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# Insulin autoantibodies in non-obese diabetic (NOD) mice

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## SUMMARY

Anti-insulin autoantibodies were detected in NOD mice using ELISA. The antibodies were detected as early as at 5 weeks of age, long before onset of clinically overt diabetes, especially in diabetes-prone female mice. The anti-insulin specificity was verified by passage on affinity chromatography insulin columns and demonstration that the anti-insulin activity was located on the $F(ab')_2$ region of the immunoglobulins. The presence of anti-insulin antibodies in prediabetic NOD mice provides a unique possibility for studying their significance and their eventual pathogenic role in the development of insulin-dependent diabetes.

**Keywords** NOD mouse anti-insulin autoantibodies (IAA) ELISA assay

## INTRODUCTION

Accumulating evidence suggests that autoimmune phenomena are responsible for the destruction of insulin-secreting cells in insulin-dependent diabetes mellitus (IDDM) (Soeldner *et al.*, 1985; Eisenbarth, 1986). Two different types of circulating autoantibodies have been reported with a high frequency at clinical onset of human IDDM: islet cell antibodies (ICA) and insulin autoantibodies (IAA). Islet cell antibodies are detected by indirect immunofluorescence (Bottazzo, Florin-Christensen & Doniach, 1974; Bottazzo *et al.*, 1980); insulin autoantibodies are detected by radioimmunoprecipitation (Palmer *et al.*, 1983) or ELISA: (Wilkin, Nicholson & Casey, 1985). Both types of antibody may be detected before onset of clinically overt diabetes, namely before starting insulin therapy, which excludes any relation with immunization by exogenous insulin. They have consequently been considered as useful predictive markers of the disease (Arslanian *et al.*, 1985; Wilkin *et al.*, 1985). Anti-insulin autoantibodies have been reported in BB rats using ELISA (Wilkin *et al.*, 1986). Pontesilli *et al.* (1987) have found IAA in non-obese diabetic (NOD) mice. Interestingly IAA were found in virtually all NOD mouse sera and at any age. In the present report we have screened sera of NOD mice for the detection of IAA before and after diabetes onset on a large set of animals. We show differences in IAA incidence depending on sex and age of diabetes-prone NOD mice.

## MATERIALS AND METHODS

*Animals*

NOD, CBA, BALB/c, DBA/2, C3H/HeN and C57BL/6 mice were bred in our animal facilities. Sera were collected from animals 5–52 weeks old and stored at −70°C. Non-overtly diabetic NOD mice and control mice were split in five groups according to age: group 1, 5–15 weeks, group II, 16–25 weeks, group III, 26–35 weeks, group IV, 36–45 weeks and group V, 46–55 weeks.

Mice were monitored for glycosuria three times a week (Glukotest, Boehringer-Mannheim, Mannheim, FRG). Glycosuric mice were checked for fasting glycaemia using test strips and a colorimetric assay (Haemoglucotest and Reflolux F, Boehringer-Mannheim). Diabetes was diagnosed when permanent fasting glycaemia above 3 g/l occurred.

*Insulin autoantibody assay*

Insulin autoantibodies were evaluated by ELISA as described by Wilkin, Nicholson & Casey, (1985) with slight modifications. Ninety-six well, flat-bottom microplates (CML, France) were coated with 1 μg/well of human recombinant insulin (Actrapid, Novo, Bagsvaerd Denmark) or rat insulin, kindly provided by Dr T. Wilkin (Southampton, England), in 200 μl of 0·05 M bicarbonate buffer, pH 9·6, overnight at 4°C. Wells were then saturated with phosphate-buffered saline 0·15 M PBS, pH 7·5) containing 1% bovine serum albumin (BSA, Cohn fraction V, Sigma Chemical Co, St Louis, Mo) for 1 h at 37°C. This blocking solution was removed and serum samples (100 μl, diluted 1:100 in PBS containing 0·05% Tween 20, PBS-Tween) were added to insulin-coated wells in duplicate for 2 h at room temperature. After washing three times with PBS-Tween, the plates were incubated at room temperature for 2 h with 200 μl of a 1:3 000 dilution of rabbit anti-mouse IgG (Biosys, Compiègne, France) conjugated to horseradish peroxidase. After three additional washes, plates were treated with 0·1 M citrate buffer, pH 5, containing 0·002% $H_2O_2$ and 0·04% *o*-phenylene

