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謹呈　渡辺賢治先生

DIABETES RESEARCH

INSULIN AUTOANTIBODIES IN MOUSE MODELS OF INSULIN-DEPENDENT DIABETES

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SUMMARY To clarify the significance of insulin autoantibodies (IAA) in insulin-dependent diabetes mellitus, we measured the IAA longitudinally in non-obese diabetic (NOD) mice, and in high-dose streptozotocin-induced diabetes (high-SZ) and EMC virus-induced diabetes (EMC) in mice, and compared the data with the occurrence of insulitis.

The IAA were detected by the polyethylene glycol (PEG) method using 125I-(Tyr A14) human insulin. The IAA were found in 38% of NOD mice and correlated with the occurrence of insulitis. The prevalence of IAA was 0% before the appearance of insulitis, 80% at 12–14 weeks of age and 30% after 20 weeks of age in female NOD mice. In male NOD mice, IAA were found in 45% at 12–14 weeks of age and 20% after 20 weeks. In high-SZ mice, IAA were detected in several mice while insulitis was not present. In EMC-virus induced diabetic mice, IAA and lymphocytic infiltration into the islets were detected 4–14 days after EMC virus infection.

These results suggest that (a) IAA are markers for islet autoimmunity in NOD mice, (b) the presence of IAA does not always reflect insulitis, (c) the presence of IAA is not sufficient for the development of overt diabetes and (d) the appearance of IAA may reflect a difference of the immune response genotype.

Key words: Insulin autoantibodies, Non-obese diabetic (NOD) mice, streptozotocin, EMC virus, insulin-dependent diabetes mellitus (IDDM), insulitis

INTRODUCTION

THE PATHOGENESIS of insulin-dependent diabetes mellitus (IDDM) seems to involve autoimmune mechanisms since: (a) there is lymphocytic infiltration of the islet (insulitis) (1); (b) autoantibodies to islet cell antigens are detected in a large population of IDDM patients at diagnosis (2–4); (c) immune-islet cell-killing mechanisms are present (5–8); and (d) there is an association with specific HLA antigens (9). NOD mice and BB rats develop diabetes in association with similar abnormalities and the development of diabetes in association with similar abnormalities and the development of diabetes is prevented by immunosuppressive agents and/or other drugs (10–14). The B-cell specific autoimmunity is not understood and it was of interest therefore that autoantibodies (IAA) in newly diagnosed IDDM patients was reported (15). More recently, it was suggested that IAA mark autoimmune B-cell destruction and development of IDDM in man (16, 17), and in BB rat (18). In NOD mice, we have reported the presence of IAA before development of diabetes (19) but that study was limited to 12–14 weeks of age only and a longitudinal study was necessary. Therefore, we investigated the presence of IAA in a longitudinal analysis of NOD mice and compared the data with the degree of insulitis. Also, we examined IAA in single high-dose streptozotocin-injected mice and EMC virus-induced diabetic mice to clarify the significance of IAA for the development of diabetes.

MATERIALS AND METHODS

The characteristics of mice used in this study are shown in Table 1. NOD mice were obtained from Japan Clea Inc., DBA/2, C57 Black, Balb/c and CD-1 mice were obtained from Charles-River Japan Inc., NOD mice were used without any treatment. In the high-SZ group,

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Table 1 Characteristics of mice used in this study

Group	*Strain*	*Sex*	*n*	*Age (week)*	*Treatment*
Female NOD	NOD	female	41	4～30	None
Male NOD	NOD	male	40	4～25	None
High-SZ	DBA/2	male	23	8	SZ 200 mg/kg i.p. × 1
	C57/BL	male	25	8	SZ 200 mg/kg i.p. × 1
	Balb/c	male	24	8	SZ 200 mg/kg i.p. × 1
	CD-1	male	30	8	SZ 200 mg/kg i.p. × 1
EMC	DBA/2	male	29	9	EMC virus diabetic strain 500 PFU i.p.
Control	DBA/2	male	8	9	None
	C57/BL	male	10	9	None
	Balb/c	male	10	9	None
	CD-1	male	25	4～14	None

DBA/2, C57/Bl, Balb/c and CD-1 mice were used and one dose of streptozotocin (Sigma Co., USA, 200 mg/kg body weight, dissolved in citrate buffer, pH 4·5) was injected intraperitoneally. For EMC diabetes, DBA/2 mice were used and 500 PFU of the diabetic strain of EMC virus (purified from EMC virus M variant by Dr. Seto, Pharmacentical Institute, School of Medicine, Keio University) were injected intraperitoneally. DBA/2, C57/Bl, Balb/c and CD-1 mice were also used for controls. None of the mice were treated with insulin.

