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Antibodies to a M_r-64000 islet cell protein in Swedish children with newly diagnosed Type 1 (insulin-dependent) diabetes

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Summary. Sera from 40 Swedish children diagnosed as having Type 1 (insulin-dependent) diabetes mellitus during a one year period along with 40 age and geographically matched control subjects were tested for antibodies to a M_r-64000 islet protein by immunoprecipitation of ^{35}S-methionine-labelled rat islet amphiphilic proteins. Of the 40 diabetic patients, 29 (73%) were found to be positive whereas all 40 control subjects were negative. Samples were also tested for titres of islet cell cytoplasmic antibodies by indirect immunofluorescence on frozen sections of human pancreas. In the diabetic group, 30 of the 40 patients (75%) were positive for islet cell cytoplasmic antibodies compared with 2 of the 40 control subjects (5%). A comparison of levels of antibodies to the M_r-64000 protein with islet cell cytoplasmic antibodies revealed a weak ($r_s = 0.46$), but significant ($p < 0.01$) correlation between the two tests. There was no effect of age or sex on levels of antibodies to the M_r-64000 protein. These results in population-based diabetic children and control subjects demonstrate a high frequency of antibodies to the M_r-64000 protein at the time of clinical onset.

Key words: Type 1 (insulin-dependent) diabetes mellitus, islets of Langerhans, autoantibodies, autoantigens, autoimmune disease.

Humoral autoimmunity to pancreatic islet cell antigens is a common feature associated with the onset of Type 1 (insulin-dependent) diabetes. The appearance of islet cell antibodies in the period preceding the clinical onset of the disease has aroused considerable interest as autoantibodies could potentially serve as markers of early phase B-cell destruction [1]. Substrates commonly used in the detection of islet cell antibodies include frozen sections of pancreas (typically islet cell cytoplasmic antibodies, ICA) [2, 3] or suspensions of islet cells (islet cell surface antibodies, ICSA) [4]. These assays suffer from the potential of antisera to bind to both specific and non-specific islet cell components. The identification of individual islet cell antigens will allow the development of accurate tests for islet cell antibodies. Characterisation of these antigens and the antibody responses to them should provide a clearer understanding of the relevance of B-cell autoimmunity in the pathogenesis of Type 1 diabetes.

A human islet cell protein of M_r-64000 (64k antigen) has been identified as one to which antibodies are frequently found in Type 1 diabetes [5]. In the original study, 8/10 recently diagnosed diabetic patients were found to be positive for antibodies to the 64k antigen, and in a subsequent study [6], antibodies were detected up to 91 months before the clinical onset of Type 1 diabetes in 11/14 individuals. Upon testing control groups of 21 healthy individuals and 41 patients with other autoimmune disorders, antibodies to the 64k antigen were only detected in one individual, a healthy sibling to a diabetic patient [6]. Thus, these studies provided evidence for a humoral response to the 64k antigen that may be specific for Type 1 diabetes. However, the frequency of antibodies to the protein in Type 1 diabetes cannot be unambiguously assessed from these studies since the sample sizes were small and were not drawn from well-defined populations. In the present study, we have tested a representative sample of children developing diabetes in Sweden together with appropriate control subjects to estimate the frequency of antibodies to the 64k antigen in children at clinical onset of Type 1 diabetes. In addition, we have examined a possible correlation between autoantibodies to the 64k antigen with those measured as ICA and have assessed possible patterns in 64k autoantibody levels with regard to age, sex and geographical origin of the diabetic patients.

Subjects and methods

Serum samples

In Sweden, all children with diabetes are referred to paediatric departments and are hospitalised for a period after diagnosis. This system allows the identification of virtually all children with newly diagnosed diabetes. The Swedish Childhood Diabetes Study, with all 44 paediatric departments in Sweden collaborating, is a case-control study designed by Dahlquist and co-workers [7]. As part of this investigation [8] serum samples have been obtained within 2 weeks of diagnosis from 389 of the 405 incident cases (aged 0–14 years) of Type 1 diabetes in Sweden during the period 1 September 1985 to 31 August 1986. In addition, sera from 321 age, sex and geographically matched control subjects were collected in parallel. From these populations study groups of 40 diabetic and 40 control individuals were selected for measurement of antibodies to the M_r-64000 protein. In view of seasonal variations in incidence rate of diabetes, we have selected every tenth individual developing diabetes to obtain an even distribution of patients throughout the study period. The mean delay after diagnosis of diabetes in the incident cases and sampling the reference subjects was 1.6 ± 0.3 months. Details of the total population and study groups are given in Table 1.

