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Autoantibodies against a novel 51 kDa islet antigen and glutamate decarboxylase isoforms in autoimmune polyendocrine syndrome type I

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Summary Beta-cell function and islet cell antibodies were studied in six patients with autoimmune polyendocrine syndrome type I. All suffered from mucocutaneous candidiasis, five had adrenocortical insufficiency and three hypoparathyroidism. All sera contained high titres of antibodies staining islets of Langerhans. Reactivity against glutamate decarboxylase, predominantly the 65 kDa isoform, was detected by immunoprecipitations and Western blots in five of the six sera, and all six sera immunoprecipitated a 51 kDa antigen from [^{35}S]-methionine labelled rat islet cell lysates. No reactivity against this latter antigen was found in sera of patients with Type 1 (insulin-dependent) diabetes mellitus ($n = 9$), Graves' disease ($n = 5$), autoimmune gastritis ($n = 4$), idiopathic Addison's disease ($n = 7$), or stiff-man syndrome ($n = 2$). The 51 kDa antigen was also detected by Western blots using homogenates of rat islets and autoimmune polyendocrine syndrome type I patient sera, whereas no such reactivity was found with homogenates of testes, adrenals, small intestine, spleen, exocrine pancreas or brain. Moreover, the 51 kDa antigen was present in the rat insulinoma cell line RINm 5F but not in the SV-40 transformed, monkey kidney cell line COS, when examined by immunoprecipitations of [^{35}S]-methionine labelled cell lysates and by Western blots. None of the patients with autoimmune polyendocrine syndrome type I had symptoms of diabetes and their insulin responses to glucose challenge were normal. The data illustrate that patients with autoimmune polyendocrine syndrome type I present an autoimmune response against islets of Langerhans, which is apparently different from that associated with classic Type 1 diabetes. As most of the autoantigens in many autoimmune diseases are enzymes involved in important functions in the affected organs, it is possible that the anti-51 kDa antibodies are directed against a protein with important functional activity in the islet. [Diabetologia (1994) 37: 61–69]

Key words Islet of Langerhans, Type 1 (insulin-dependent) diabetes mellitus, autoimmunity, carboxypeptidase H, beta cell.

The autoimmune basis for Type 1 (insulin-dependent) diabetes mellitus is illustrated by the lymphocyte infiltration of the islets of Langerhans [1] accompanied by the presence of circulating autoantibodies [2] and the response of peripheral T lymphocytes to islet antigens in vitro [3]. The autoantibodies are known to react against several targets in the islets, although few have so far been identified. Glutamate decarboxylase (GAD), an enzyme responsible for the conversion of glutamic acid to γ-aminobutyric acid (GABA), is the most well-characterized islet cell antigen [4]. Antibodies against GAD are detected in up to 80 % of patients with recent-onset Type 1 diabetes [5], and pre-diabetic individuals can exhibit reactivity against the enzyme many years before the appearance of clinical symptoms [6].

Autoimmune polyglandular syndrome type I (APS I) is an autosomal recessive condition, usually manifested in early childhood [7, 8]. A failure of several endocrine organs is often accompanied by chronic mucocutaneous candidiasis, dystrophy of nails, vitiligo and keratopathy [9]. In most reports, hypoparathyroidism is the leading endocrine manifestation, affecting approximately 80 % of the patients [9–11]. Adrenocortical insufficiency and gonadal failure are also common fea-

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Table 1. Clinical profile, and results of insulin response to glucose challenge in the patients with polyendocrine syndrome type I

Patient #	1	2	3	4	5	6
Sex	F	F	F	M	M	M
Age (years)	17	16	7	6	19	14
Duration of disease (years)	11	13	5	5	17	11
Candidiasis	+	+	+	+	+	+
Addison's disease	+	–	+	+	+	+
Gonadal insufficiency	+	+	–	–	–	–
Hypoparathyroidism	+	+	–	–	–	+
Diabetes	–	–	–	–	–	–
First phase insulin release[a]	200	300.5	320	651.6	705	360

+ and – denote the presence or absence of clinical and laboratory findings of disease.
[a] Results of 0–10 min area insulin in $mU \cdot l^{-1} \cdot min^{-1}$ according to [29] (normal range 200–1870 $mU \cdot l^{-1} \cdot min^{-1}$)

tures [10]. Autoantibodies in sera of patients with APS I have been reported with reactivity against parathyroid glands [12], adrenal cortex [12], islets of Langerhans [2], gastric parietal cells [2] and the thyroid gland [13, 14].

Although Type 1 diabetes is a condition previously reported to occur in only 4% to 12% of patients with APS I [9, 10], Ahonen et al. [9] demonstrated that approximately 50% of the patients will develop diabetes before the age of 50 years. Eisenbarth and Jackson [15] report that figures as low as 4% to 12% are underestimations, due to the fact that most of the studies have been carried out on young patients with APS I.