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**Table 1.** Frequency of anti-insulin autoantibody (IAA) in different age groups of non-diabetic NOD and control mice

| | Group (weeks) | | | | |
|---|---|---|---|---|---|
| | I (5–15) | II (16–25) | III (26–35) | IV (36–45) | V (46–55) |
| Non-diabetic | (46) | (51) | (35) | (23) | (9) |
| NOD mice | 23·9 | 37·3 | 62·9 | 39·1 | 66·7 |
| | (22) | (25) | (5) | (32) | (18) |
| Control mice | 22·7 | 8·0 | 0·0 | 0·0 | 0·0 |

Results are expressed as percentage IAA-positive, and the number of animals tested is given in parentheses.

diamine (Sigma Chemical Co.). The absorbance in each well was read at 492 nm using an MR600 microplate reader (Multiscan Dynatech, Sarl France). Each sample was studied in duplicate.

*Purification of anti-insulin antibodies in mouse sera*

Pooled sera were purifed using affinity chromatography on CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with insulin. Anti-insulin autoantibodies were eluted using 0·1 M glycine- HCl, pH 2·8. Two-hundred-microlitre fractions were collected, neutralized with Tris buffer saline (1 M TBS, pH 9) and assayed by ELISA as described above. The same pools were run in parallel through a CNBr-activated Sepharose 4B coupled with BSA (Sigma Chemical Co.) as control.

*Preparation of $F(ab')_2$ fragments*

Two milligrams of immunoglobulins from either non-diabetes-prone (DBA/2, CBA, BALB/c, C3H/HeN) mice or high IAA titre NOD mice were purified by affinity chromatography on a protein A-Sepharose CL-4B (Pharmacia) column. They were then treated with 60 μg of pepsin (Sigma Chemical Co.) in 3 ml 0·1 M acetate buffer, pH 3·9, for 14 h at 37°C. The reaction was stopped by adding 150 μl of TBS. The preparation was dialysed against PBS and the Fc fragments were removed by running through a protein A-Sepharose CL-4B column.

*Expression of results*

Anti-insulin activity was expressed as absorbance read at 492 nm ($A_{492}$). Positivity was defined as absorbance above mean of normal controls + 2 s.d. Statistical evaluation was performed using the $\chi^2$ test and Student's $t$-test.

The intra- and inter-assay C.V. were 1·8% ($n = 7$) and 15·9% ($n = 13$) respectively, using human insulin and a standard NOD mouse serum.

The expression of data did not take into account non-specific binding of individual sera. Standard samples included a NOD mouse serum pool, a NOD monoclonal anti-insulin autoantibody and a DBA/2 mouse serum pool. The standard samples were tested for non-specific binding on uncoated wells and those coated with insulin. The absorbance difference

