Antibodies to a 64,000 M_r Human Islet Cell Antigen Precede the Clinical Onset of Insulin-dependent Diabetes

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Abstract

Antibodies in sera from newly diagnosed insulin-dependent diabetes mellitus (IDDM) patients are directed to a human islet cell protein of relative molecular mass (M_r) 64,000. Since IDDM seems to develop after a prodromal period of β-cell autoimmunity, this study has examined whether 64,000 M_r antibodies could be detected in 14 individuals who subsequently developed IDDM and five first degree relatives who have indications of altered β-cell function. Sera were screened by immunoprecipitation on total detergent lysates of human islets and positive sera retested on membrane protein preparations. Antibodies to the 64,000 M_r membrane protein were consistently detected in 11/14 IDDM patients, and in all 5 first degree relatives. 10 IDDM patients were already positive in the first samples, obtained 4–91 mo before the clinical onset of IDDM, whereas 1 patient progressed to a high 64,000 M_r immunoreactivity, at a time where a commencement of a decline in β-cell function was detected. 64,000 M_r antibodies were detected before islet cell cytoplasmic antibodies (ICCA) in two patients. In the control groups of 21 healthy individuals, 36 patients with diseases of the thyroid and 5 SLE patients, the 64,000 M_r antibodies were detected in only one individual, who was a healthy sibling to an IDDM patient. These results suggest that antibodies against the M_r 64,000 human islet protein are an early marker of β-cell autoimmunity and may be useful to predict a later development of IDDM.

Introduction

The clinical onset of insulin-dependent diabetes mellitus (IDDM)[1] is associated with a specific loss of the β-cells in the islets of Langerhans and autoimmune phenomena may play a role in the pathogenic process. A majority of newly diagnosed IDDM patients have islet cell surface antibodies (ICSA) and/or cytoplasmic antibodies (ICCA) (1). Infiltrating lymphocytes are present in the pancreatic islets (2) and there is evidence for hypersensitivity towards pancreatic antigens (3).

The nature of the primary β-cell target antigen(s) remains to be elucidated. Immunoprecipitation of lysates of [^{35}S]methionine labeled human islets showed that newly diagnosed diabetic children had antibodies to a relative molecular mass (M_r) 64,000 protein (4). In the spontaneously diabetic BB rat, which develops IDDM similar to the human disease, antibodies to a rat islet cell 64,000 M_r protein were also found to appear between 12 and 22 d of age (5, 6), which is before the detection of both insulitis (around day 65) (7), decrease in β-cell mass and function (day 45–50) (8, 9) and the subsequent onset of IDDM at or beyond 60–70 d of age.

IDDM in man seems to develop after a long latency period, which is reflected both in a decreased first-phase insulin release after intravenously administered glucose (10–13) and in the appearance of ICCA (14–16), which precedes the clinical onset of IDDM up to several years. Our hypothesis is that the 64,000 M_r protein is a primary target autoantigen. In this study we have therefore tested whether carefully documented individuals followed before their clinical onset of IDDM (10–15, 17) and first degree relatives with signs of impaired β-cell function, had 64,000 M_r antibodies. Control groups including healthy siblings to IDDM patients, healthy individuals with no family history of IDDM and patients with other autoimmune diseases were tested as well.

Methods

Isolation of islets. Human islets of Langerhans were isolated from the pancreata of 18 cadaver kidney donors at the University Hospitals of Copenhagen (male/female ratio 9:9, 14–60 yr of age). HLA-typing showed HLA-DR 1 in 3, DR 2 in 6, DR 3 in 4, DR 4 in 4, DR 5 in 2, DR 6 in 3, and DR 7 in four pancreata. One pancreas was not tissue typed. Isolation of human islets by collagenase digestion and selection of individual islets under a stereomicroscope was carried out as described (18). The islets were cultured for 1 d in RPMI 1640 medium supplemented with 0.35 g per liter $NaHCO_3$, 20 mM Hepes, 10% (vol/vol) newborn calf

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1. *Abbreviations used in this paper:* FBS, fasting blood sugar; FITC, fluoresceine isothiocyanate; ICCA, islet cell cytoplasmic antibodies; ICSA, islet cell surface antibodies; IDDM, insulin dependent diabetes mellitus; IVGTT, intravenous glucose tolerance test; NCS, newborn calf serum; NHS, normal human serum; OGTT, oral glucose tolerance test; PAGE, polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosis; TBTE, 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine/HCl, 1% Trasylol, 5 mM EDTA, 0.1 mM *p*-chloromercuribenzenesulfonic acid; TNMB buffer, 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine/HCl; TN-114, Triton X-114.

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serum (NCS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. After being reselected and transferred individually to fresh medium supplemented with 0.5% normal human serum (NHS) instead of NCS, the islets were kept in culture (100 islets/4 ml) for 5–10 d. The release of insulin was measured by radioimmunoassay using porcine insulin (Nordisk Gentofte, Gentofte, Denmark) as standard.

Preparation of labeled cell extracts. Cultured islets were washed twice by centrifugation (2 min, 200 *g*) in methionine-free RPMI 1640 medium supplemented with 16 mM glucose, 2% NHS, penicillin, and streptomycin. Islets (500 islets/ml) were first incubated in this medium for 60 min before [^{35}S]methionine (0.5 mCi/ml, New England Nuclear, Boston MA; > 500 Ci/mmol) was added. After incubation for 4–6 h at 37°C, the labeled islets were harvested by centrifugation, washed twice in RPMI 1640 medium containing 0.5 mM methionine and once in TNMB buffer. The islets were resuspended (1,000 islets/ml) in TNMB supplemented with 1% Trasylol (Novo Industries, Bagsværd, Denmark), 5 mM EDTA and 0.1 mM *p*-chloromercuribenzenesulfonic acid (Sigma Chemical Co., St. Louis, MO) (TBTE buffer) and 1% Triton X-114 (TN-114) (Sigma Chemical Co.) precondensed as described by Bordier (19). The suspension was incubated on ice for 2–4 h to solubilize the islets and then centrifuged at 100,000 *g* for 30 min. The lysate supernatant was either used for immunoprecipitation directly or after separation of amphiphilic membrane proteins from hydrophilic proteins by phase separation (19).

