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Insulin autoantibodies, islet cell surface antibodies and the development of spontaneous diabetes in the BB/Edinburgh rat

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SUMMARY

The presence of insulin autoantibodies (IAA) and islet cell surface antibodies (ICSA) was sought in two longitudinal studies, involving BB/Edinburgh rats of high (BB/E/H, $n=157$) and low (BB/E/L, $n=61$) susceptibility to diabetes development. Both studies were designed to correlate pancreatic morphology with cellular and humoral immunity. In Study I, groups of eight male and eight female non-diabetic rats of the BB/E/H line were killed at 15 day intervals from 30–105 days and plasma samples were obtained by cardiac puncture. In study II, 61 BB/E/H and 41 BB/E/L rats underwent pancreatic biopsy 1–3 times from 30 days of age until onset of diabetes or 150 days, plasma samples being taken from the tail vein at biopsy. Both studies revealed a higher prevalence for ICSA than IAA in BB/E rats. Whereas a highly significant association of ICSA with diabetes development was observed in study II ($\chi^2=8{\cdot}30$, $P<0{\cdot}005$), IAA were associated with diabetes development only weakly ($P<0{\cdot}03$, Mann-Witney U-rank test). No correlation between the presence of ICSA and IAA in individual rats was observed and IAA were not significantly associated with BB/E/H in preference to BB/E/L rats, although positive IAA values were significantly elevated in the former compared with the latter ($P<0{\cdot}01$). These observations support the concept that IAA form part of a background of heightened autoimmunity against which frank diabetes develops in some animals.

Keywords BB rats insulin autoantibodies islet cell surface antibodies spontaneous diabetes

INTRODUCTION

The spontaneously diabetic BB rat provides a good animal model for human Type I diabetes, a disease with autoimmune features (Nakhooda *et al.*, 1977). Animals characteristically develop glycosuria and hyperglycaemia between 60 and 150 days of age, the incidence of diabetes being equivalent in the male and female animals. Evidence for an immunological basis of the diabetes in this model (reviewed by Yale & Marliss, 1984) is provided by the observed insulitis and selective β-cell destruction in the pancreas and the ability to prevent the disease by immunosuppressive treatments such as anti-lymphocytic serum, neo-natal thymectomy, anti I-E antibody administration or cyclosporine therapy.

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As in man, diabetes develops in the animals against a background of heightened autoreactivity, multiple autoantibodies being detectable during the prediabetic period. These include antibodies to islet cell surface (ICSA) and gastric parietal cells, thymocytotoxic antibodies and antibody to smooth muscle (Dyrberg *et al.*, 1982; Elder *et al.*, 1982). The precise relationship of these autoantibodies to the development of diabetes, however, is uncertain. Recently, insulin autoantibodies (IAA) have been detected in newly diagnosed untreated patients and in normoglycaemic potentially diabetic individuals (Palmer *et al.*, 1983; Arslanian *et al.*, 1985; Srikanta *et al.*, 1985). The predictive value of IAA for clinically overt diabetes is at present in dispute as published reports conflict (Karjalainen *et al.*, 1986; Dean *et al.*, 1986; Atkinson *et al.*, 1986).

One value of a model such as the BB rat is that it is possible to carry out longitudinal studies in which various parameters can be assessed for their predictive power. We report here the results of two such longitudinal studies in which plasma samples have been assayed for the presence of both IAA and ICSA and their significance for diabetes development analysed.

MATERIALS AND METHODS

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. This colony now has two components which have been created by selective breeding over the past 3 years; high susceptibility litters (BB/E/H) in which the average incidence of diabetes is 60–70%, with the mean age at onset 96 days, and low susceptibility litters (BB/E/L), in which the incidence of diabetes at the time of experimentation was below 10%.

Plasma samples

The plasma samples tested in this study were derived from rats used in two longitudinal studies designed to correlate pancreatic morphology with cellular and humoral immunity. Study I involved 96 BB/E/H animals with batches of eight male and eight female rats being killed at 15 day intervals from 30–105 days. In this group, samples were obtained by cardiac puncture at killing. For comparative values, plasma samples were obtained likewise from 20 BB/E/L animals (aged 90–120 days), 12 normal Wistar rats (90–150 days), and 32 diabetic BB/E/H rats (90–150 days) treated daily with insulin (Ultratarde, Novo, Copenhagen).

Study II consisted of 102 animals (61 BB/E/H; 41 BB/E/L rats) which were biopsied 1–3 times between 30 and 150 days of age and also at onset of diabetes if symptoms developed. Plasma samples were obtained from the tail vein at the time of pancreatic biopsy.

