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Rapid communications

Pre-diabetes in the spontaneously diabetic BB/E rat: lymphocyte subpopulations in the pancreatic infiltrate and expression of rat MHC class II molecules in endocrine cells

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Summary. Use of monoclonal antibodies specific for rat lymphocyte subsets and an anti-insulin marker has allowed us to document the following sequence of events leading to the development of clinical diabetes in this animal model. The first change observed in the pancreas is increased expression of MHC class II molecules on vascular endothelium and this precedes lymphocytic infiltration. Next, T cells of the T helper phenotype infiltrate the pancreas around blood vessels. Many of the infiltrating T cells show class II expression indicating that they are activated. A few cytotoxic and suppressor cells and B lymphocytes are also present and their numbers increase proportionately with rat age. Some macrophages are also seen. Finally, at a late stage class II MHC molecules can be detected in partially destroyed islets on β cells which are still actively synthesising insulin. We have never observed expression of class II molecules on glucagon or somatostatin secreting cells which are invariably well preserved.

Key words: Pre-diabetes, BB rat, lymphocyte subsets, β cell, MHC class II expression.

The BB rat is probably the best available animal model of human Type 1 (insulin dependent) diabetes mellitus [1]. In these animals islet cell surface antibodies and a mononuclear cell pancreatic infiltrate precede the development of the disease which has been shown to be associated with the RTI^u haplotype of the rat MHC [2].

As part of a longitudinal study designed to show the relationship between autoimmunity markers, islet morphology and β-cell function [3] we have examined the distribution of subpopulations of lymphocytes infiltrating the pancreas. Since there is some evidence in organ specific autoimmune states that affected glands aberrantly express class II molecules [4, 5] the pancreas were also extensively screened for the presence of Ia molecules on endocrine cells.

Materials and methods

Animals

The animals used in this study were from the Edinburgh colony (BB/E), the nucleus of which was kindly donated in 1982 by Dr P. Thibert, Animal Resources Division of Canada, Ottawa. The average incidence of diabetes in this colony is 60-70% and the mean age at onset ~96 days. A cohort of 96 BB/E rats was studied prospectively with eight male and eight female normoglycaemic rats being selected for study every 15 days between 30 and 105 days of age.

Protocol

At each time point, selected rats received an IP injection of ^3H thymidine (0.5 μCi/g body weight) 1 h before being killed under light halothane anaesthesia. The pancreases were rapidly excised sand serum samples were taken and stored at $-20\,^\circ C$ for determination of glucose and insulin concentrations and autoantibodies to thyroglobulin, gastric parietal cells, smooth muscle, islet cell surface and cytoplasm and insulin. Pancreatic tissue was divided into three portions which were either (a) immediately snap-frozen in isopentane at $-70\,^\circ C$ for examination of lymphocytic infiltration and expression of class II rat MHC molecules, (b) fixed in Bouin's solution for autoradiography, or (c) used to prepare isolated islets for insulin secretion studies. This report describes only the observations relating to lymphocytic infiltration and class II MHC antigen expression on frozen sections.

Antisera

The following monoclonal antibodies were generously provided by Dr. D. Mason, Sir William Dunn School of Pathology, Oxford. OX6, specific for rat Ia; W3/25 which recognises T helper (T_H) cells and macrophages; OX8, a T cytotoxic/suppressor (Tc/s) cell marker and OX19 (pan T). B lymphocytes were recognised using OX12 which is specific for rat Kappa chains. Novo, Copenhagen, supplied the following monoclonals specific for endocrine cells: anti-human insulin (HU1 018); anti-glucagon (GLU 001) and anti-somatostatin (SOM 018) and rabbit anti-glucagon sera and a guinea pig antibody (M 1183) used to detect insulin containing cells. Rabbit anti-somatostatin sera was generously provided by Dr. Patel, Montreal.

Immunofluorescence

Serial sections (4 μm) of snap-frozen pancreas were scanned for lymphocytic infiltration, endocrine cells and aberrant Ia expression using the above monoclonal antibodies. Clone supernatants were used at