Phase separation. The TN-114 lysate was layered over a cushion of TBTE buffer containing 6% (wt/vol) sucrose and 0.2% TN-114 in a siliconized tube. The TN-114 was condensed by incubation at 30°C for 3 min and collected by centrifugation (3 min, 1,500 *g*) at 30°C as a 17% detergent droplet below the sucrose cushion. The aqueous phase was reextracted twice with 0.5% TN-114 and layered over the original sucrose cushion. TBTE buffer was added to the combined detergent droplets to give a final 1% detergent concentration.

Immunoprecipitation. Total TN-114 lysates or phase separated lysates were pretreated for 1 h on ice with NHS followed by 30 min incubation with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The immunosorbent was removed by centrifugation and aliquots of the supernatant (100–200 μl containing 5–19 $\times$ 10^6 cpm in total extracts or 5–8 $\times$ 10^6 cpm in samples prepared by detergent phase separation) incubated with 25 μl serum for 16 h followed by incubation with 100 μl preswollen protein A-Sepharose. The immunoprecipitates were washed six times by centrifugation in 2 ml TNMB-buffer containing 0.5% TN-114 and once in ice-cold double distilled and autoclaved H_2O containing 0.02% (wt/vol) Trasylol. Bound proteins were eluted from the immunosorbent by 1 min boiling in 80 mM Tris/HCl, (pH 6.8), containing 3% SDS, 15% sucrose, 5% 2-mercaptoethanol and 0.006% bromphenol blue.

Gel electrophoresis. Immunoprecipitated proteins were separated according to molecular weight by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (20) using 7.5–15% linear acrylamide gradient gels. The gels were stained with Coomassie Brilliant Blue and processed for fluorography using Enlightning (New England Nuclear) and Kodak X-omat AR film. M_r standards (Pharmacia Fine Chemicals) used were phosphorylase b (94,000), bovine serum albumin (67,000), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lactalbumin (14,400).

Human sera. Sera were obtained from 14 IDDM patients (Table I) who had been carefully studied before the clinical onset by being monozygotic twins or a monozygotic triplet (patients 1, 3, 6, 11, 12, 14, and 5, respectively) (10, 11, 17), or healthy siblings to IDDM patients (patients 2, 8, 9) (15).[2] Patients 4 (15), 7, 10, and 13 were referred due to a history of transient hyperglycemia and/or glucosuria. IDDM was diagnosed according to the World Health Organization (WHO) criteria of clinical onset (21). Sera were also obtained from five first degree relatives: siblings, including one twin, and three parents of IDDM patients having signs of altered β-cell function (Table II). The control group consisted of sera from 16 healthy siblings to IDDM patients, including 2 twins and 1 triplet and from 5 healthy laboratory staff members without a family history of IDDM (Table III). 10 of the controls (C 12–21) were siblings to the IDDM patients participating in this study (Table I). Four control individuals (C 1–4) were selected from a group of 16 tissue-typed siblings to IDDM children participating in a Swedish family study on the basis of HLA-DR identity to at least one of the IDDM patients studied (Table I). Two control siblings (C 5 and 6) were selected on a random basis from a group of nontissue-typed siblings to Danish IDDM patients. Disease specificity was tested by immunoprecipitation with sera from five patients with systemic lupus erythematosus (SLE) having nuclear antibody titers of 32–1024 as measured in an immunofluorescence assay and DNA-antibody titers of 12–45% measured in a Farr immunoprecipitation assay (information and sera kindly provided by Dr. V. Andersen, University of Copenhagen). Furthermore sera from 34 patients with Hashimoto's or Graves' disease were tested. 18 sera were from Copenhagen and included 12 Graves' and 6 Hashimoto's disease patients having microsomal antibody titers of 0–640 as measured by immunofluorescence on frozen sections of human thyroid tissue (in which a titer of 640 is the highest measured) and some having thyroglobulin antibody titers $\geq$ 200,000 measured in an agglutination assay (sera and information kindly provided by Drs. K. Bech and U. Feldt-Rasmussen, Frederiksberg Hospital, Copenhagen). 16 sera were from Cardiff and included 6 Graves' and 10 Hashimoto's disease patients. The Graves disease patients had thyrotropin-stimulating hormone receptor antibodies varying from low to very high. The Hashiomoto's patients had microsomal antibody titers of 1.6–1.9 in an enzyme-linked immunosorbent assay (in which a titer of 1.8 corresponds to an agglutination assay titer $\geq$ 10,000) (sera and information kindly supplied by Dr. Allan McGregor, University of Cardiff). Furthermore sera were tested from one patient with atoxic diffuse and one patient with toxic adenom of the thyroid.

All sera were centrifuged at 10,000 *g* for 30 min, aliquoted and kept at −20°C. Coded sera from 5 monozygotic twins/triplets (P1, 3, 5, 11, 12), 5 first degree relatives (R 1–5), 16 thyroiditis patients, and 3 controls (C8, 9, 14) were initially scored in a "blinded" fashion with the subject category assigned after analysis of the assay results.

