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Rapid communication

Insulin autoantibodies in the pre-diabetic period: Correlation with islet cell antibodies and development of diabetes

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Summary. IgG and IgM class insulin autoantibodies were measured by an enzyme-linked immunosorbent assay in sera from members of the Barts-Windsor-Middlesex prospective family study for Type 1 (insulin-dependent) diabetes. One hundred and twelve individuals from 28 families were selected for study on the basis of a clearly defined islet cell antibody status. IgG insulin autoantibodies were found to be significantly associated with islet cell antibody positive ($n=30$) versus islet cell antibody negative ($n=57$) first degree family relatives ($p=0.002$), with increased significance ($p=0.0003$) if complement-fixing (CF)-islet cell antibody individuals ($n=20$) only were considered. In addition, a significant association of IgG insulin autoantibodies with subsequent development of diabetes was observed within the CF-islet cell antibody positive group ($p<0.0003$). No such associations were found for IgM insulin autoantibodies, but a higher prevalence of these autoantibodies was observed in islet cell antibody negative first degree relatives ($n=57$) compared with a control group of 73 Blood Bank donors ($p=0.00007$), and they were significantly associated with siblings ($n=48$) rather than parents ($n=39$), ($p=0.001$). We conclude that the presence of IgG insulin autoantibodies and CF-islet cell antibodies confer more risk for future development of diabetes than the presence of either marker alone.

Key words: Insulin autoantibodies, pathogenesis.

The initial report by Palmer et al. [1] of the presence of insulin autoantibodies (IAA) in a subset (32%) of newly diagnosed Type 1 (insulin-dependent) diabetic patients prior to therapy has subsequently been confirmed by similar studies [2, 3]. More recently, Soeldner et al. [4] have demonstrated the simultaneous appearance of IAA and islet cell antibodies (ICA) in the serum of a patient 6 years before the development of diabetic symptoms. Whilst these data suggest that IAA could provide an additional marker for individuals at risk for the development of diabetes, other findings [5] indicate that their presence is more compatible with genetic individual susceptibility rather than established disease.

The Barts-Windsor-Middlesex Family Study (BWM-FS), started in 1978, is a prospective study of 207 Type 1 diabetic probands diagnosed under the age of 20 and their families [6]. All participants have been HLA-A, B, C and DR phenotyped, and their sera screened at regular intervals (3–6 months) for autoantibodies to islet and other endocrine cells. During the past 7 years, 12 first degree relatives of the probands have become diabetic, all being ICA positive prior to diagnosis. Others are regarded as "high risk" candidates for the development of diabetes because of the presence of complement-fixing (CF)-ICA in their sera and their HLA haplo- and/or identity with the proband. The availability of multiple sequential serum samples from well-documented family members has prompted the development of a new rapid screening assay for insulin antibodies. A sensitive micro enzyme linked immunosorbent assay (Elisa) has been devised to detect specifically IgG and IgM class insulin-binding antibodies.

The method is described in detail here, together with the results obtained from insulin-naive and insulin-dependent members of the BWM-FS.

Subjects and methods

Family members

One hundred and twelve subjects from 28 families in the Barts-Windsor-Middlesex Family Study (BWM-FS) were chosen for this study. Selection was based primarily on a clearly defined ICA status; i.e. for inclusion in the ICA positive group a patient was required to have at least 5 sequential serum samples scored positive by immunofluorescence employing an anti-IgG conjugate. A corresponding number of positive observations with an anti-C3 conjugate were necessary for inclusion in the CF-ICA positive category. HLA-A, B, C and DR phenotypes were not considered among the criteria for inclusion of individuals in the study. Eighty-seven first degree relatives were selected. Twenty (15 siblings, 5 parents) were CF-ICA positive and 11 (8 siblings, 3 parents) from this group developed Type 1 diabetes since entering the study. The ICA positive but CF-ICA negative group included 1 parent and 9 siblings; one of the latter subsequently became diabetic. The remaining 57 (24 siblings, 33 parents) were constantly scored as both ICA and CF-ICA negative. All first degree relatives and 25 Type 1 (insulin-dependent) diabetic family members (22 probands, 3 parents) were screened for IAA at entry tc the study. In addition, a control group of 73 normal individuals aged 20–40 (Blood