Insulin antibodies

A micro-enzyme-linked immunosorbent assay (ELISA) as described for the measurement of human IAA (Dean *et al.*, 1986) was used with the following modifications:

Insulins. Microwells were coated using 100 ng purified rat, human or pig insulin (kindly donated by Novo).

Conjugate. Rabbit anti-rat immunoglobulin horse radish peroxidase (Miles Scientific, Slough, UK) was employed at $1:10^3$ dilution in 0·015 M PBS containing 0·1% Tween 20 (poly-oxyethylene sorbitan monolaurate, Sigma, Dorset, UK) and 10% heat-inactivated normal rabbit serum (NRS, Sera Laboratories, UK).

Test plasma and blocking for non-specific binding. Rat plasma were screened at 1:50 dilution in 0·015 M PBS containing 5% Tween 20 and 10% NRS. All microwells were blocked for non-specific binding by overnight incubation in PBS containing 20% NRS.

Substrate. Reactions with the substrate 3,3′-5,5′-tetramethyl benzidine were terminated after 30 min incubation at room temperature. Samples were screened in triplicate in both antigen-coated and control wells. The latter were pre-treated with coating buffer alone (0·05 M Na carbonate-bicarbonate buffer, pH 9·6) and blocked for non-specific binding in the same manner as the antigen-coated wells. It was established that the presence of heparin or insulin in the physiological/diabetic range did not affect the assay.

Calculation of results

For each sample, the mean specific Optical Density at 450 nm (Δ OD) was calculated by subtraction of the mean non-specific reading in the corresponding control wells. Reference plasma from normal Wistar and diabetic BB/E rats were included in each assay as controls. (The intra- and inter-assay C.V. were 5·6% ($n=5$) and 15·2% ($n=3$) respectively, using rat insulin and a standard diabetic rat plasma.

The specific binding observed for plasma samples from normal Wistar rats ($n=12$, age 90–150 days) was Δ OD = 0·014 ± 0·018, 0·071 ± 0·015 and 0·070 ± 0·013 (mean ± s.d.) for rat, human and pig insulin respectively. Readings for other samples were considered positive when OD values were at least twice the corresponding mean non-specific binding value and greater than mean + 3 s.d. of the normal Wistar rat group for the relevant species of insulin.

Islet cell surface antibodies

ICSA were estimated using an ^{125}I-protein A radioligand assay essentially as described by Dyrberg *et al.* (1982). Dispersed islet cell suspensions were prepared from β-cell-rich fetal rat islets (Bone & Swenne, 1982) and 5×10^4 cells were incubated with plasma samples (10 μl; final dilution 1:20) for 60 min at 4°C. The cells were then washed in PBS and incubated for a further 30 min at 4°C with ^{125}I-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 ct/min/10^5 cells. Intra and interassay variation was 6% and 14% respectively ($n=8$), with non-specific binding (islet cells incubated in the absence of plasma) contributing < 0·02% of total counts.

Statistical analyses

The Mann–Witney *U*-rank test has been employed for the analysis of associations of IAA within the various groups studied, $P=0·05$ being chosen as the level of statistical significance.

RESULTS

The incidence of insulin autoantibodies in rats. Both studies showed the spontaneous occurrence of IAA in untreated diabetes prone BB/E rats. The results of study I are summarized in Table 1, from which it can be seen that a low incidence of IAA was observed from 30 days of age, with the peak incidence of activity occurring at 90 days, close to the mean age of onset of diabetes in the BB/E/H rat colony (96 days). IAA were also found in BB/E/L rats aged 90–120 days, but were never observed in outbred Wistar rats of comparable age. The presence of IAA in BB/E rats was confirmed in the serial biopsy study (study II). Only 4/39 (10%) and 1/31 (3%) of the high and low incidence lines respectively were positive for IAA before 60 days of age. Thereafter, 16/48 (33%) of biopsied BB/E/H rats were IAA positive, temporal variation in the presence of IAA occurring in eight of these animals; 3/29 (~10%) of the BB/E/L rats biopsied after 60 days of age were IAA positive, but ΔOD values were low in these three rats (3–4 s.d. > mean of Wistar rat values).

Table 1 also shows the species binding specificity of the insulin antibodies. Most of the rat IAA appeared to recognize shared epitopes on rat, human and pig insulin. Although human and pig insulin appeared more reactive than rat insulin in this assay system, among the 18 IAA positive BB/E/H rats (study I), six plasma samples bound preferentially to rat insulin, three to human and one to both pig and human insulins. As expected, a higher incidence of insulin antibodies was seen in diabetic animals maintained on injections of heterologous insulin.