Islet cell cytoplasmic antibodies. ICCA were analyzed in all the European patients and controls (Tables I–III), using cryostat sections of human blood group O pancreata and a novel, highly sensitive two-color immunofluorescence assay with Texas red labeled proinsulin antibody and fluorescein isothiocyanate (FITC)-labeled antibody to human IgG (22). All the United States patients and controls had been tested previously by the FITC-labeled protein A-monoclonal antibody method (23), and sera were not available for retesting by the two-color assay.

Results

Membrane protein characteristics of the M_r 64,000 protein. The M_r 64,000 protein partitioned into the detergent phase of TN-114 after phase transition at 30°C. The detergent phase containing amphiphilic membrane proteins (24) constituted 8–10% of total TCA precipitable counts in human islet cell lysates. As shown in Fig. 1, the partition could be used to purify the 64,000 M_r protein before immunoprecipitation, resulting in an effective removal of background proteins.

Human islet preparations and the immunoprecipitation assay. The islet preparations released 3.8±2.9 ng insulin/islet per d (mean±SD). A positive correlation between the expression of the 64,000 M_r antigen and the insulin releasing capacity of the islet preparations was found (data not shown). Therefore, all sera from each individual were tested in parallel at least once on the same islet preparation, and the intensity of immunoprecipitated 64,000 M_r antigen on autoradiograms compared. Sera

2. De Beaufort, C. E., N. C. Den Boer, G. J. Bruining, R. van Strik, and T. Weterings. Submitted for publication.

Table I. IDDM Patients Analyzed for 64,000 Antibodies in the Prediabetic Period

Patient	Origin	Sex	HLA-DR	Age at clinical onset of IDDM	Indication for observation	Prediabetic period followed	ICCA*	α64,000‡
						mo		
1	USA	M	3, x	48	Monozygotic twin	78–5	2+	3+
2	NL	F	3, 4	14	IDDM family study	32–0	≥ 64	3+
3	USA	F	3, 4	11	Monozygotic twin	21–2	4+	3+
4	NL	M	3, 4	3	Glucosuria	4–0	≥ 64	3+
5	USA	M	4, x	21	Monozygotic triplet	91–7	2+	2+
6	DK	M	3, 7	26	Monozygotic	25–7	≥ 64	2+
7	DK	F	4, w6	13	Glucosuria	4–0	≥ 64	2+
8	NL	M	1, 4	19	IDDM family study	28–0	≥ 64	1+
9	NL	M	3, w9	17	IDDM family study	16–4	≥ 64	1+
10	DK	M	4	24	Glucosuria	24–0	≥ 64	—
11	USA	F	1, 7	13.5	Monozygotic twin	12–1	—	±
12	USA	F	NT	28	Monozygotic twin	84–48	±	±
13	DK	M	NT	18	Glucosuria	22–0	—	2+
14	S	M	1, 4	18	IDDM family study	25–0	≥ 64	2+

* ICCA in the USA patients are expressed on a scale 0–4+. ICCA in sera from Denmark (DK), Sweden (S), and the Netherlands (NL) are expressed as the highest dilution at which sera were still positive. ‡ Positivity of 64,000 M_r antibodies was designated very strong: 3+, strong: 2+, and weak: 1+. The positivity of the strongest positive serum during the observation period is listed.

from different patients were also tested in parallel and compared to positive and negative control sera. All serum samples were screened on total detergent lysates from at least two different islet preparations. Fig. 2 shows the immunoprecipitation of crude islet cell lysates using sera from IDDM patients 6, 7, 9, 13, and 14 together with sera from healthy controls (C1, 3) and a first degree relative with indications of a decreased β-cell function (R3). Differences in immunoreactivity of 64,000 M_r antibody positive sera are clearly apparent and were consistently detected. Immunoreactivity of sera was estimated as intensity of the 64,000 M_r band on autoradiograms and scored on a 0–3+ scale. All positive sera were further analyzed using TN-114 detergent phase purified membrane proteins to increase the specificity of the assay and verify that they recognized the 64,000 M_r membrane protein.

Analyses of 64,000 M_r antibodies in IDDM patients. Sera from 11 of 14 IDDM patients consistently immunoprecipitated the 64,000 M_r protein (Table I) when tested on both total lysates and a TN-114 detergent phase purified membrane protein fraction.

Four of the IDDM patients (patients 1–4) had antibodies that repeatedly precipitated a very strong 64,000 M_r band designated 3+, five patients (patients 5–7 and 13, 14) immunoprecipitated a strong band, designated 2+, two patients (patients 8 and 9) immunoprecipitated a weak 64,000 M_r band designated 1+, patients 11 and 12 were either weakly positive or negative in the multiple testings, and patient 10 was negative.

Analyses of 64,000 M_r antibodies in first degree relatives of IDDM patients having inaications of a decreased β-cell function and/or ICCA. Individuals 1–5 (Table II) are first degree relatives of IDDM patients obtained from the prospective Joslin prediabetes study on the basis of ICCA positivity (25). Relatives 1, 2, 4, and 5 exhibited first phase insulin release in response to intravenously administered glucose lower than the 95th percentile. Four individuals in this group immunoprecipitated a very strong (3+) 64,000 M_r band (Fig. 2, lane *K*, Fig. 3, lanes *B–E*), and one a weak (1+) 64,000 M_r component (Fig. 3, lane *A*).

Analyses of 64,000 M_r antibodies in healthy individuals. This group was comprised of 10 healthy siblings of the IDDM patients included in this study, 6 healthy siblings to other IDDM patients, and 5 individuals without IDDM in the family (Table III). Sera from a healthy sibling to patient 10 immunoprecipitated a very strong (3+) 64,000 M_r band from both crude lysates and TN-114 detergent phase purified proteins. This individual was tested and found to be positive for ICCA, but IVGTT showed no abnormalities.