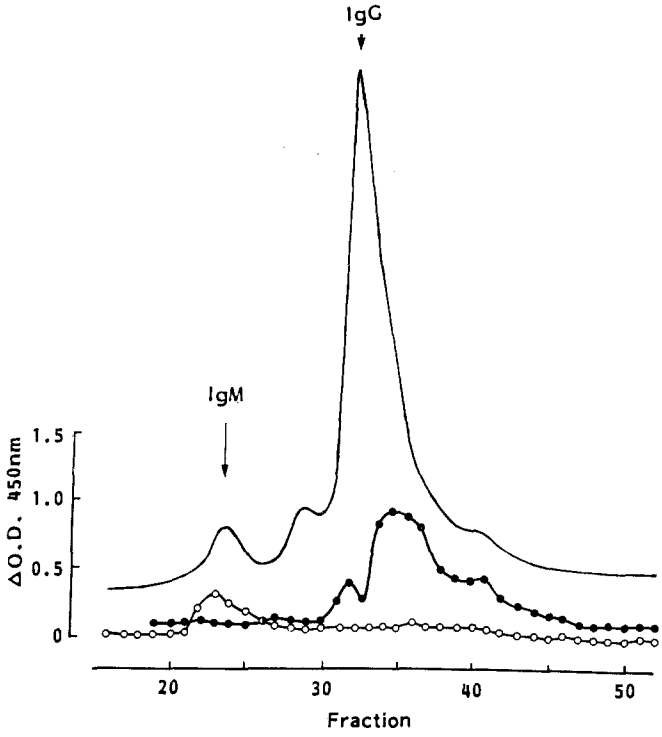

Fig. 1. Separation of IgG and IgM insulin autoantibodies (IAA) from the serum of a non-diabetic child by high performance liquid chromatography. Serum (150 µl) was applied to a TSKG 4000 SW column (7.5 × 600 mm) and the column was eluted with phosphate buffered saline (1 mol/l) at 0.5 ml/min, with separation of 1 ml fractions (LKB, Surrey, UK). The protein elution trace (—) is shown, with arrows indicating peak fractions for total IgM and IgG. (○—○), (●—●) denote IgM and IgG IAA measurements respectively by Elisa in the separated fractions (50 µl)

Bank donors) were investigated for the presence of IAA. The control group formed part of an ongoing study of the immune response to tetanus toxoid vaccination, and all were ICA negative.

Class-specific insulin antibody Elisa

The method was essentially as described recently [7, 8], but the conditions have been optimised to enable simultaneous screening for IgG and IgM class IAA. Recrystallised semi-synthetic human insulin (kindly donated by Novo, Copenhagen, Denmark) was dissolved in 0.05 mol/l Na carbonate-bicarbonate buffer, pH 9.6 (1 µg/ml), and 100 µl aliquots were pipetted into alternate rows of a 96-well, flat bottomed microplate (Nunc Immunoplate II, Gibco, Paisley, Scotland); the remaining rows (control wells) received 100 µl of coating buffer alone. After 5-h incubation, the plates were emptied and washed (3 × 3 min, 220 µl/well) with 0.015 mol/l phosphate buffered saline (PBS) containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma, Dorset, UK). These and all subsequent incubations and washings were carried out at room temperature. All wells were blocked for non-specific binding by incubation with PBS containing 20% heat-inactivated normal goat serum (NGS, Sera Labs, Sussex, UK) for 18 h (120 µl/well). The blocking solution was removed and serum samples (100 µl, diluted 1:100 in PBS containing 5% Tween 20 and 10% NGS) were added to antigen-coated and control wells in triplicate and incubated for 2 h. After removal of the test solutions and a further controlled washing with PBS/0.1% Tween 20 as above, plates were incubated for 1 h with affinity purified goat antihuman horseradish peroxidase conjugates (Tago, Burlingame, CA, USA). For detection of IgG class antibodies, the γ-chain specific conjugate (Code No 2390) was used at 1:2000 dilution (100 µl/well). For IgM antibodies the μ-chain specific conjugate (Code No 2392) was used (100 µl/well) at 1:500 dilution (batch 92.13.01) or 1:1000 dilution (batch 92.15.01). The conjugates were removed, and, after washing the plates × 3 as described previously, 100 µl substrate was added to each well. Substrate solutions were prepared freshly by dissolving 3,3′-5,5′-tetra methyl benzidine (Aldrich, Gillingham, Dorset, UK) in dimethyl sulphoxide (10 mg/ml) in 0.05 mol/l citric acid, 0.1 mol/l phosphate buffer (pH 5.0, 100 ml) and adding 20 vol H_2O_2 (100 µl) immediately prior to use. The reactions were stopped after 12 min (IgG) and 20 min (IgM) by addition of 12% H_2SO_4 (50 µl/well) and plates were read at 0.D.450 nm on an automated MR 580 Microelisa reader (Dynatech, Santa Monica, CA, USA). For each assay the instrument was set at zero using a well containing PBS-0.1% Tween 20 (150 µl).