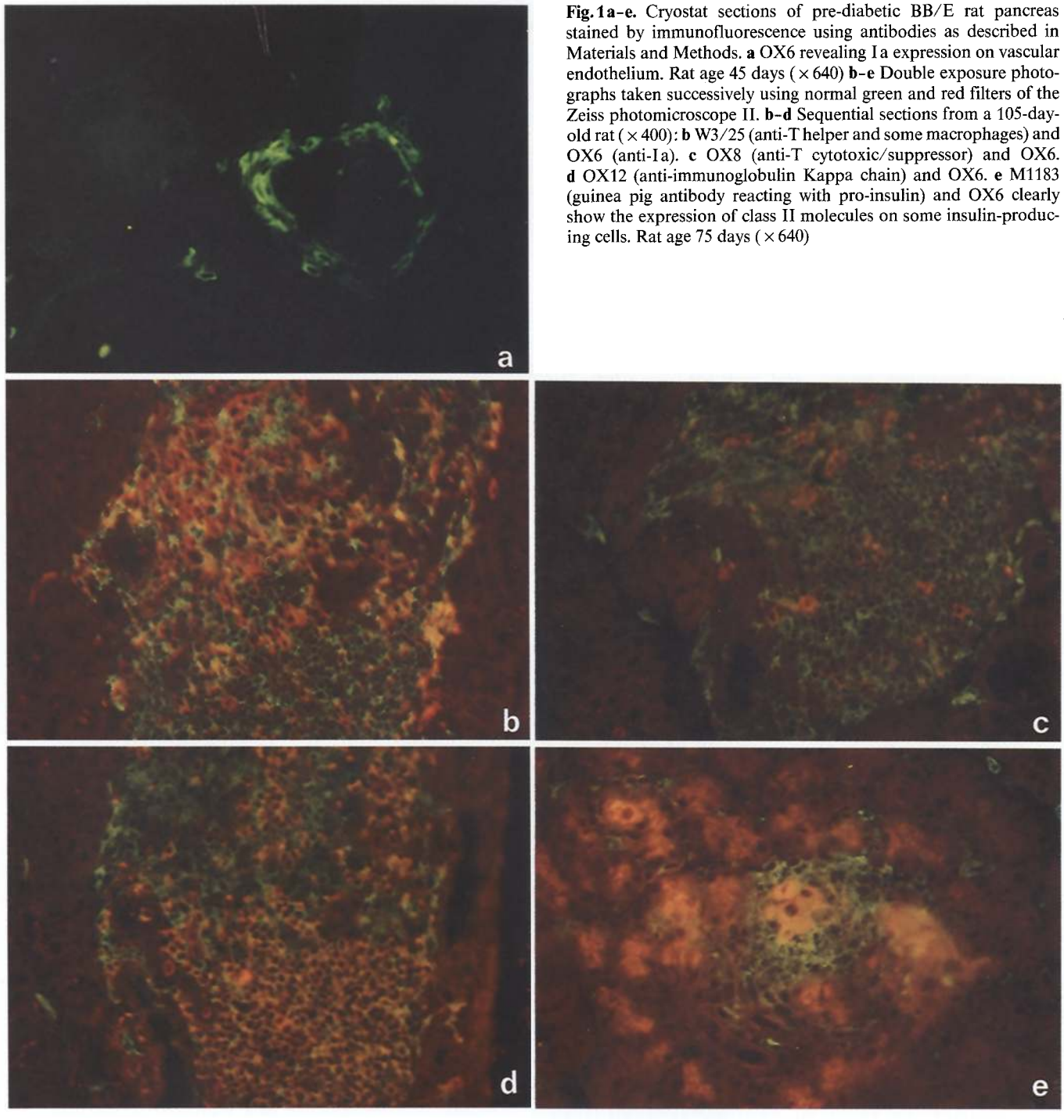

Fig. 1a–e. Cryostat sections of pre-diabetic BB/E rat pancreas stained by immunofluorescence using antibodies as described in Materials and Methods. **a** OX6 revealing Ia expression on vascular endothelium. Rat age 45 days (×640) **b–e** Double exposure photographs taken successively using normal green and red filters of the Zeiss photomicroscope II. **b–d** Sequential sections from a 105-day-old rat (×400): **b** W3/25 (anti-T helper and some macrophages) and OX6 (anti-Ia). **c** OX8 (anti-T cytotoxic/suppressor) and OX6. **d** OX12 (anti-immunoglobulin Kappa chain) and OX6. **e** M1183 (guinea pig antibody reacting with pro-insulin) and OX6 clearly show the expression of class II molecules on some insulin-producing cells. Rat age 75 days (×640)

1:1 dilution in PBS (0.05 mol/l, pH 7.6) containing 20% normal rabbit serum, except for OX12 which was in PBS only. Ascitic fluids and polyclonal antibodies were employed at 1:20 dilution in PBS. Sequential acetone fixed sections of prancreas were incubated with individual antibodies for 20 min at 25 °C and reactions were revealed after a similar incubation with either fluoresceinated rabbit antimouse immunoglobulin (Dako, High Wycombe, Bucks, UK) or biotinylated horse anti-mouse immunoglobulins followed by fluoresceinated or rhodaminated avidin (Vector, Burlinghame, California, USA). A 15-min washing period in PBS followed each application and to prevent non-specific binding, rabbit or horse antisera were adsorbed with rat liver acetone powder (Sigma, Poole, Dorset, UK) prior to use.