Table II. First-Degree Relatives to IDDM Patients With Signs of Impaired β-cell Function

Relative	Origin	Relation to IDDM proband(s)	Sex	Age	ICCA*	Insulin release (percentile)‡	α64,000§
1	USA	Sib and daughter	F	34	4+	3	1+
2	USA	Parent	F	58	4+	≪ 1	3+
3	USA	Sib	M	30	4+	53	3+
4	USA	Parent	F	66	4+	<1	3+
5	USA	Parent	M	26	4+	3	3+

* ICCA were measured on a scale from 0–4+. ‡ References 11–14. § α64,000 immunoreactivity was measured on a scale from 0 to 3+.

Table III. Healthy Controls Tested for 64,000 M_r Antibodies

Control	Sex	Age	HLA D/DR	Sibling to patient no.	ICCA	α64,000
1	M	16	3.7	—	—	—
2	F	13	3, 4	—	—	—
3	F	15	4	—	—	—
4	F	19	3, 4	—	—	—
5	F	11	NT	—	—	—
6	F	15	NT	—	—	—
7	F	44	NT	—	—	—
8	M	37	NT	—	—	—
9	M	34	NT	—	—	—
10	F	24	NT	—	—	—
11	F	23	NT	—	—	—
12	M	16	3, 5	2	—	—
13	M	14	3, 4	2	—	—
14	M	17	4, X	5	—	—
15	F	31	NT	6	—	—
16	F	10	NT	7	—	—
17	M	18	1, 4	8	—	—
18	M	17	1, w6	8	—	—
19	F	14	1, w6	8	—	—
20	F	23	NT	9	—	—
21	M	21	NT	10	≥ 64*	3+‡

Controls 1–6 were healthy siblings to IDDM patients not included in this study. Controls 2 and 5 are monozygotic twins to an IDDM patient. Controls 7–11 were laboratory staff members. Controls 12–21 were healthy siblings to the IDDM patients included in this study. Control 14 is a triplet to patient 5. Sera from controls 15 and 21 were obtained 34 and 7 mo, respectively, after onset of IDDM in their sibling. All the other sera were obtained before or at onset of IDDM in the sibling. * ICCA expressed as dilution at which serum was still positive. ‡α64,000 immunoreactivity expressed on a scale from 0 to 3+.

All other individuals in this group were M_r 64,000 antibody negative (Table III, Fig. 1, lanes *C, D, G, H,* Fig. 2, lanes *A, B,* Fig. 3, lane *F*).

Analyses of 64,000 M_r antibodies in sera from SLE patients and patients with diseases of the thyroid. Five SLE patients were tested and found to be negative for 64,000 M_r antibodies.

Sera from 16 Hashimoto's patients, 18 patients with Graves' disease, one patient with atoxic diffuse, and one patient with toxic adenom were tested and found to be negative for 64,000 M_r antibodies. All these individuals were also tested for ICCA. Only one of these patients was ICCA positive. Immunoprecipitates with some sera containing high titers of thyroid microsomal antibodies and crude human islet cell lysates had intensive background of nonspecifically precipitated proteins. Six of those sera immunoprecipitated a fuzzy band at a slightly lower M_r than the 64,000 protein band in the 64,000 M_r region (data not shown). However, prolonged gel electrophoretic analyses separated this band from the 64,000 M_r antigen, immunoprecipitated by IDDM sera. Furthermore, the sera were negative on TN-114 detergent phase purified membrane proteins demonstrating that the protein they detected in crude lysates was not the 64,000 M_r membrane protein immunoprecipitated by IDDM sera. These sera were tested in a group of coded sera and were scored negative on the basis of those analyses. Those results demonstrate the importance of using purified membrane proteins for 64,000 M_r antibody analyses to avoid false positives.

Prospective analysis of 64,000 M_r antibodies in IDDM patients. The 14 IDDM patients included in this study had been followed up to 4–91 mo before clinical onset of IDDM. Fig. 4 shows the 64,000 M_r antibody immunoreactivity and ICCA analyses in the observation period of each patient. Patients 1–6, 8, 10, 11, and 12 have been described in detail elsewhere with regard to clinical parameters (10–15, 17).[2]

Case studies

Patient 1 (Fig. 4, Table I) (10, 11, 14) was the only 64,000 M_r antibody positive patient which progressed from a negative to 64,000 M_r antibody positive state during the observation period. When tested on total islet lysates, samples obtained 78 and 70 mo before the clinical onset were 64,000 M_r antibody negative.