Blood was sampled by heart aspiration without anaesthesia after testing for glycosuria by Tes-tape (Eli-Lilly, Co., USA), the mice were killed and the pancreata were removed for histological examination.

Autoantibodies to insulin were determined in 100 µl serum samples which were incubated for 24 hr at 4°C with 100 µl 125I-(Tyr A14) human insulin (0·03 ng, Amersham Lab., UK) and 300 µl of 1/15 molar phosphate buffer supplemented with 0·25% bovine serum albumin. Free insulin was separated from antibody-bound insulin by polyethylene glycol precipitation (20). Serum autoantibodies to insulin were expressed by the following formula:

$$\text{IAA} = ((\text{CPM (sample)} - \text{CPM (NSB)})/\text{total CPM}) \times 100\%$$

NSB (non-specific binding) was defined as the counts in the absence of the serum sample. Standard reference sera (normal and diabetic sera, known to contain IAA) were included in each assay for controls. The intra- and inter-assay coefficients of variation (C.V.) were found to be 4·0 and 8·3% respectively. Confirmation that binding proteins were likely to be IAA was obtained by displacement of binding in high dose human insulin.

The pancreata were fixed in Bouin's solution, embedded in paraffin, stained with hematoxylin-eosin and examined by light microscopy. The extent of lymphocytic infiltration (intensity of inuslitis) in an islet was scored 0 to 3, with 0 indicating absence of insulitis, and 1 less than 25%, 2 less than 50% and 3 greater than 50% lymphocytic infiltration. The grade of insulitis in a mouse was expressed as the average score calculated by the following equation: grade of insulitis = total score/number of islets. About 10 different islets were examined per pancreas.

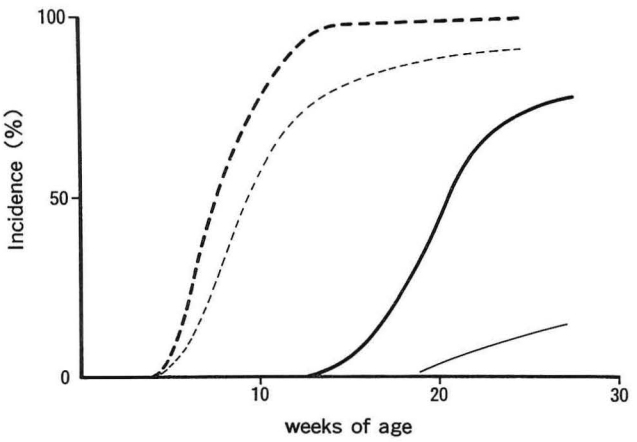

Figure 1. Cumulative incidence of diabetes and insulitis in our NOD mice; – – –: cumulative incidence of insulitis in female NOD mice, – – –: that in male NOD mice, ——: cumulative incidence of diabetes in female NOD mice, ——: that in male NOD mice.

Results

Mean insulin binding of sera from control mice was 1·10 ± 0·35 (mean ± SD)%. There were no differences between strains and the insulin binding in this group. We regarded sera as IAA positive when bound 125I-insulin levels were above the mean + 2SD value of controls.

In our NOD mice colony, insulitis was not found before four weeks of age, insulitis developed after 4–5 weeks of age, and diabetes developed after 14 weeks of age. The incidence of diabetes was about 80% in male and 20% in females (Figure 1). The prevalence of IAA was 0% at four weeks of age, 80% at 12–14 weeks of age and 30% after 18 weeks of age in female NOD mice. In male NOD mice, IAA were found in 45% at 12–20 weeks of age and 20% after 23 weeks of age (Figure 2). The 125I-insulin bound showed the highest titer at 12–14 weeks of age in both sexes of NOD mice. The IAA were only found in the sera from NOD with insulitis and not in those from NOD mice without insulitis. Though there was no correlation between the grade of insulitis and 125I-insulin binding, the prevalence rate of IAA and bound 125I-insulin levels were highest at 12–14 weeks of age which was the period when lymphocytic infiltration into islets was intense. Also, there was no correlation in 125I-insulin binding between NOD mice with diabetes and those without diabetes.

In high-SZ mice, IAA were detected in several sera but insulitis was not found in any mouse. Bound of 125I-

insulin in sera from high-SZ mice were low except for several sera from DBA/2 mice (Figure 3).