Islet cell surface antibodies, insulin autoantibodies and diabetes development. The prevalence of ICSA with age in BB/E/H rats is shown in Table 1. Unlike insulin and other autoantibodies (such as parietal cell and smooth muscle antibodies) measured in this group (study I), ICSA were not observed before 60 days of age, but were found with increasing incidence rising to > 75% at 105 days. In study I, there was no correlation ($r=0·104$) between ICSA and IAA in individual BB/E/H rats.

The distribution of ICSA and IAA in relation to diabetes development in study II is presented in Fig. 1. As in study I, no animal younger than 60 days old was positive for ICSA, so only data from rats aged 60–105 days have been included for analysis. Among this group 28 (58%) of the BB/E/H and four (14%) of the BB/E/L rats subsequently developed diabetes. In the high incidence line, 20/

Table 1. Incidence of antibodies to insulin and islet cell surface (ICSA) in BB/Edinburgh rats (study 1)

Age (days)	*n*	Insulin			ICSA
		Rat	Human	Pig	(%positive)
		(% positive per group)			
BB/E/H rats					
30	16	13	13	6	0
45	16	0	0	0	0
60	16	13	13	38	25
75	16	31	25	38	25
90	16	38	63	56	56
105	16	19	31	31	75
BB/E/L rats					
90–120 days	20	35	45	45	NT
Insulin treated diabetic BB/E rats					
90–150 days	32	56	88	91	NT

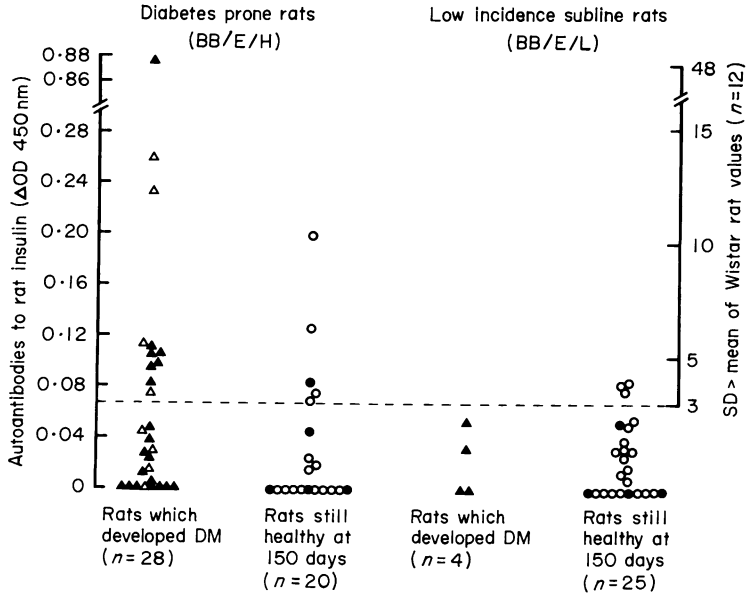

Fig. 1. Insulin autoantibodies (IAA) in relation to diabetes development. The presence of islet cell surface antibodies is indicated by solid symbols in high (BB/E/H) and low (BB/E/L) susceptibility rats (study II). The highest ΔOD values observed for IAA (rat insulin) in individual animals at any age between 60 and 150 days (or diabetes onset) is shown in relation to the number of s.d. above the mean of normal Wistar rat values ($n=12$), values > 3 s.d. (---) being regarded as IAA positive.

28 (71%) of the rats that became diabetic had ICSA, whereas only seven (35%) of these rats were also IAA positive. By contrast, in the group of 20 BB/E/H rats which did not develop diabetes, four had ICSA, a further four were IAA positive and one animal alone was positive for both antibodies. In the BB/E/L rats, all four animals which developed diabetes were ICSA positive/IAA negative, but among the 25 animals of this group that remained healthy at 150 days, four had ICSA and a further three rats had IAA (albeit of low value).

A strong association of ICSA with diabetes development was thus observed in the biopsy study ($\chi^2 = 8{\cdot}30$, $P < 0{\cdot}005$). Insulin autoantibodies were less significantly associated with diabetes development ($P < 0{\cdot}03$), using Mann–Witney *U*-rank test analysis of values from all study II rats which developed diabetes ($n = 32$) versus those remaining healthy ($n = 45$), and this low level of significance was confirmed ($P < 0{\cdot}05$) if BB/E/H rats alone were considered in the analysis.