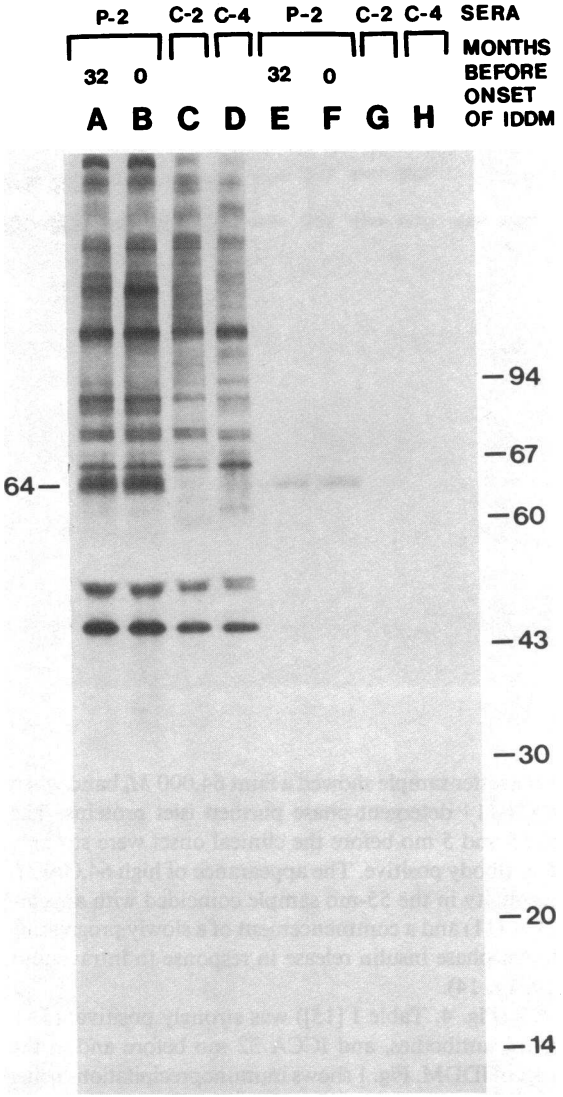

Figure 1. Fluorograph showing immunoprecipitation of human islet cell proteins before and after TN-114 detergent phase purification with sera from an HLA-DR 3/4 positive IDDM patient and two control individuals. Total islet cell lysates (lanes *A–D*) or an amphiphilic membrane protein fraction purified by TN-114 phase separation of islet cell lysates (lanes *E–H*) were immunoprecipitated with sera from patient 2, 32 mo before (lanes *A* and *E*) and at the clinical onset of IDDM (lanes *B* and *F*) and sera from controls 2 (lanes *C* and *G*) and 4 (lanes *D* and *H*).

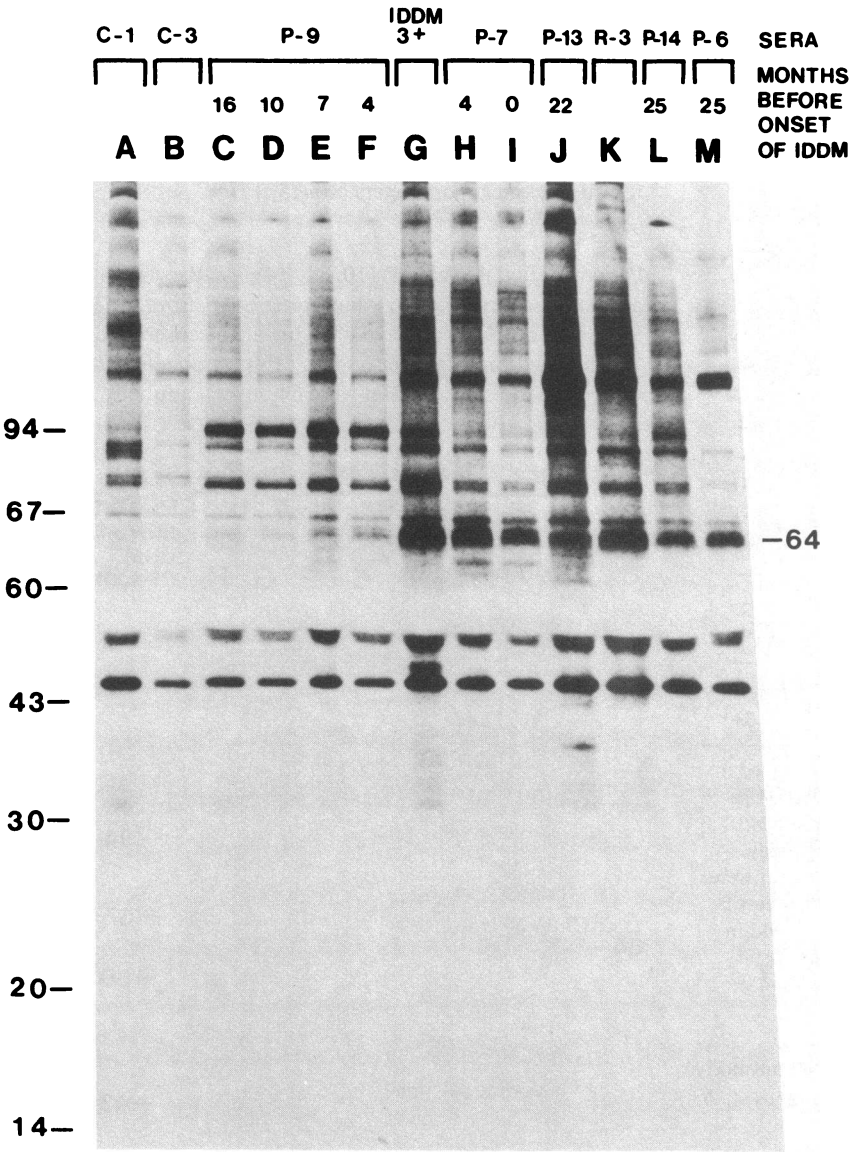

Figure 2. Fluorograph showing variations of 64,000 M_r immunoreactivity in sera from several IDDM patients and a first degree IDDM relative. Total islet cell lysates were immunoprecipitated with sera from controls 1 (lane *A*) and 3 (lane *B*), sera from patient 9 (lanes *C–F*) obtained 16, 10, 7, and 4 mo before clinical onset of IDDM, patient 7 (lanes *H, I*) 4 mo before and at clinical onset of IDDM, patient 13 (lane *J*) 22 mo before, patient 14 (lane *L*) 25 mo before, patient 6 (lane *M*) 25 mo before clinical onset of IDDM and IDDM relative 3 (lane *K*). A 3+ positive IDDM serum used as a control is shown in lane *G*.