In EMC mice, lymphocytic infiltration in the islets was found in all mice, both four and seven days after EMC virus infection (Figure 4). The insulitis of this model of diabetes differed from that of NOD mice and that of low-SZ mice, since, mononuclear cells were found only sparsely in the islets. IAA were detected from four days after EMC virus infection and at 14 days the prevalence rate was high and also 125I-insulin bound was at a high level.

Discussion

We have previously reported that IAA are found in NOD mice at 12–14 weeks of age (19).

In this study, we had examined the IAA in NOD mice, high SZ and EMC mice, longitudinally.

In NOD mice, IAA could not be detected at four weeks

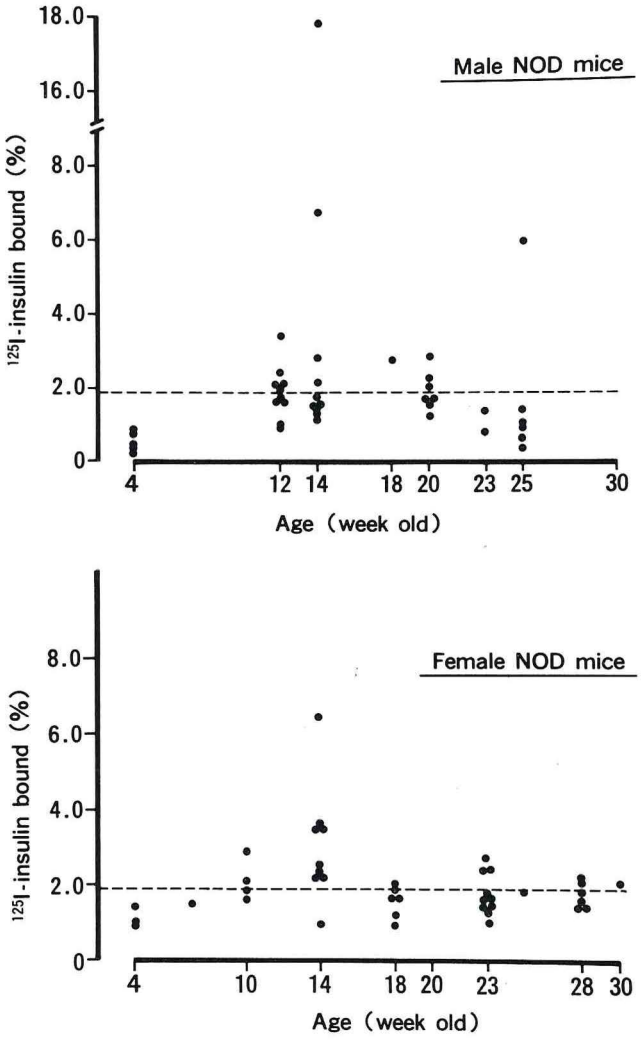

Figure 2. Distribution of insulin autoantibodies in sera from NOD mice of both sexes.

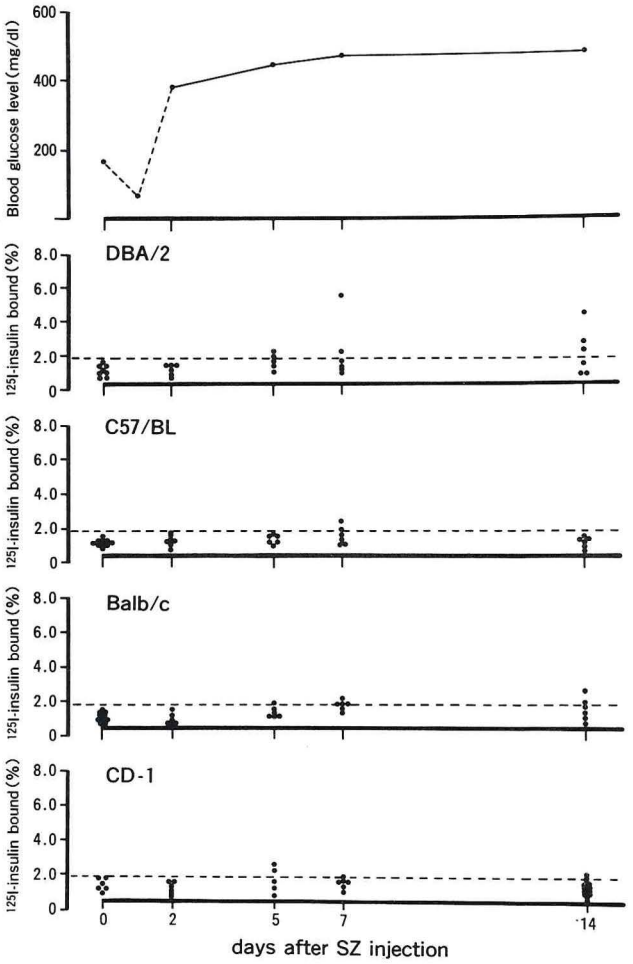

Figure 3. Blood glucose level and distribution of insulin autoantibodies in single high dose streptozotocin-induced diabetes in DBA/2, C57/Black, Balb/c and CD-1 mice.