Sera from a recently diagnosed diabetic patient previously shown to be strongly positive for antibodies against the M_r-64000 protein (patient 3, reference 5), and from a normal, healthy blood donor were included in each analysis of antibodies against the M_r-64000 protein.

The study was approved by the Ethics Committee of the Karolinska Institute, Stockholm, Sweden.

Measurement of islet cell cytoplasmic antibodies

End point titres of ICA were determined using a two colour-immunofluorescence test [9, 10] on serial dilutions (1:2, 1:4, 1:8, etc.) of the 80 serum samples. To facilitate interlaboratory comparison, titres have been converted to Juvenile Diabetes Foundation (JDF) units by comparison with a standard curve constructed with a standard reference serum as previously described [11].

Measurement of antibodies to the M_r-64000 islet protein

Levels of antibodies to the M_r-64000 islet cell protein were measured by immunoprecipitation of rat islet cell extracts using a modification of methods previously described [6].

Islets were isolated from 5-7-day old Wistar rats by collagenase digestion of pancreata [12]. Islets were individually selected under the dissecting microscope and maintained in tissue culture for 1–3 days in RPMI-1640 medium containing 4.2 mmol/l $NaHCO_3$, 20 mmol/l Hepes, 10% (w/v) newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. After washing twice by centrifugation (1 min, 200 *g*) in methionine-free RPMI-1640 containing 16 mmol/l glucose, 4.2 mmol/l $NaHCO_3$, 20 mmol/l Hepes, 2% (v/v) Nuserum (Teknunc, Roskilde, Denmark), 100 U/ml penicillin and 100 µg/ml streptomycin, islets (batches of 2000) were incubated for 5 h in 4 ml of the same medium supplemented with 2 mCi of ^{35}S-methionine (Amersham International, Amersham, UK; >500 Ci mmol^{-1}). After labelling, islets were washed twice in 5 ml of Hanks Solution containing 2% (v/v) Nuserum and 0.1 mmol/l methionine and once in 150 mmol/l NaCl, 10 mmol/l Hepes (pH 7.4), 0.5 mmol/l methionine and 10 mmol/l benzamidine.

The labelled islets were homogenised for 30 s in 300 µl of 0.25 mmol/l sucrose, 10 mmol/l Hepes (pH 7.4), 0.5 mmol/l methionine, 10 mmol/l benzamidine, 0.1 mmol/l p-chloromercuribenzenesulphonic acid and 0.5% (w/v) Trasylol (Novo, Bagsværd, Denmark) using a Polytron homogeniser. The homogenate was centrifuged at 36,000 *g* for 30 min at 4° C. The pellet was resuspended in 150 µl of 2% Triton X-114 in Hepes buffer (10 mmol/l Hepes (pH 7.4) supplemented with 150 mmol/l NaCl, 0.5 mmol/l methionine, 10 mmol/l benzamidine and 0.5% (w/v) Trasylol). Proteins were extracted on ice for 2 h. Clumps of particulate matter were disrupted by repetitive pipetting and insoluble matter after extraction removed by centrifugation at 16,000 *g* for 20 min at 4° C.

Detergent extracts (150 µl) were overlaid onto 225 µl of a cushion of 6% (w/v) sucrose in Hepes buffer in a siliconised test tube. The tube was incubated at 30° C for 3 min followed by centrifugation at 300 *g* for 3 min to sediment aggregated detergent micelles. The upper, aqueous phase was removed, Triton X-114 added to a concentration of 1% (v/v) and incubated for 10 min on ice. The re-extracted aqueous phase was again overlaid onto the sucrose cushion and the phase separation performed as before. The upper phases were discarded and the detergent phase suspended in 1 ml of Hepes buffer with a final Triton X-114 concentration of 1% (v/v).