However, the latter sample showed a faint 64,000 M_r band when tested on TN-114 detergent phase purified islet proteins. The samples at 55 and 5 mo before the clinical onset were strongly 64,000 M_r antibody positive. The appearance of high 64,000 M_r immunoreactivity in the 55-mo sample coincided with appearance of ICCA (11) and a commencement of a slowly progressing decline in first-phase insulin release in response to intravenous glucose (10, 11, 14).

Patient 2 (Fig. 4, Table I [15]) was strongly positive, (3+), for 64,000 M_r antibodies, and ICCA 32 mo before and at the clinical onset of IDDM. Fig. 1 shows immunoprecipitations using sera from this patient and two HLA-DR matched controls (DR 3, 4) on total lysates (lanes *A–D*) and TN-114 detergent phase purified membrane proteins (lanes *E–H*).

Patient 3 (Fig. 4, Table I) (11, 13, 14) was strongly positive, (3+), for 64,000 M_r antibodies 21 and 2 mo before the clinical onset. ICCA (11, 13, 14) was also positive.

Patient 4 (Fig. 4, Table I) (15) was strongly 64,000 M_r antibody and ICCA positive in samples obtained 4, 2, and 1 mo before and at the clinical onset of IDDM (15).

Patient 5 (Fig. 4, Table I) (10, 11, 14) was strongly 64,000 M_r antibody positive, (2+), in a sample obtained 91 mo before clinical onset of IDDM, but became weakly positive in later samples. He was ICCA positive during the whole observation period (11). Compared with his nondiabetic triplet mate, he showed a decrease of the first phase insulin release in response to intravenous glucose at 91 mo and a further progressive loss became apparent during the following years (10, 11).

Patient 6 (Fig. 4, Table I) (17) was strongly (2+) positive for 64,000 M_r antibodies already in the first sample (Fig. 2, lane *M*) obtained when his twin brother developed IDDM, which occurred 25 mo before the diagnosis of his own IDDM. He remained strongly positive in four samples obtained before and at clinical onset of IDDM. He progressed from an ICCA negative to a positive state in the third sample obtained 9 mo before clinical onset of IDDM.

Patient 7 (Fig. 4, Table I) had intermittent glucosuria in connection with a febrile infection 3 yr before and developed persistent mild glucosuria 5 mo before the clinical onset of IDDM. She was strongly (2+) positive for 64,000 M_r antibodies

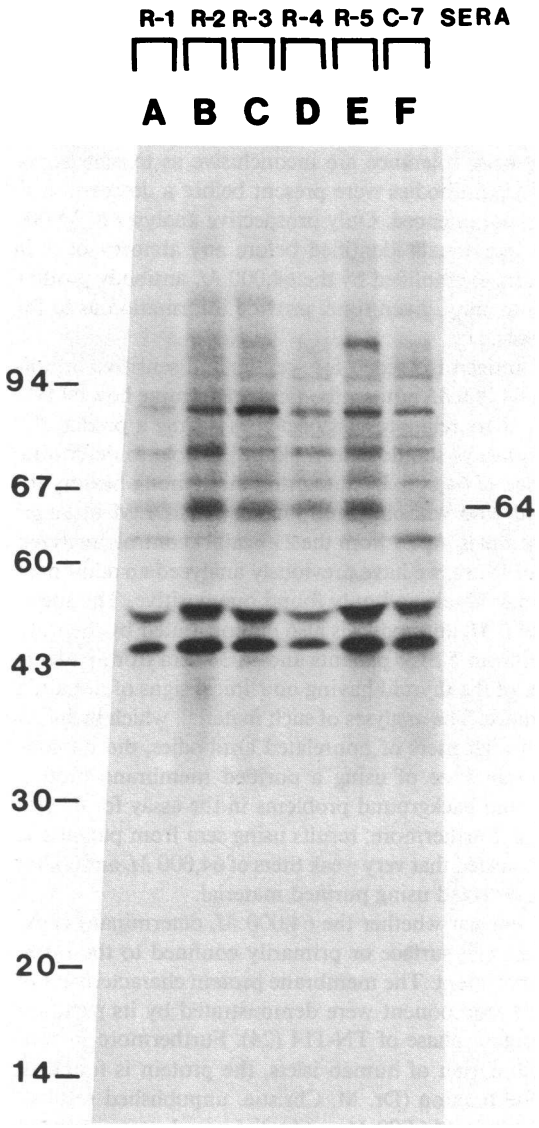

Figure 3. Fluorograph showing immunoprecipitation of the 64,000 M_r band with sera from first degree relatives of IDDM patients. Total islet cell lysates were immunoprecipitated with sera from relatives 1–5 (lanes *A–E*) and with serum from control 7 (lane *F*).