In double fluorochrome experiments to recognise lymphocyte subsets, first layer antibodies W3/25, OX8 or OX12 were applied to sequential sections followed by biotinylated anti-mouse immunoglobulin and rhodaminated avidin. Second layer antibodies OX6 or OX19 were then applied and revealed using fluoresceinated rabbit anti-mouse IgG. To identify endocrine cells expressing Ia antigens, the first layer antibody M1183 or polyclonal rabbit antibodies to glucagon or somatostatin were revealed with the corresponding rhodaminated antibody to guinea pig or rabbit immunoglobulins (Nordic, Maidenhead, Berks, UK). The second layer antibody OX6 was revealed with fluoresceinated rabbit anti-mouse immunoglobulin. After mounting, sections were examined under a Zeiss photo microscope II fitted with epi-illumination. Photographs were taken on Kodachrome ASA 200 film with automatic exposure and development at ASA 400.

Results and discussion

This preliminary paper is based on scanning studies using at least 8 pancreases at each time point. The first change observed was an increased expression of class II molecules occurring on what appeared to be vascular endothelium (Fig. 1 a); This preceded lymphocytic infiltration of the gland, which was not apparent until 60 days of age in these animals. Insulitis was observed by scanning adjacent sections with monoclonal anti-Ia and it was confirmed using Bouin-fixed tissue stained with haematoxylin and eosin. Twenty five percent of the pancreases were infiltrated by 60 days and 62% by 105 days. These lymphocytic infiltrates, mainly consisting of cells which were $OX19^+$ and $W3/25^+$ appeared to emanate from the vessels and converge on islets. By double fluorochrome staining on sequential sections it was established that the majority of invading lymphocytes were also Ia^+, suggesting recent activation (Fig. 1 b). It has been shown that Ia expression on rat T cells is an early and perhaps transient indication of activation (P. Chisholm, personal communication).

A few isolated cells were double stained with W3/25 and OX6 and had a morphological resemblance to macrophages. The latter have been observed in pancreatic infiltrates by electron microscopy [6]. There is unfortunately as yet no marker in the rat for differentiating Tc from Ts, thus we are unable to determine whether the few $OX8^+$ cells found in infiltrates represent mainly cytotoxic effectors or whether they are suppressor cells acting to dampen the immune response. Most of the infiltrating $OX8^+$ cells were Ia^- (Fig. 1 c).

B lymphocytes were identified in all pancreatic infiltrates, their numbers increasing proportionally with rat age. A few plasma cells could also be identified among the infiltrating B lymphocytes by their cytoplasmic immunoglobulin staining (Fig. 1 d). It is interesting that T and B lymphocytes appeared to occupy distinct areas in the infiltrates.

Coincident with infiltration by activated lymphocytes, occasional endocrine-like cells were seen to express Ia. When sections were double stained with antibody to insulin, some β cells were identified containing insulin and expressing Ia (Fig. 1 e). This is the first time this has been demonstrated in the BB rat and is strikingly similar to the observations of Bottazzo and Dean of aberrant expression of class II MHC molecules in β cells in the pancreas of a diabetic child [5]. We have never observed expression of class II molecules on glucagon or somatostatin secreting cells, which are invariably well preserved. The cytoplasmic nature of the β-cell staining with OX6 and lack of $OX6^+$ glucagon or somatostatin secreting cells would seem to preclude passive acquisition of Ia molecules. However, proof of active synthesis of these antigens by insulin-producing cells can come only from studies employing in situ hybridisation for messenger RNA class II MHC probes. Ia^+ positive β cells have been observed mainly in partially disrupted islets. Several workers have shown either increased Ia expression or de novo Ia expression on cells exposed to γ interferon [7, 8] and it is not inconceivable that this or another lymphokine is being produced locally by the Ia^+ T cells. In the prediabetic rat the finding of some islets with full insulin expression surrounded by Ia^+ lymphocytes while the endocrine cells were Ia negative, suggests that β-cell Ia expression does not precede lymphocytic infiltration and may be a secondary rather than a primary phenomenon. The trigger initiating the whole process remains obscure.

Elucidation of the precise aetiology of Type 1 (insulin dependent) diabetes and detailed examination of the effects of possible therapeutic intervention is clearly difficult in man. This animal model offers the possibility of such studies.

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