Extracts representing detergent-phase purified amphiphilic proteins from 200 islets were used for each sample. The preparations were initially precleared by incubation for 7 h at 4° C with a pool of normal human serum (25 µl per sample) and binding to preswollen protein A-Sepharose (100 µl per sample; Pharmacia, Uppsala, Sweden). After centrifugation (30 s, 100 *g*), the supernatant was dispensed in 100 µl aliquots and incubated with 25 µl of test serum (unless otherwise indicated) for 18 h at 4° C. Immune complexes were isolated by incubation with pre-swollen protein A-Sepharose (100 µl per sample) for 2 h at 4° C with continuous shaking. Immunoprecipitates were washed 5 times with 1.6 ml of 10 mmol/l Hepes (pH 7.4) supplemented with 0.5% (v/v) Triton X-114, 150 mmol/l NaCl, 0.5 mmol/l methionine, 10 mmol/l benzamidine and 0.5 mg/ml bovine serum albumin and once with 1.6 ml of water. Proteins were eluted from the immunosorbent as previously described [6], and run on 10% sodium dodecyl sulphate-polyacrylamide gels according to Laemmli [13]. Gels were processed for autoradiography as previously described [6].

Analysis of autoradiograms

All autoradiograms were analysed independently by two observers without knowledge of sample identity. Samples were ranked positive or negative after comparison with the positive and negative controls run with every experiment. A quantitative estimate of M_r-64000 protein antibody positivity in samples was obtained by densitometric scanning of bands corresponding to the protein on autoradiograms using an LKB Ultroscan densitometer. The mean from 2–3 independent experiments was calculated for each sample. Peak height was taken as a measure of band density and results are expressed as a proportion of the height of peaks corresponding to the positive controls in each experiment.

Statistical analysis

The significance of correlations between observations were tested by the Spearman Rho test of rank correlation with correction, where appropriate, for large values of n. The significance of differences between groups was tested by the Mann-Whitney test. All tests were two-tailed. A p value of <0.05 was considered significant.

Results

Immunoprecipitation of the M_r-64000 protein by a standard diabetic serum

Serum from a diabetic patient of recent onset previously shown [5] to have high titres of antibodies to the M_r-64000 protein was used as a positive control in

all experiments. Dilutions of this serum were performed to characterise the immunoprecipitation reaction (Fig. 1). The quantity of protein immunoprecipitated, as assessed by the density of the corresponding band on autoradiograms, was dependent on the volume of serum used with half-maximal recovery of protein using 2.7 µl of serum and maximum recovery at 20-25 µl. In order to maximise detection of antibodies to the protein in low titre sera, subsequent experiments on the frequency of antibodies were performed using 25 µl of test serum. This standardisation provides for an accurate and reliable assay with which to evaluate the presence of 64k antibodies in random serum samples.

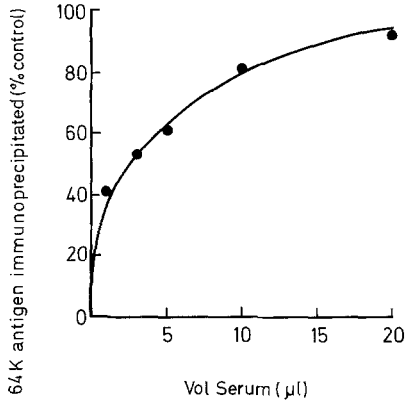

Fig. 1. *Concentration dependence of immunoprecipitation of an M_r-64000 protein by serum from a diabetic patient.* Precleared detergent phase purified islet amphiphilic proteins (100 µl) were incubated with 1-20 µl of serum from a diabetic patient for 18 h at 4° C. Immune complexes were purified on protein A-Sepharose, eluted and separated by SDS polyacrylamide gel electrophoresis. Islet proteins immunoprecipitated were detected by autoradiography. The quantity of M_r-64000 protein immunoprecipitated was assessed by densitometric scanning of autoradiographs expressing results as a proportion of the maximum band density corresponding to the M_r-64000 protein seen in each experiment. Results shown are the mean of three independent experiments