In the present study, patients with APS I were investigated with respect to a humoral immune response against pancreatic islets, since the appearance of beta-cell damage apparently differs from that found in classic Type 1 diabetes. Evidence of a different antibody reactivity against GAD was obtained, and reactivity against a novel 51 kDa islet cell autoantigen was observed.

Subjects, materials and methods

Patient sera and specific antisera

Sera from six patients with APS I, three females and three males, between 6 and 19 years of age, were used in the study. All patients had mucocutaneous candidiasis, three suffered from hypoparathyroidism, and five had adrenocortical insufficiency. None of the patients were diabetic at the time of serum collection (Table 1). Investigation of the insulin response to an intravenous glucose challenge was performed in all patients according to Bingley et al. [16]. Sera from nine patients with recent-onset Type 1 diabetes, all investigated for the presence of islet cell antibodies (ICA) [2] and GAD antibodies [17], were used. Also, sera from seven patients with Addison's disease, five patients with Graves' disease, four patients with autoimmune gastritis, two patients with stiff-man syndrome, and six healthy blood donors were employed. The NIMH-1440 antiserum raised in sheep against purified rat brain GAD was a gift from Dr. I. J. Kopin and co-workers [18] at the National Institute of Health (Bethesda, Md., USA). This antiserum reacts against the two known isoforms of GAD (GAD-65 and GAD-67), but exhibits preference for the low molecular weight isoform. A rabbit antiserum against porcine glucagon was purchased (Dako Corporation, Carpinteria, Calif., USA), as was a guinea pig antiserum against porcine insulin (Dako-immunoglobulins a/s, Glostrup, Denmark). A rabbit antiserum against rat carboxypeptidase-H [19] was a generous gift from Dr. P. Guest at the Department of Clinical Biochemistry, University of Cambridge (Cambridge, UK). A rabbit antiserum (K2) against feline GAD-67 was a gift from Dr. A. Tobin, UCLA (Los Angeles, Calif., USA) [20].

Immunohistochemistry

For immunoperoxidase staining, unfixed cryostat sections of Wistar-Furth rat pancreas and cerebellum were submitted to a mild fixation at +4°C in 50% acetone in water [volume/volume (v/v)] for 30 s and then in 100% acetone for 5 min. After air-drying at room temperature the sections were rinsed in phosphate-buffered saline (PBS) composed of 137 mmol/l NaCl, 2.7 mmol/l KCl, 4.3 mmol/l Na_2HPO_4 and 1.4 mmol/l KH_2PO_4, pH 7.3 for 5 min, incubated in 0.3% H_2O_2 in PBS for 15 min and again rinsed in PBS. Incubation with a normal rabbit serum for 5 min at room temperature preceded the incubation with patient sera (in serial dilution), glucagon antiserum in a dilution of 1:75 or NIMH-1440 in 1:1,000 dilution for 24 h at +4°C. Following washings with PBS, each section was covered with a 1:200 dilution of peroxidase-conjugated rabbit anti-human or anti-sheep IgG antibody (Dako) and the incubations were continued for another 30 min at room temperature. After washing, the chromogenic reaction was started by the addition of a mixture containing 10 mg 3-amino-9-ethylcarbazol dissolved in 6 ml dimethyl sulphoxide, 50 ml 0.02 mol/l NaAc pH 5.5 and 4 µl 30% H_2O_2. The reaction was continued for 15 min at room temperature and stopped by rinsing in PBS. Counterstaining was performed with haematoxylin.

Immunofluorescence double staining was performed using cryostat sections of Wistar-Furth rat pancreas fixed in 1% (weight/volume) paraformaldehyde (Sigma Chemical Co., St. Louis, Mo., USA) in PBS. The primary antibody was glucagon antiserum which was detected using rodamine immunoglobulin-conjugate against rabbit IgG (Dako). The third antibody was either the APS I patient serum (tested in serial dilution) or the insulin antiserum, which were detected by fluorescein isothiocyanate (FITC) conjugated rabbit IgG against human IgG or guinea pig IgG, respectively. Analysis were performed using a Nikon Microphot FXA fluorescence microscope.

Isolation and culture of islets of Langerhans, RINm 5F cells and COS cells

Islets of Langerhans from Wistar-Furth rats were manually isolated after collagenase digestion [21], and used either freshly after isolation or after free-floating culture for 48 h in RPMI medium (NordVacc, Skärholmen, Sweden) containing 28 mmol/l glucose and 10% fetal calf serum. For immunoprecipitations, the islets were labelled with [^{35}S]-methionine (> 1,100 Ci/mmol, Amersham International, Bucks., UK) and lysed using a method previously described [22]. For use in Western blots islets were lysed without previous radiolabelling. The RINm 5F cell line [23] was cultivated in Ham F12 medium (NordVacc) sup-