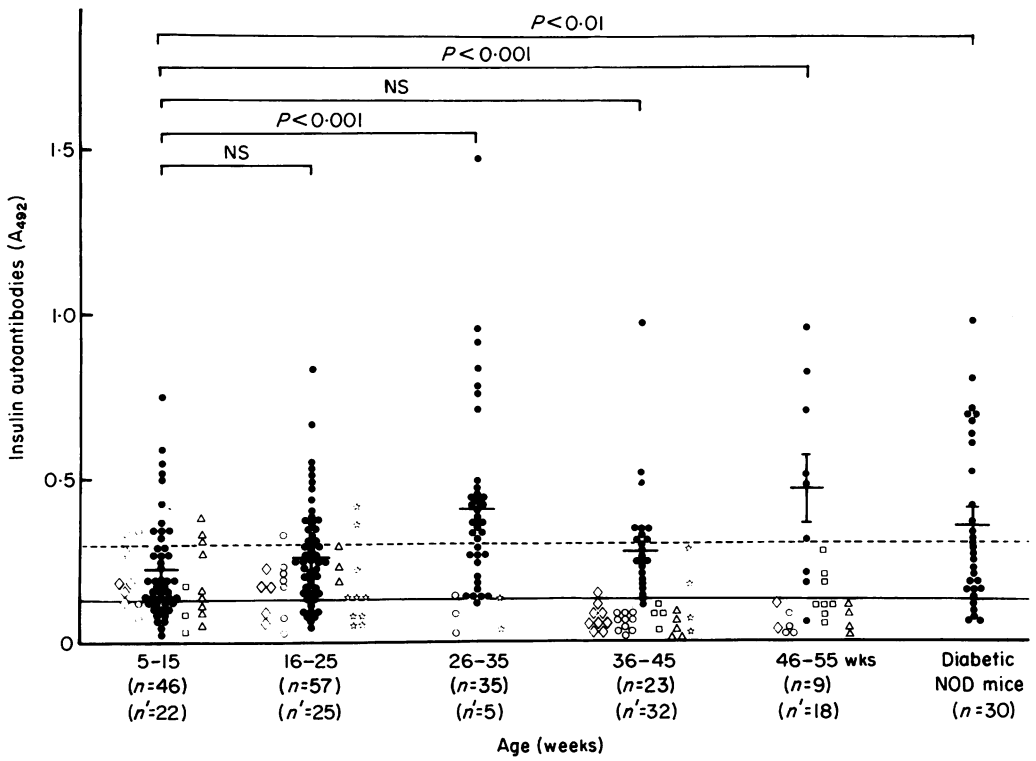


**Fig. 1.** Anti-insulin autoantibody in NOD and control mice. Each point indicates the absorbance of an individual serum: NOD (●); DBA/2 (○); C3H/HeN (△); C57BL/6 (☆); BALB/c (◇); CBA (□). Groups are as indicated in the *Materials and Methods* section. Continuous and broken lines represent mean of control mouse results and mean + 2 s.d., respectively. The statistical evaluation applies to comparisons between NOD group I and group II, III, IV, V and diabetic mice, respectively. NS not significant.

**Table 2.** Comparison of anti-insulin autoantibodies between male and female non-diabetic NOD mice in the five age groups

| | Group (weeks) | | | | |
|---|---|---|---|---|---|
| | I (5–15) | II (16–25) | III (26–35) | IV (36–45) | V (46–55) |
| $A_{492}$ | | | | | |
| Males | 0·209±0·063 | 0·181±0·031 | 0·289±0·093 | 0·208±0·009 | 0·233±0·091 |
| Females | 0·261±0·032 | 0·301±0·027 | 0·440±0·054 | 0·315±0·049 | 0·664±0·11 |
| IAA-positive (%) | | | | | |
| Males | 0·0 | 25·0 | 42·9 | 14·3 | 25·0 |
| Females | 29·7 | 41·0 | 67·9 | 50·0 | 98·0 |

The values of $A_{492}$ are expressed as mean±s.d.

between insulin-coated wells and uncoated wells was: 0·74±0·06 for the NOD serum standard, 0·65±0·04 for the NOD monoclonal standard and 0·049±0·02 for the DBA/2 serum standard (mean±s.e.m., $n=12$). Specific binding could also be evaluated as 74·7%, 85·5% and 32% of total binding for the NOD serum standard, the NOD monoclonal standard and the DBA/2 serum standard, respectively.

## RESULTS

*Anti-insulin reactivity in NOD mouse sera*

Sera from 164 non-diabetic NOD mice, 30 diabetic NOD mice, 30 diabetic NOD mice and 102 control DBA/2, C3H/HeN, C57BL/6, BALB/c and CBA mice (mean age 25±1 weeks, range 5–55 weeks), were studied. Anti-insulin antibodies were detected in 41% of non-diabetic NOD mice, 46% of diabetic NOD mice and 6·9% of control mice, with a statistically significant difference between NOD and control mice ($P<0·001$).

Table 1 shows that the difference in IAA incidence between non-diabetic, NOD and control mice is significant in groups II ($P<0·01$), III ($P<0·01$), IV ($P<0·001$) and V ($P<0·001$), but not in younger animals (group I). The age-dependency of IAA production in NOD mice is also indicated by the significantly higher percentage of positive sera within non-diabetic mice in groups III and V than in group I ($P<0·001$). Similar differences appear when considering means of IAA serum levels as defined by absorbance at 492 nm (Fig. 1). Interestingly, in all groups showing the presence of IAA (groups II–V), females had a higher incidence ($P<0·01$) and a higher level ($P<0·05$ in groups II and V) of antibodies than males (Table 2).