Frequency of antibodies to the M_r-64000 protein in diabetic and control children

A representative autoradiogram showing polypeptides immunoprecipitated by sera from 8 diabetic patients (even numbers on gel) together with their age, sex and geographically matched control subjects (odd numbers) is shown in Figure 2. The M_r-64000 protein was the only amphiphilic islet cell membrane protein to be specifically immunoprecipitated by the majority of diabetic sera. On this gel samples 2, 4, 6, 10, 12 and 16 were found to be positive for antibodies to the 64k antigen whereas samples 8 and 14 were negative. All 8 control subjects included in this analysis were negative.

The entire sample of 80 individuals was tested on

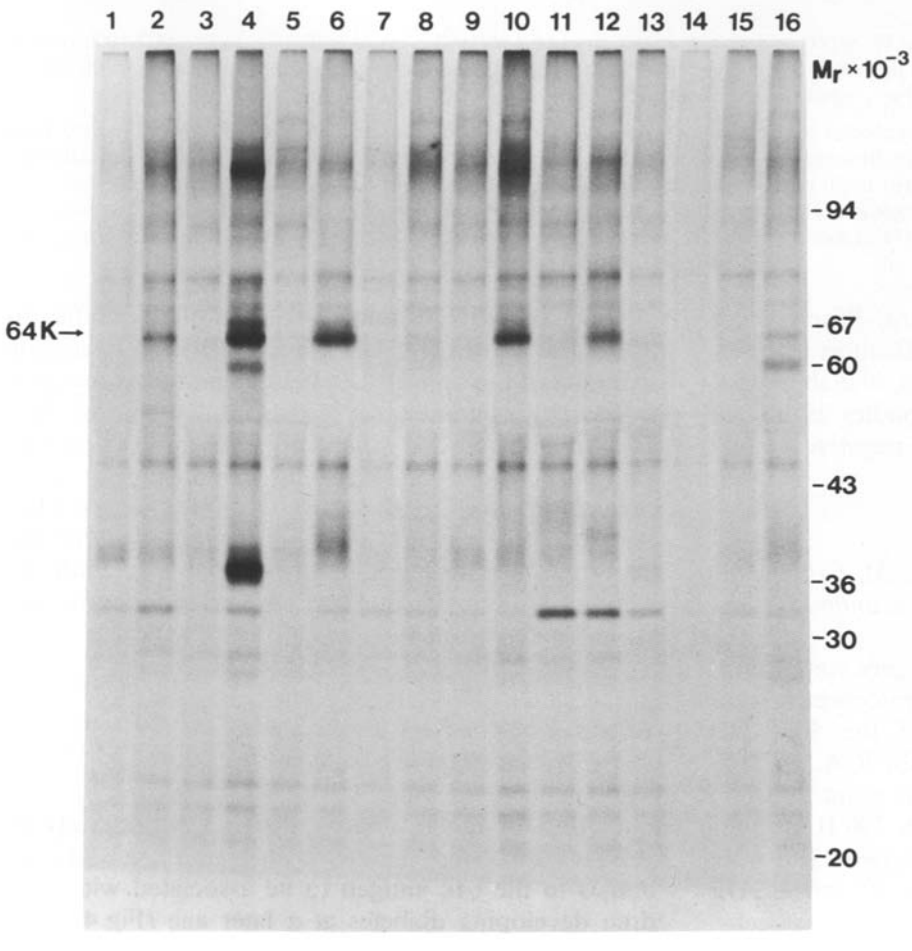

Fig. 2. *Immunoprecipitation of islet proteins by sera from diabetic and control children.* Precleared detergent phase purified islet amphiphilic proteins (aliquots of 100 µl) were incubated with 25 µl of serum from diabetic patients or their matched control subjects for 18 h at 4° C. Immune complexes were purified on protein A-Sepharose, eluted and separated on SDS polyacrylamide gels. The figure shows a representative autoradiogram showing peptides immunoprecipitated by sera from 8 diabetic patients (even numbered lanes on gel) together with their appropriate control subjects (adjacent left hand lane)