Similar analysis of the data from BB/E/H rats (Fig. 1) revealed no significant association ($P = 0{\cdot}4$) of IAA with ICSA positive ($n = 25$) versus ICSA negative animals ($n = 23$), confirming the earlier observation in study I. Likewise, no significant association ($P = 0{\cdot}15$) was found for the presence of IAA in BB/E/H rats ($n = 48$) compared with BB/E/L rats ($n = 29$), presumably due to the low incidence of IAA in these animals. A further analysis, based solely on all positive IAA values recorded in the biopsy study, however, shows that higher ΔOD values for IAA are significantly associated ($P < 0{\cdot}01$) with the BB/E/H observations ($n = 47$) compared with those for the BB/E/L rats ($n = 19$).

DISCUSSION

Tolerance to self antigens can be broken following administration of an antigen crossreactive with self (Weigle, 1965), thus thyroglobulin or insulin autoantibodies are induced in genetically susceptible mice or humans following injection of rat thyroglobulin or pig insulin. These induced autoantibodies arise as the result of the provision of a new carrier determinant on the immunogen to which the recipient is not tolerant. This situation generates carrier specific T helper cells, which can help autoreactive B cells, recognizing shared determinants on the cross-reacting antigen. The occurrence of insulin autoantibodies in established diabetic BB/E rats could therefore be viewed as a breach of self-tolerance by this process, since these animals are maintained on heterologous insulin. However, the presence of autoantibodies in untreated BB/E rats and their absence in age-matched Wistar rats requires further explanation. Although tolerant to their own insulin, healthy rats would not be expected to be tolerant to pro-insulin, which is normally sequestered in the β-cell. In the prediabetic period of the BB/E rat, we have shown that insulitis (and attendant β-cell destruction) occurs from 60 days onwards. Thus, it is not inconceivable that insulin precursors (pre-proinsulin and proinsulin) come into contact with the immune system during this period, and this could generate an autoantibody response, which by definition would be wholly cross-reactive with pro-insulin. The occurrence of these autoantibodies may be restricted to animals of a certain genetic background.

Insulin autoantibodies have been detected in the sera of non-diabetic individuals. In man, complement fixing islet cell antibodies are thought to have some predictive value for insulin-dependent diabetes. Wilkin *et al.* (1985) have found no association of human insulin autoantibodies with the presence of islet cell antibodies and have suggested that their presence is more compatible with individual genetic susceptibility rather than established disease. However, Dean *et al.* (1986) have examined the class distribution of these autoantibodies and have found that the presence of both IgG insulin autoantibodies and complement fixing islet cell antibodies confers increased risk for future diabetes development than the presence of either marker alone. In the BB/E rat, although we observed a preferential association of antibodies to rat insulin with the group of animals which subsequently became diabetic, the finding was of low significance and there was no correlation between the presence of these antibodies and islet cell surface antibodies, which proved a much better indicator for diabetes development. However, subclass analysis of the spontaneous rat insulin autoantibodies will have to be performed before any definitive statement can be made.

Autoantibodies can arise not only through antigenic cross reactivity but also via perturbations of the idiotype network. For example, studies by Wasserman and his colleagues (1983) have shown in animals that administration of BISQ (an acetyl choline agonist) results in the production of autoantibodies to the acetyl choline receptor. Additionally Cohen and his coworkers (1984) have shown that administration of insulin to genetically susceptible mice leads to the production not only of insulin autoantibodies, but also to antibodies to the insulin receptor, presumably via perturbation of the idiotype network. We have previously already discussed the ways in which

autoantibodies to the insulin receptor or to insulin could arise following viral infection (Cooke, Lydyard & Roitt, 1985) and a recent report (Bodansky *et al.*, 1986) has demonstrated a high incidence of IgM class insulin autoantibodies following such common viral infections as mumps, measles, chickenpox and rubella. Whether these arise as a consequence of viral mediated destruction of the β-cell with concomitant release of insulin precursors leading to insulin autoantibodies as described above or via perturbation of the idiotype network as hypothesized (Cooke *et al.*, 1984), remains to be elucidated. If the autoantibodies prove to be wholly cross reactive with insulin precursors, β-cell attack by a virus would appear to be the initiating event. A viral aetiology of the disease in the BB rat cannot be excluded. Examination of serial plasma samples for autoantibodies to insulin and the insulin receptor should at least allow us to decide which autoantibody arises first and which develops as a consequence of the idiotype network.

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