of age when insulitis had not yet developed but they were found later in sera from mice with insulitis. The prevalence rate and titer of IAA were highest at 12–14 weeks of age which was the period when lymphocyte infiltration into islets was intense. These results suggest that IAA are markers of insulitis in NOD mice. However, it is difficult to regard IAA as a marker which can predict the development of diabetes because the prevalence of IAA decreased gradually after 20 weeks of age, when the incidence of diabetes increased, and because the prevalence rate of IAA at 12–14 weeks of age was higher than the incidence rate of diabetes in NOD mice of both sexes.

Recently, Pontesilli *et al.* (21) and Reddy *et al.* (22) have reported that IAA were detected by an ELISA assay in NOD mice before insulitis developed. A small quantity of IAA may appear before the development of insulitis as the ELISA assay has been reported to have a higher sensitivity than the radiobinding assay in some sera (23, 24). However, if IAA can be detected by ELISA assay

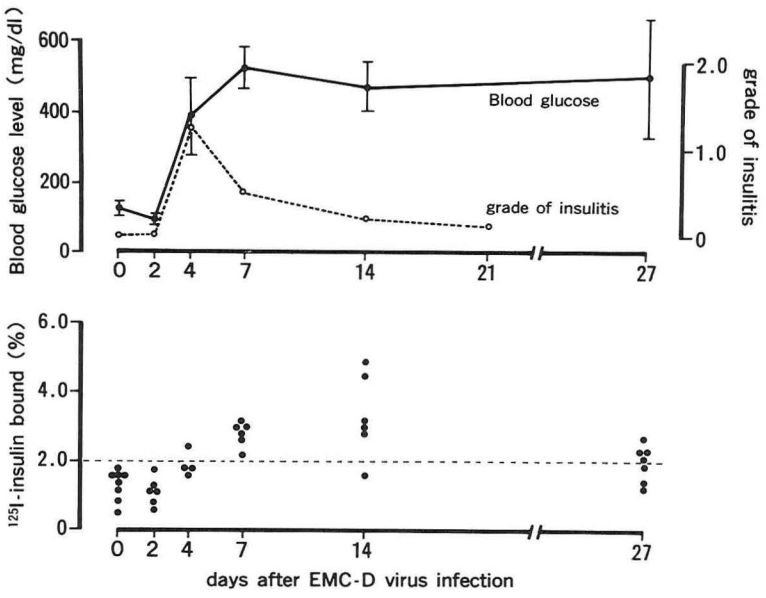

Figure 4. Blood glucose level, grade of insulitis and distribution of insulin autoantibodies in EMC virus-induced diabetes in DBA/2 mice.

in NOD mice without insulitis, then IAA detected by this assay are not sufficient to be a marker of the presence of insulitis. Further studies are necessary because the significance of IAA detected by an ELISA assay may differ from that of IAA detected by a radiobinding assay (23, 24). We have reported that in low-SZ mice IAA could not be detected while insulitis was found in all mice (19). Therefore, IAA may not always be a marker of insulitis or a predictor of diabetes but may, instead, reflect the immune response genotype.

In high SZ mice, IAA were detected in several sera while insulitis was not present. Although we have reported that IAA was not found in high-SZ mice, seven days after SZ injection (19), in the present study, high levels of 125I-insulin bound were found in DBA/2 mice and low levels of 125I-insulin bound were found in C57/BL, Balb/c and CD-1 mice. In EMC mice, both IAA and insulitis were detected. The pathogenesis of high-SZ diabetes in mice is direct chemical damage of pancreatic beta cells by streptozotocin and autoimmunity does not play any role in the development of this diabetes. Also, in EMC mice, autoimmunity does not play any pathogenetic role while insulitis is present (25). Therefore, our results suggest that the IAA appear as a result of destruction of islet beta cells and that the presence of IAA in serum from a diabetic patient does not always mean that autoimmune mechanisms have played some role in the pathogenesis of diabetes in that patient.

In conclusion, these results suggest that IAA alone are not sufficient as a marker of insulitis or the development of diabetes. However it may be possible to predict the development of diabetes by using IAA in combination with other markers, i.e., ICA, HLA and so on. Further investigations are necessary to clarify the significance of IAA.

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