Table 1. Description of study groups

	Type 1 (insulin-dependent) diabetic patients		Control subjects	
	Total	Sample	Total	Sample
Number of individuals	389	40	321	40
Male/female	203/186	21/19	170/151	24/16
Median (range) age (years) at sampling	9 (0-14)	8 (1-13)	10 (0-15)	8 (1-14)
ICA positive	316 (81%)	30 (75%)	9 (3%)	2 (5%)
ICA levels (range)[a]	70 (3-8200)	70 (6-290)	27 (17-1200)	17,43

[a] ICA values of positive samples only are expressed in JDF units [11]

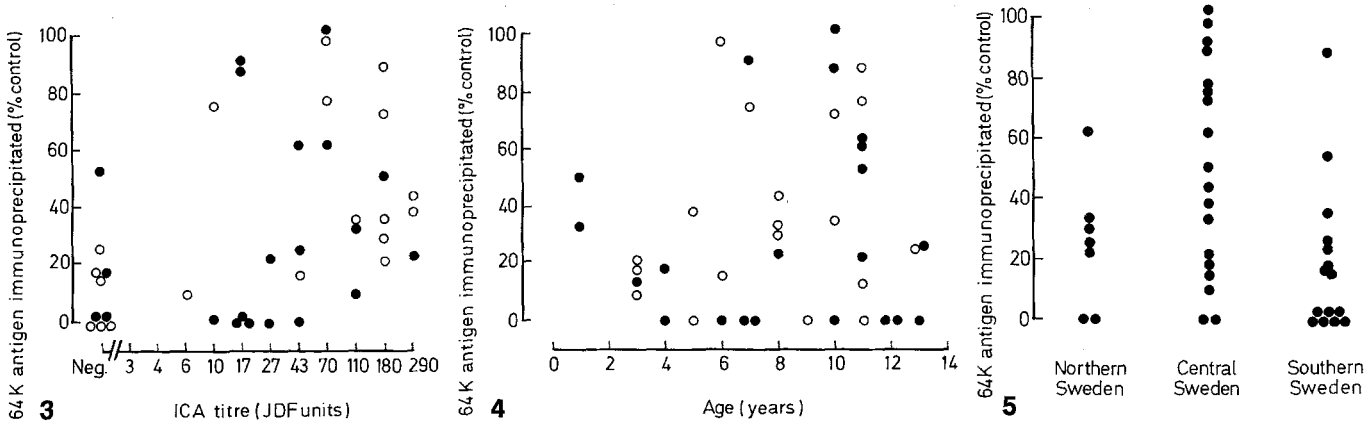

Fig. 3. *Comparison of levels of antibodies to the M_r-64000 protein with levels of ICA.* Antibodies to the M_r-64000 protein in sera from diabetic patients were detected as described in the legend to Figure 2. Antibody levels were quantified by densitometric scanning of autoradiograms, expressing data as a proportion of the protein immunoprecipitated by 25 µl of a positive control serum run in each experiment. The data are presented as a scatter plot relative to units of ICA, measured by indirect immunofluorescence on frozen sections of human pancreas. Closed circles (•) are boys, open circles (o) are girls

Fig. 4. *Relationship of levels of antibodies to the M_r-64000 protein with age at onset of diabetes.* Levels of antibodies to the M_r-64000 protein were analysed in sera from diabetic patients as described in the legend to Figure 3. Results are shown as a scatter plot relative to the age of onset of each individual. Closed circles (•) are boys, open circles (o) are girls

Fig. 5. *Geographical variation in the levels of antibodies to the M_r-64000 protein.* Levels of antibodies to the M_r-64000 protein in sera from diabetic patients were determined as described in the legend to Figure 3. Data were grouped according to the region of origin of each diabetic patient. Northern Sweden was defined as the area north of, and including the counties of Koppaberg and Gävleborg. Southern Sweden was defined as the area south of and including Östergötland, Jönköping and Halland. Central Sweden was defined as the intermediate region. The median of antibody levels in Southern Sweden (15%) was significantly lower than that in Central Sweden (47%; $p \leq 0.01$, Mann Whitney test)

at least two separate islet preparations. Where there was a doubt as to a positive identification samples were tested an additional time. Of the 40 diabetic patients, 29 (73%) were positive for antibodies to the 64k antigen. All 40 control samples were negative in two repetitions of the assay.