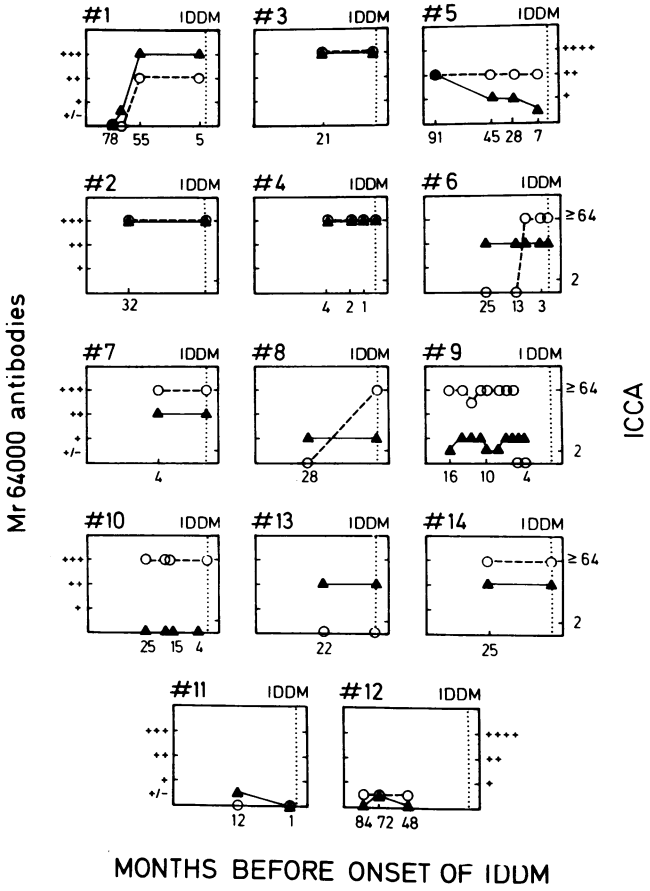

Figure 4. Time course of 64,000 M_r antibodies and ICCA in the subclinical phase of 14 IDDM patients. ICCA (*open circle*) were measured either in a double immunofluorescence assay and results expressed as the highest dilution at which sera were positive (patients, 2, 4, 6–10, 13, 14) or measured in a protein A immunofluorescence assay and results expressed on a scale from 0 to 4+ (patients 1, 3, 5, 11, 12) (10, 11, 13, 14). 64,000 M_r antibody immunoreactivity (*solid triangles*) was estimated on the basis of intensity of the 64,000 M_r antigen band on autoradiograms and expressed on a scale from 0 to 3+.

and ICCA in samples obtained 4 mo before and at the clinical onset of IDDM (Fig. 2, lanes *H, I*).

Patient 8 (Fig. 4, Table I) (15) was weakly positive for 64,000 M_r antibodies in two samples obtained 28 mo before and at the clinical onset of IDDM. He progressed from an ICCA negative to a positive state in the period between the first sample and the clinical onset.

Patient 9 (Fig. 4, Table I)[2] was followed at regular intervals during a 1-yr period, starting 16 mo before the clinical onset of IDDM. 64,000 M_r antibodies were positive in samples 2–4, 7–10, while samples 1, 5, and 6 only immunoprecipitated a faint 64,000 M_r component (Fig. 2, lanes *C–F*). All samples precipitated an additional strong band at ~ 94,000 M_r (Fig. 2, lanes *C–F*), which was detected as only a faint band in immunoprecipitates with other IDDM sera as well as control sera (Fig. 2, lanes *A, B,* and *G–M*) and was absent when sera were tested on TN-114 detergent phase purified membrane proteins. This patient was positive for ICCA in all but the last two samples.

Patient 10 (Fig. 4, Table I) came to our attention due to intermittent glucosuria, hyperglycemia and impaired glucose tolerance during a febrile infection 25 mo before clinical onset of IDDM. An oral glucose tolerance test showed impaired glucose tolerance. A 10,000-kJ diabetes diet was initiated, and the condition normalized. Fasting blood sugar was normal 2 wk later and remained within normal range during the rest of the observation period. IVGTT performed 8 mo later showed a total loss of first-phase insulin response. This patient was 64,000 M_r antibody negative but ICCA positive during the observation period.

Patient 11 (Fig. 4, Table I) (11, 14) was ICCA negative during the observation period. Sera obtained 12 and 1 mo before clinical onset of IDDM were negative when tested on total islet cell lysates. However, the first sample was weakly positive, when tested on TN-114 detergent phase purified islet cell proteins.

Patient 12 (Fig. 4, Table I) (11, 14) had been followed at 84, 72, and 48 mo before clinical onset of IDDM. 64,000 M_r antibodies were negative when the sera were tested on total islet cell lysates. The second serum sample was weakly positive, and the

first and third serum samples were negative, when tested on TN-114 detergent phase purified proteins.

Patient 13 (Fig. 4, Table I) had intermittent glucosuria and showed impaired glucose tolerance 22 mo before the clinical onset of IDDM. Serum samples obtained 22 mo before (Fig. 2, lane *J*) and at the clinical onset were ICCA negative but strongly positive for 64,000 M_r antibodies.

Patient 14 (Fig. 4, Table I) was the only ICCA positive individual among 57 siblings to IDDM children in a Swedish family study. He was tested and found to be strongly 64,000 M_r antibody positive (Fig. 2, lane *L*) IVGTT showed a lowered β-cell function. 25-mo later, when he developed clinical symptoms of IDDM and insulin was administered, he was still ICCA and 64,000 M_r antibody positive.

Correlation between ICCA and 64,000 M_r autoantibodies. Comparison of 64,000 M_r antibody and ICCA assays in individual patients (Fig. 4) shows that patients 1–9, and 14 were positive in assays for both 64,000 M_r antibodies and ICCA. Patients 10 and 13 were discordant; patient 10 was ICCA positive but 64,000 M_r negative, and patient 13 was 64,000 M_r antibody positve and ICCA negative during the observation period. 64,000 M_r antibodies were clearly detected before ICCA in patients 6 and 8. Patients 11 and 12 were negative or weakly positive in both assays indicating either that autoantibodies did not develop in these patients or that they were present earlier (patient 11) or later (patient 12) than the observation period. All 1st degree relatives (Table II) and controls, except one Hashimoto's disease patient, were concordant in both assays.

Discussion

Our results provide substantial evidence that antibodies to a 64,000 M_r human islet cell membrane protein (4) are associated with development of IDDM.

All IDDM patients included in this study except patient 10 had a family history of IDDM. In fact several of them had been followed before the clinical onset of IDDM for that reason. This approach to study prodromal autoimmunity in IDDM was motivated by the fact that the prevalence of IDDM is only 0.4%. However, the majority of new patients (87%) has no IDDM family members.