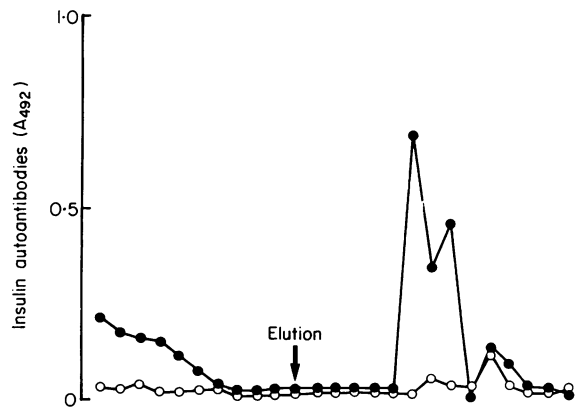


**Fig. 2.** Specificity of anti-insulin antibodies. A pool of 10 NOD mouse sera (●), selected for their high anti-insulin activity ($A_{492}>0·7$), was purified on an insulin-CNBr-Sepharose 4B. Each fraction was tested in ELISA. A pool of control mouse sera (○) was purified in parallel and the fractions tested in ELISA.

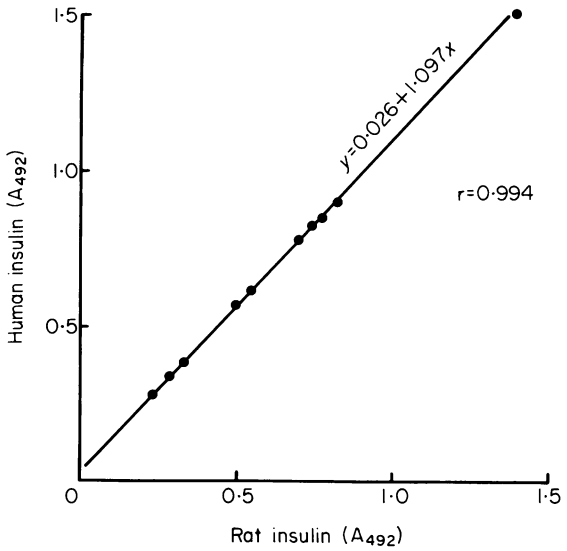


**Fig. 3.** Comparison of NOD mouse IAA reactivity with human and rat insulins. Ten sera from either diabetic or non-diabetic NOD mice were tested in ELISA using human insulin or rat insulin. The IAA serum levels (as defined by absorbance at 492 nm) are represented. There is no significant difference in ELISA using either human or rat insulin.

*Specificity of anti-insulin antibodies*

A pool of 10 NOD mouse sera selected for their high anti-insulin activity ($A_{492}\geq 0·7$) was purified on an insulin-CNBr-Sepharose 4B column. The anti-insulin antibody activity was present in fractions 16–19 obtained after fixation and elution from the

column, as indicated on Fig. 2, whereas fractions passed through the insulin column were devoid of IAA activity.

The passage of the same serum pool on a BSA-CNBr-Sepharose 4B column was performed in parallel. Fractions 1–10 passed through the BSA-column contained a high anti-insulin activity ($A_{492}=1{\cdot}12$) whereas fractions 11–24 non-specifically fixed and then eluted from the column contained a low anti-insulin activity ($A_{492}=0{\cdot}35$) (data not shown).

*Anti-insulin activity of F(ab′)$_2$ fragments*

The F(ab′)$_2$ fraction prepared from a pool of IAA containing NOD mouse sera was positive in ELISA ($A_{492}=0{\cdot}515$) whereas F(ab′)$_2$ fractions prepared from a pool of non-diabetes-prone mouse sera remained negative ($A_{492}=0{\cdot}195$). The 25 kD molecular weight of the F(ab′)$_2$ fractions obtained in these experiments was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis performed in reducing conditions.

*Species specificity of IAA*

Sera from either diabetic or non-diabetic NOD mice detected as positive in ELISA using human insulin were shown to be also positive when using rat insulin, known to possess the same amino acid sequence as mouse insulin. These data indicate that, in ELISA, IAA autoantibodies are not species specific (Fig. 3).

## DISCUSSION

This study demonstrates the presence of anti-insulin antibodies in NOD mice. The insulin specificity of the antibody activity detected was assessed by absorption experiments using an insulin immunosorbant. The antibodies detected may be considered as autoantibodies, since the mice had not received any injection of exogenous insulin at the time of blood collection. Surprisingly IAA were detected in 22·7% of the young control mice tested (group I), raising the issue of their significance in non-diabetes-prone animals. This observation may relate to previous study of natural autoantibodies in neonatal mice (Dighiero *et al.*, 1985).