Correlation of levels of antibodies to the M_r-64000 protein with titres of islet cell cytoplasmic antibodies

The collection of diabetic and control sera were examined for ICA by indirect immunofluorescence on pancreatic sections. In this assay 30 of the 40 (75%) diabetic patients were found positive for ICA. The median level of ICA, determined by end point titration, was 70 JDF units and the range 6-290 JDF units (Table 1). Among the control subjects two (5%) were ICA positive; their levels of ICA were 17 and 43 JDF units respectively.

A quantitative estimate of levels of antibodies to the M_r-64000 protein was obtained by densitometric scanning of the appropriate bands on autoradiograms, expressing results relative to the positive control. This analysis allowed a comparison between levels of 64k antibodies and ICA (Fig. 3) to demonstrate a weak correlation between the two tests ($r = 0.46$, $p \leq 0.01$; Spearman's Rho test). Thus, 24/40 (60%) of the patients had serum autoantibodies capable of both detecting ICA in pancreatic sections and immunoprecipitating the 64k protein.

Effects of age and sex on levels of antibodies to the M_r-64000 protein

The effect of age at onset of diabetes on antibody levels showed a slight tendency for higher levels of antibodies to the 64k antigen to be associated with children developing diabetes at a later age (Fig. 4). This

was not, however, statistically significant. No significant differences were observed between boys and girls in the frequencies or levels of antibodies to the 64k antigen. Thus 13/21 (62%) of boys and 16/19 (84%) girls were found to be positive for antibodies to the 64k antigen with median antibody levels of 21% and of 29% respectively.

Regional variations in levels of antibodies to the M_r-64000 protein

To analyse possible regional variations in antibody levels, Sweden was divided into three regions: Northern Sweden was defined by the area north of and including the counties of Koppaberg and Gävleborg; Southern Sweden was defined by the area south of and including the counties of Östergötland, Jönköping and Halland; and Central Sweden was considered to be the region between these two. In the study group only 7 diabetic children inhabited the sparcely populated Northern region of Sweden making it difficult to analyse possible regional differences with respect to this area. However, patients from the Southern area of Sweden had significantly lower median antibody levels than patients from the central region (Fig. 5, $p \leq 0.01$; Mann Whitney test).

Discussion

Earlier studies on the diabetes-associated M_r-64000 islet cell antigen have employed human islets as the source of antigen [5, 6]. Where islets from rodents have been used, detection of the protein has been hampered by a heavy background on autoradiograms of non-specifically bound protein [14]. In the present work we have used a preparation of amphiphilic membrane proteins from rat islets as source of antigen. The procedure described removes much of the background, thus reducing the possibility of assigning false positive values to test samples. The ability to detect the M_r-64000 protein in the more readily available rat islet should facilitate studies on the possible role of the protein in the pathogenesis of Type 1 diabetes. Here, in an extension to previous work and using a semiquantitative test, we have tried to obtain an estimate of the frequency and levels of antibodies to the protein in children with recently diagnosed Type 1 diabetes. A control group of age and geographically matched healthy individuals was available for comparison [7, 8].