Calculation of results

The specific Optical Density (Δ O. D.) for each sample was calculated by subtraction of the non-specific value recorded for the corresponding control well. Standard reference sera (normal and diabetic, known to contain either IgG or IgM antibodies) were included in each assay for controls. The intra-assay coefficients of variation (C.V.) were found to be 3.2% and 7.2% for these IgG and IgM standards respectively ($n=15$). Because the inter-assay C.V. were somewhat higher (13% and 20% respectively, $n=13$), due mainly to the use of different batches of conjugates, all Δ O. D. values have been corrected for inter-assay variation using the appropriate IgG or IgM class factor as follows: $\frac{\Delta \text{ O.D. of standard reference initially observed}}{\Delta \text{ O.D. of standard reference in a particular assay.}}$

Statistical analysis

Because the different groups studied have shown skewed distributions of IAA, non-parametric statistics have been preferred [9]. The Mann-Whitney U rank test has been used throughout, with $p<0.05$ being chosen as the level of statistical significance.

Results

A log-linear relationship was observed for the dilution curves of the standard reference sera covering Δ O.D. values in the positive range (0.08–1.4 for IgG and 0.15–1.4 for IgM). Competitive inhibition studies using purified human insulin have shown that physiological levels of insulin do not affect the binding of either class of antibody to the antigen-coated wells (50% inhibition of antibody binding was observed at 5.5, 15 and 20 mU in 3 sera with IgG IAA and at 150, 300 and 380 mU insulin, respectively, in a further 3 sera with IgM IAA). Confirmation that the γ- and μ-chain conjugates employed are not cross-reactive and detect IgG or IgM insulin antibodies was obtained by high performance liquid chromatography separation of these immunoglobulins in the serum of a non-diabetic child found to contain both classes of IAA (Fig. 1).

IgG insulin autoantibodies

The prevalence of IgG IAA in the various groups studied is shown in Figure 2 a, together with the results obtained by this assay for the group of 25 insulin-dependent patients (duration of treatment 1–22 years). No significant difference was observed for the distribution of IAA in the 57 ICA negative first degree relatives and the control group of 73 Blood Bank donors ($p=0.130$). However, a significant difference was found between the ICA positive group ($n=30$) and the ICA negative group ($p=0.002$), with increased significance ($p=0.0003$) if CF-ICA positive individuals ($n=20$) only