Anti-insulin autoantibodies (IAA) were found in diabetic NOD mice but also, and interestingly, in non-diabetic NOD mice, as described already by Pontesilli *et al.* (1987). Although non-overtly diabetic NOD females had a higher incidence and a higher level than males, no difference was found between female and male control mice. It seems therefore that age (> 15 weeks) and sex (female), but not overt diabetes, are associated with IAA production in NOD mice. IAA production before diabetes onset has been reported also in BB rats using ELISA (Wilkin *et al.*, 1986) and in humans using a radioimmunoprecipitation assay (Palmer *et al.*, 1983). The species specificity of IAA appears controversial in the light of our data. Although the reactivity of IAA in some BB rats has been shown to be surprisingly higher using pig or human insulin as substrate rather than using rat insulin (Dean *et al.*, 1987), we observed a similar reactivity in NOD mice using either human or rat insulin.

Several interpretations may be proposed to explain these observations. IAA production could be secondary to aggression against $\beta$ cells by the autoimmune process, directed against islet cell membrane antigens, resulting in release of particularly immunogenic insulin (or pro-insulin). They would then only represent a marker of $\beta$ cell destruction as suggested in humans for cytoplasmic anti-islet cell antibodies, detected by indirect immunofluorescence (Bottazzo *et al.*, 1974; 1980).

Alternatively IAA could participate in the onset of autoimmunity, notably by interaction with the idiotypic network. In any case, NOD mice provide a very useful model to investigate further the significance of IAA in prediabetic states.

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## REFERENCES

Arslanian, S.A., Becker, D.J., Rabin B., Atchison, R., Eberhart, M., Cavender, D., Dorman, J. & Drash, A.L. (1985) Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. *Diabetes*, **34**, 926.

Bottazzo, G.F., Florin-Christensen, A. & Doniach, D. (1974) Islet-cell antibodies in diabetes mellitus with autoimune polyendocrine deficiencies. *Lancet* **ii**, 1279.

Bottazzo, G.F., Dean, B.M., Gorsuch, A.N., Cudworth, A.G. & Doniach, D. (1980) Complement fixing islet-cell antibodies in type I diabetes: Possible monitors of active beta-cell damage. *Lancet* **i**, 668.

Dean, B.M., Bone, A.J., Varey, A.M., Walker, R., Baird, J.D. & Cooke, A. (1987) Insulin autoantibodies, islet cell surface antibodies and the development of spontaneous diabetes in the BB/Edinburgh rat. *Clin. exp. Immunol.* **69**, 308.

Dighiero, G., Lymberi, P., Holmberg, D., Lundquist, I., Coutinho, A. & Avrameas, S. (1985) High frequency of natural autoantibodies in normal newborn mice. *J. Immunol.* **134**, 765.

Eisenbarth, G.F. (1986) Type I Diabetes Mellitus. A chronic autoimmune disease. *N. Engl. J. Med.* **314**, 1360.

Palmer, J.P., Asplin, C.M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P.K. & Paquette, T.L. (1983) Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science*, **222**, 1337.

Pontesilli, O., Carotenuto, P., Gazda, L.S., Pratt, P.F. & Prowse, S.J. (1987) Circulating lymphocyte populations and autoantibodies in non-obese diabetic (NOD) mice: a longitudinal study. *Clin. exp. Immunol.* **70**, 84.

Soeldner, J.S., Tuttleman, M., Srikanta S., Ganda O.P. & Eisenbarth, G.S. (1985): Insulin dependent diabetes mellitus and autoimmunity: islet-cell auto-antibodies, insulin auto antibodies and beta-cell failure. *N. Engl. J. Med.* **313**, 893.

Wilkin, T., Nicholson, S. & Casey, C. (1985) A microenzyme-linked immunosorbent assay for insulin antibodies in serum. *J. Immunol. Meth.* **76**, 185.

Wilkin, T., Armitage, M., Casey, C., Pyke, D.A., Hoskins, P.J., Rodier, M., Diaz, J.L. & Leslie R.D.G. (1985) Value of insulin antibodies as serum markers for insulin-dependent diabetes mellitus. *Lancet* **i**, 480.

Wilkin, T., Kiesel, U., Diaz, J.L., Burkart, V. & Kolb, H. (1986) Autoantibodies to insulin as serum markers for autoimmune insulitis. *Diab. Res.* **3**, 173.