The assay employed involves immunoprecipitation of metabolically radiolabelled endogenous proteins from purified islet preparations followed by electrophoresis and autoradiography. This procedure has the advantage of high specificity with regard to the islet antigen that is not encountered with other detection methods such as radioiodination and autoradiography or immunofluorescence where exogenous antigen structures may be recognised [15]. However, there are clearly limitations to the procedure. The assay is not a high capacity assay. The laborious nature of islet isolation and immunoprecipitation together with the expense of radiolabelling islets at the high specific activity required for antigen detection precluded a study on more than 80 of the 710 samples available. In order to maximise the sensitivity of the test, we have used a serum dilution of 1:5. With this dilution, as illustrated by the experiments with the positive control, samples with high titres of antibodies to the protein will immunoprecipitate virtually all of the protein in the islet extract. Thus, the antibody levels of high responders may be underestimated in this test. Furthermore, in order to be detected in this assay the 64k antibodies have to be immunoprecipitating, bind to protein A (i.e. IgG and not IgM) and in the present study crossreact with the rat protein. False negative sera could contain antibodies failing to meet one or more of those criteria. Subject to such limitations, the results presented here provide novel information concerning humoral autoimmunity against islet cell antigens in a localised and carefully matched population [16].

Of the 40 diabetic patients selected, 29 (73%) were positive for antibodies to the M_r-64000 protein. This figure is consistent with earlier studies using human islets where antibodies were detected in 80% (8/10) [5] or 78% (11/14) [6] of the diabetic patients tested. Antibodies were not detected in any of the 40 control individuals. It is clear, therefore, that humoral autoimmunity to the 64k protein is a frequent phenomenon in Type 1 diabetes.

The semi-quantitative nature of the assay has enabled us to investigate possible correlations with ICA. ICA were detected in 30 (75%) of the diabetic patients and in 2 (5%) of the 40 control subjects. The frequency of antibodies to the M_r-64000 protein in the diabetic patients was thus approaching that of ICA. In the 40 diabetic patients there was a significant but weak correlation between levels of antibodies to the M_r-64000 protein and ICA. This could be interpreted in two ways. The immunofluorescence detected on frozen sections of pancreas in the ICA assay could, in part, be due to a reaction with the M_r-64000 protein. However, recent studies have indicated that the antigen recognised on frozen sections of pancreas by diabetic sera may have the properties of a sialoglycoconjugate [17]. Treatment of sections with the protease, pronase, did not reduce ICA immunofluorescence. Thus, unless resistant to pronase treatment, the M_r-64000 antigen may not contribute significantly to antigens detected by indirect immunofluorescence. Quantities of purified M_r-64000 protein are required for absorption studies to clarify this point. The low r-value found by Spearman's Rho test is, rather, an indication of an indirect than direct correlation between the two tests. An alternative explanation for correlation between the two antibody tests may be that certain indi-

viduals have enhanced humoral autoimmunity to a number of islet cell components. This may be related to the severity of autoimmune aggression against islet cells and possibly to the severity of the disease. High titres of ICA have indeed been associated with a more rapid loss of endogenous C-peptide after diagnosis [18]. Whether this also holds for antibodies to the 64k antigen remains to be established.

It is noteworthy that 5 patients (13%) did not show detectable levels of either ICA or antibodies to the 64k antigen at diagnosis (Fig. 3). This lack of a humoral response to islet cell antigens might be related to a considerable loss of B-cell mass and thus of stimulating antigen. However, in previous studies [6, 19], individuals have been identified with weak or no antibody responses to islet antigens in the prediabetic period. It can be speculated that those patients may have been positive only before the observation period or that humoral autoimmunity to islet cell antigens is not involved in the development of diabetes in some patients.

The number of samples tested was too small to examine seasonal effects on antibody levels. The data did provide some evidence for regional variation in 64k antibody levels suggesting that patients from the southern part of Sweden had significantly lower levels of antibodies than those living in the central region. Much caution must be taken in the interpretation of data from such a small sample in a study performed over a short period. However, these results call for further investigation into possible regional heterogenity in immune responses to islet cell antigens and to identify factors that may influence such differences.

In conclusion, the high frequency of antibodies to the M_r-64000 protein and the correlation with ICA point to the M_r-64000 protein being a likely candidate antigen to complement or perhaps supersede indirect immunofluorescence on pancreatic sections in the assessment of anti-islet autoimmunity in Type 1 diabetes.

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