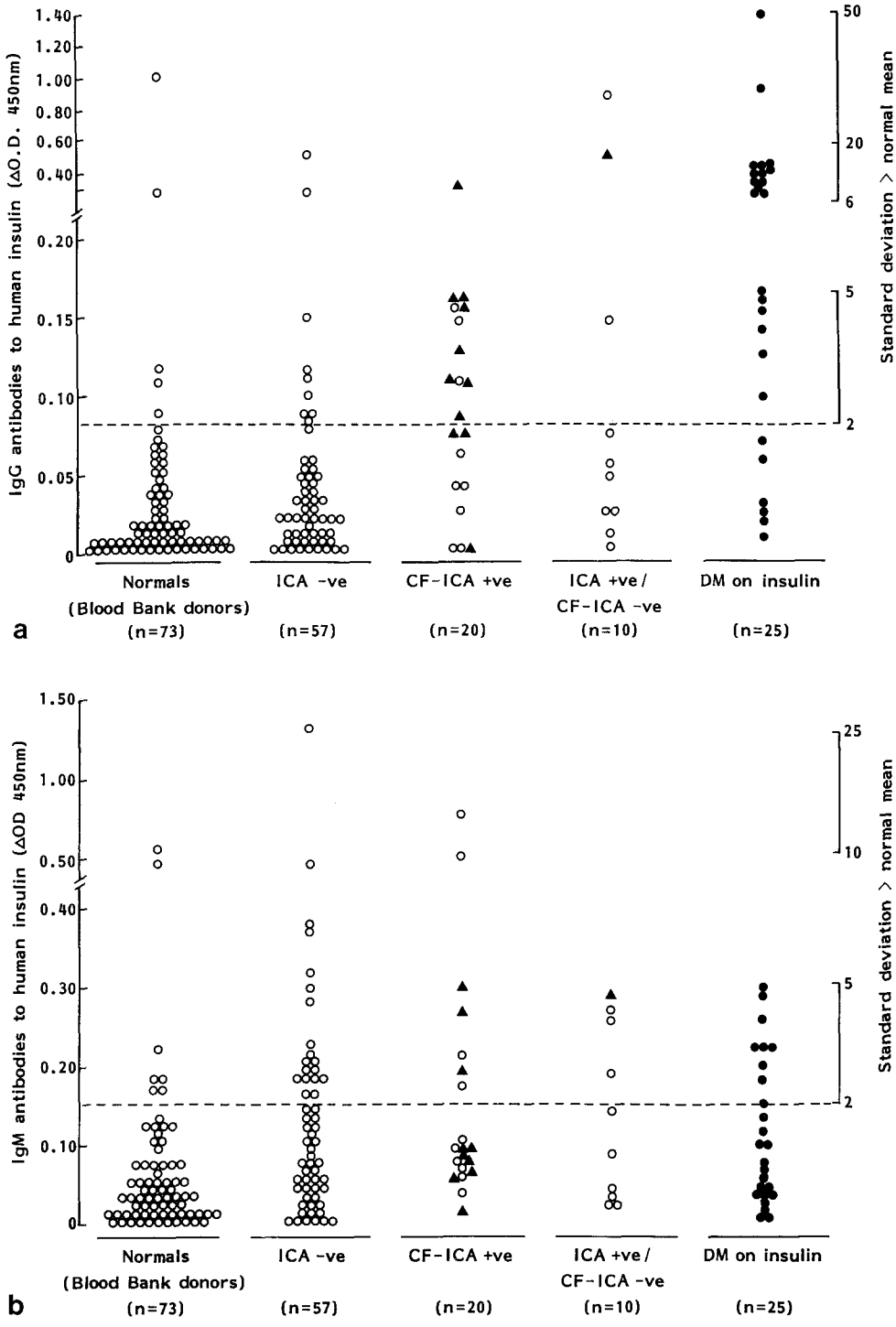

Fig. 2 a and b. Distribution of **a** IgG and **b** IgM class insulin antibodies for normal subjects, first degree relatives (○) and Type 1 diabetic (DM) family members (●). Subsequent development of diabetes is denoted by (▲). Significant differences between groups: **a** ICA positive ($n=30$), CF-ICA positive ($n=20$) versus ICA negative group ($n=57$), $p=0.002$, $p=0.0003$ respectively. **b** ICA negative ($n=57$) versus normal subjects ($n=73$), $p=0.00007$