In those highly selected individuals it was demonstrated that the 64,000 M_r antibodies preceded the clinical onset of IDDM by several years and sometimes even preceded the detection of ICCA.

The detection of 64,000 M_r antibodies several years before IDDM was clinically manifest lends support to the concept that an autoimmune attack on β-cells may start long before the clinical onset (25). Experiments in mice indicate that a critical threshold of 80–90% loss of β-cells is needed before clinical symptoms of diabetes develop (26). As described (11–13) for some of the patients studied here it is possible that a similar progressive loss of β-cell function over a long period of time has also occurred before the clinical onset of IDDM.

The expression of the 64,000 M_r antibodies did not progress linearly with time except in one patient. Therefore it is not known how early the antibodies developed in the other positive individuals. In fact, the one patient followed for the longest period had the strongest positive reaction in a sample obtained 91 mo before clinical onset of IDDM and became less positive in later samples. Since the immune reaction seemed to diminish closer to onset we speculate that a low 64,000 M_r immunoreactivity detected in some patients may, in fact, reflect the end of an autoimmune process, since antigen in sufficient amounts may not be present to maintain an immune response. The data available for the IDDM patients and the first degree relatives with abnormal glucose tolerance are inconclusive as to whether or not 64,000 M_r antibodies were present before a decrease in β-cell function commenced. Only prospective analyses of 64,000 M_r positive individuals identified before any abnormalities in β-cell function, exemplified by the 64,000 M_r antibody positive control sibling, may, given time, provide information as to the order of events.

Isolated antigen is needed for specific and sensitive immunoassays for 64,000 M_r autoantibodies to determine how 64,000 M_r antibody titers relate to loss of β-cells during a prediabetic period. Such assays should also make it possible to determine the prevalence of 64,000 M_r autoantibodies among healthy individuals with and without family history of IDDM in larger population samples. Apart from the 21 healthy controls analyzed in the present study, we have previously analyzed an additional 13 healthy individuals and only found one positive. The specificity of 64,000 M_r antibodies is also demonstrated by their absence in sera from 5 SLE patients and in 36 sera from patients with diseases of the thyroid having no clinical signs of impaired glucose tolerance. The analyses of such material, which included patients with high titers of nonrelated antibodies, did demonstrate the importance of using a purified membrane protein fraction to avoid background problems in the assay for 64,000 M_r antibodies. Furthermore, results using sera from patients 1, 11, and 12 indicated that very weak titers of 64,000 M_r antibodies may only be detected using purified material.

It is still unclear whether the 64,000 M_r determinant is expressed on the cell surface or primarily confined to the intracellular compartment. The membrane protein characteristics of the 64,000 M_r component were demonstrated by its partition into the detergent phase of TN-114 (24). Furthermore in subcellular fractionation of human islets, the protein is found in the particulate fraction (Dr. M. Christie, unpublished results). It was found that the 64,000 M_r antibodies were better correlated to ICSA detected on purified rat islet β-cells than to ICCA (4). The demonstration of a plasma membrane expression of the 64,000 M_r protein by surface iodination was not possible due to difficulties in disrupting human islets without damaging the fragile endocrine cells. An unequivocal assignment of the 64,000 M_r antigen to the plasma membrane or intracellular membranes has therefore not as yet been feasible.

In the present study the introduction of a novel highly sensitive double fluorescence assay resulted in the detection of ICCA in some individuals that had been negative using more conventional assays. Comparison of ICCA and 64,000 M_r antibody assay data showed discordance in two IDDM and one Hashimoto's disease patients. In theory since 64,000 M_r antibodies are directed to a human islet cell protein they should also be detectable by the ICCA assay, providing firstly, that it is sensitive enough to detect antibodies to a minor membrane protein and secondly that 64,000 M_r antigenic epitopes have not been destroyed during preparation of pancreatic sections. However, by the nature of immunofluorescence assays, sera may be positive in an ICCA assay without containing 64,000 antibodies.

It was noted that 64,000 M_r antibodies were detected in early samples of two patients which later became positive in the ICCA assay, demonstrating that in these patients, the 64,000 M_r an-

tibodies were the first detectable sign of an adverse immune reaction to islet cells. The opposite, that patients were positive in the ICCA assay before developing 64,000 M_r antibodies, was not observed.

The function of the 64,000 M_r protein is presently unknown. It was neither detected in 11 endocrine and nonendocrine human cell lines (27), nor in freshly isolated human peripheral lymphocytes (4) and thyrocytes (S. Bækkeskov, unpublished results) indicating that its expression may be restricted to islet cells. 64,000 M_r antibodies were detected in all β-cell specific, ICSA positive sera, tested on rat islets in a previous study (4) indicating that the protein is expressed in β-cells. Furthermore, a protein of the same molecular mass has been specifically immunoprecipitated with IDDM sera from β-cell tumors of transgenic mice (S. Bækkeskov and D. Hanahan, unpublished results). An unequivocal assignment of the protein to human β-cells requires analyses of purified human β-cells and such material is presently not available.

The present data indicate that the 64,000 M_r protein antibodies may appear long before the clinical onset of IDDM. It is suggested that this component may represent the primary target protein in an autoimmune reaction against the β-cells. A recent controlled trial with cyclosporin A suggests that the length and prevalence of remission in newly diagnosed IDDM patients are increased (28). It is speculated that immune intervention may rather result in a prevention of IDDM if started before a major loss of β-cells has occurred. Therefore, a simple, reliable, and rapid assay using purified target autoantigen(s) are urgently needed to detect islet cell antibody positive persons who are potentially at risk of developing IDDM. We suggest that the 64,000 M_r protein may be a likely candidate for use in such assays.

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