm the previously reported presence of ICSA in potentially diabetic BB rats (Dyrberg et al. 1982, 1984). On the other hand, our evidence would not appear to support the hypothesis that IAA are potentially useful markers for either autoimmune insulitis or imminent development of diabetes in BB rats (Diaz et al. 1986).

The present islet insulin secretion studies, whilst not showing full statistically significant differences between ICSA-positive and negative rats, did indicate a perturbed pattern of biphasic insulin release in the ICSA-positive animals. A lowered pancreatic insulin release has been reported in young potentially diabetic BB rats (Svenningsen et al. 1986) but the pattern of release was normal and was shown to occur as a result of a lowered pancreatic insulin content. Such an explanation cannot account for the present findings, since results are expressed per unit islet protein and islets from ICSA-positive and negative rats have a similar relative insulin content (Bone et al. 1983).

The observed differences in the pattern of insulin release between these two studies could, however, be explained either by different experimental technique (isolated islets vs perfused pancreas) or by age difference of the study animals. Interestingly, a progressive decline in first phase insulin release during an IVGTT has recently been reported in pre-diabetic BB rats 25–50 days prior...
to onset of overt IDDM (Reddy et al. 1986). These authors also noted a similar impairment of β-cell function in some BB rats which remained normoglycaemic. Taken altogether, these studies suggest that diabetes-prone BB rats with circulating ICSA and/or abnormal islet secretory function often go on to develop overt IDDM, but this is not always the case.

Whilst previous studies have investigated islet growth regulation in response to the hyperglycaemia of diabetes (for a review see Hellerstrom 1977), nothing is known about islet cell replication during development of IDDM. We have therefore investigated islet cell replication in groups of normoglycaemic but potentially diabetic BB/E rats. Low diabetes-incidence and young (<75 days of age) diabetes-prone BB/E rats showed a rate of islet cell replication comparable to that of normal rats (Blum et al. 1963). However, as diabetes-prone rats approached the mean age for onset of IDDM (approximately 96 days), a number of animals showed a marked increase in islet cell labelling. All of these animals showed some areas of mononuclear cell infiltration within the pancreatic sections although it should be noted that both the periphery and overall integrity of their islets remained intact. Increased islet cell labelling was not always associated with mononuclear cell infiltration, since 7 diabetes-prone rats with a pancreatic infiltrate had a normal islet cell labelling index. This finding provides indirect evidence precluding a possible contributory effect of actively proliferating infiltrating cells towards an elevated labelling index value. Indeed, examination of cryostat sections from a subsequent biopsy study confirmed that infiltrating cells expressing class II antigens do not possess radio-labelled nuclei and were therefore unlikely to be responsible for the increased islet cell labelling observed in diabetes-prone rats.

From the present study it is not possible to determine whether increased islet cell replication may have a protective or preventative effect on the onset of overt IDDM in the BB rat. However, since pancreatic infiltration was observed in the absence of a raised islet labelling index, it is tempting to speculate that autoimmune attack of the pancreas may, in some animals, be able to trigger an increase in the rate of islet cell replication. Such an increase may help to explain why some BB rats showing impaired glucose tolerance and histological evidence of insulitis are able to maintain normoglycaemia (Reddy et al. 1986). It is equally possible, however, that animals remained non-diabetic because the autoimmune attack was less severe rather than because of a compensatory increase in islet growth.

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