were considered. Two of the control group had Δ O.D. values > mean + 5 SD of the group, so their values have been omitted in calculation of the normal mean and SD (Δ O.D. = 0.024 ± 0.028, $n=71$; corresponding mean non-specific binding = O.D. 0.045 ± 0.03, $n=72$). Regarding values > normal mean + 2 SD as positive, 14 (47%) of the ICA positive group versus 9 (16%) of the ICA negative first degree relatives were IAA positive. Eleven (55%) of the CF-ICA positive group were thus positive for IAA and 8 (73%) of these subsequently developed Type 1 diabetes at intervals ranging from 3 months to 7 years since the initial IAA screening. Likewise, 3 (30%) of the 10 who were ICA positive but CF-ICA negative were IAA positive (1 has since become diabetic) and 19 (76%) of the insulin-dependent patients had antibodies reactive with human insulin. None of the 9 IAA positive/ICA negative individuals, as yet, has developed diabetes, and the three subjects who were CF-ICA/IAA positive remain healthy 7 years after the initial IAA detection.

IgM insulin autoantibodies

The results of the screening for IgM IAA are presented in Figure 2 b. No significant difference in the distribution of these antibodies was observed, either for the

ICA positive versus the ICA negative group ($p=0.240$) or the CF-ICA positive versus ICA negative group ($p=0.087$). A significant difference was observed, however, for IgM IAA in ICA negative first degree relatives versus the control subjects ($p=0.00007$), and a higher prevalence of these antibodies was associated with siblings ($n=48$) rather than parents ($n=39$) ($p=0.001$) in contrast to the results obtained for IgG IAA ($p=0.051$). As for IgG IAA, 2 values > mean + 5 SD of the control group were discarded for calculation of normal mean + SD (Δ O.D. = 0.053 + 0.051, $n=71$; corresponding mean non-specific binding = O.D. 0.053 + 0.032, $n=71$). In this case, regarding Δ O.D. values > normal mean + 2 SD as positive, 11 (37%) of the ICA positive group were IAA positive, but only 4 of these (3 CF-ICA positive, 1 CF-ICA negative) progressed to diabetes. On the same basis, 19 (33%) of the ICA negative first degree relatives and 8 (32%) of the diabetic group were IAA positive.

Discussion

The use of class-specific conjugates in an Elisa has revealed that IgG IAA are significantly associated with CF-ICA, and that possession of both serological markers indicates increased risk for Type 1 diabetes than that associated with the presence of either marker alone. IgM IAA, on the other hand, did not show such an association. Although the present study is based on screening of initial samples collected many months before the onset of diabetic symptoms, sequential analysis of follow-up sera from this cohort (unpublished) has confirmed our initial data. The IgG class specific results obtained here by Elisa support recent observations by Srikanta et al. [3], which suggests that IAA measured by liquid phase radioimmunoassay (RIA) could be preferentially of IgG class. However, the incidence of IAA in populations at risk appears lower when measured by RIA [1-4] than by the enzymatic method. Nevertheless, our conclusions differ from those of Wilkin et al. [6] who found no association of IAA with ICA using an Elisa. This latter discrepancy is perhaps accounted for by our separate analysis of IgG and IgM IAA.

A clue to the lack of association of IgM IAA with ICA and subsequent development of diabetes is provided by the higher incidence of IgM IAA in siblings than parents. This phenomenon is particularly emphasised in the 57 ICA negative first degree relatives, where 33% had IgM IAA. Recently, we have observed elevated levels (> 10 SD above normal mean) of IgM but not IgG IAA in a high proportion of patients (mainly children) with serologically proven recent viral infections [10]. The significance of these findings is now under investigation, but they may reflect an immune mechanism triggered by a classical carrier-hapten system with the final production of IAA. Whether the same mechanisms account for the unexpected finding of raised IAA levels in a small proportion of adults undergoing vaccination remains to be established.

The main message, however, is that in the group of 30 ICA positive first degree relatives studied here, only 5 of 14 individuals (3 CF-ICA positive, 2 CF-ICA negative) with IgG IAA have not developed diabetes during the prospective study. We strongly support regular screening for IAA in conjunction with ICA in genetically predisposed invididuals [3]. The data, collected in such a prospective manner, should provide new clues for early detection of B cell metabolic dysfunction.

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