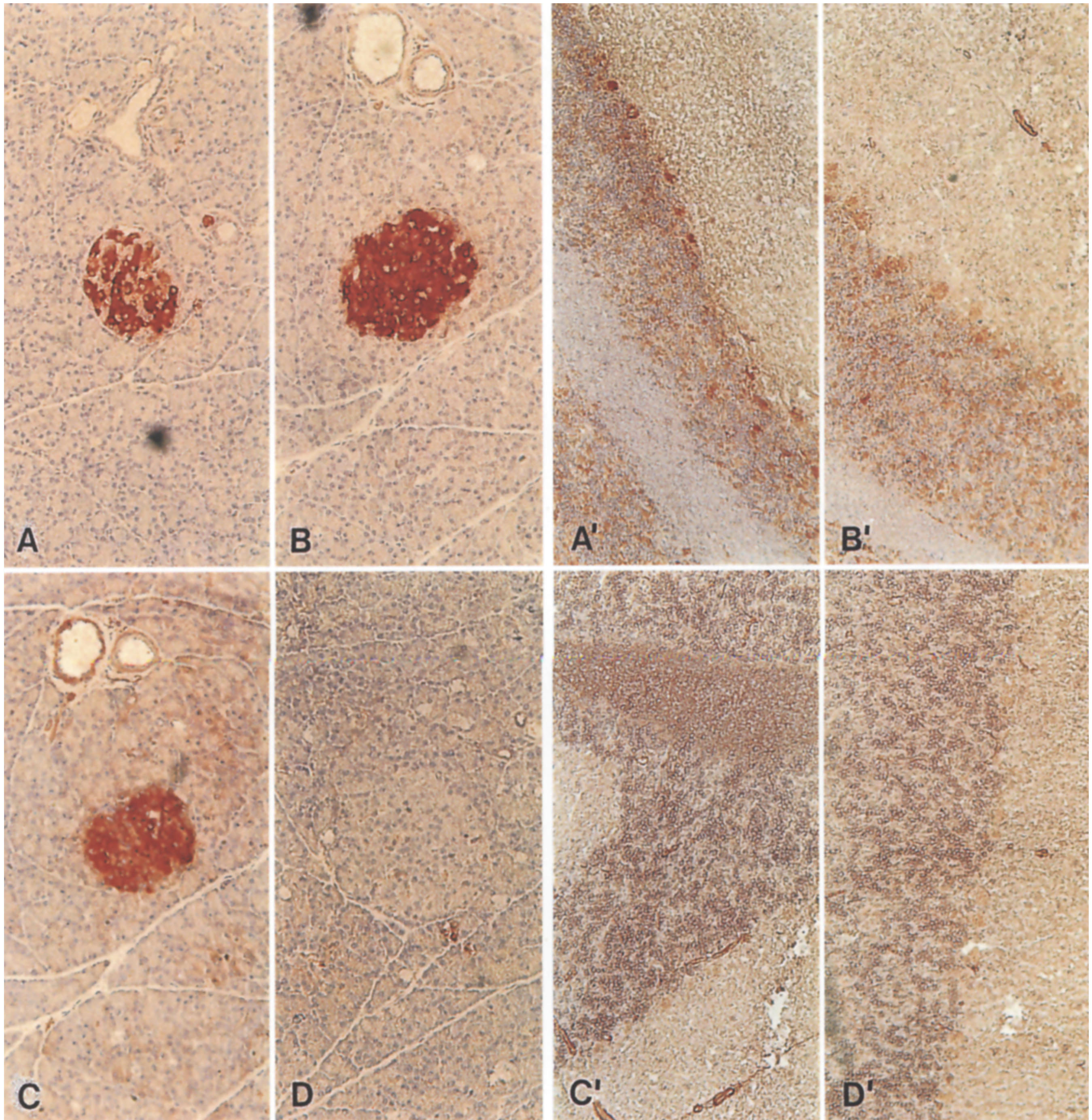

Fig. 1. Immunoperoxidase staining of frozen sections of rat pancreas (A, B, C, D) and cerebellum (A′, B′, C′, D′). The GAD antiserum NIMH-1440 (A and A′), sera from APS I patient # 1 (B and B′), APS I patient # 3 (C and C′), and a healthy blood donor (D and D′) were used. In pancreas, a positive staining of the islets is seen with the GAD antiserum NIMH-1440 and with both APS I patients (A, B and C). In the cerebellum, only GAD NIMH-1440 and APS I patient # 1 stain the neurons of the Purkinje and granular cell layer (A′ and B′), whereas no staining is observed with APS I patient # 3

plemented with non-essential amino acids and 10 % fetal calf serum. The SV-40 transformed, monkey kidney cell line COS [24] was cultivated in DMEM (NordVacc) with 10 % fetal calf serum. As described for the islets of Langerhans [22], the RINm 5F cells and the COS cells were either lysed, or labelled with [^{35}S]-methionine and lysed, for use in Western blots and immunoprecipitations, respectively.

Homogenates from rat tissues

Brain, testes, small intestine, adrenals, spleen and exocrine pancreas of Wistar-Furth rats were homogenised in ice-cold 10 mmol/l Tris/Cl buffer, pH 7.4 containing, 0.25 mol/l sucrose, 1 mmol/l EDTA and 1 mmol/l phenylmethyl sulphonylfluoride

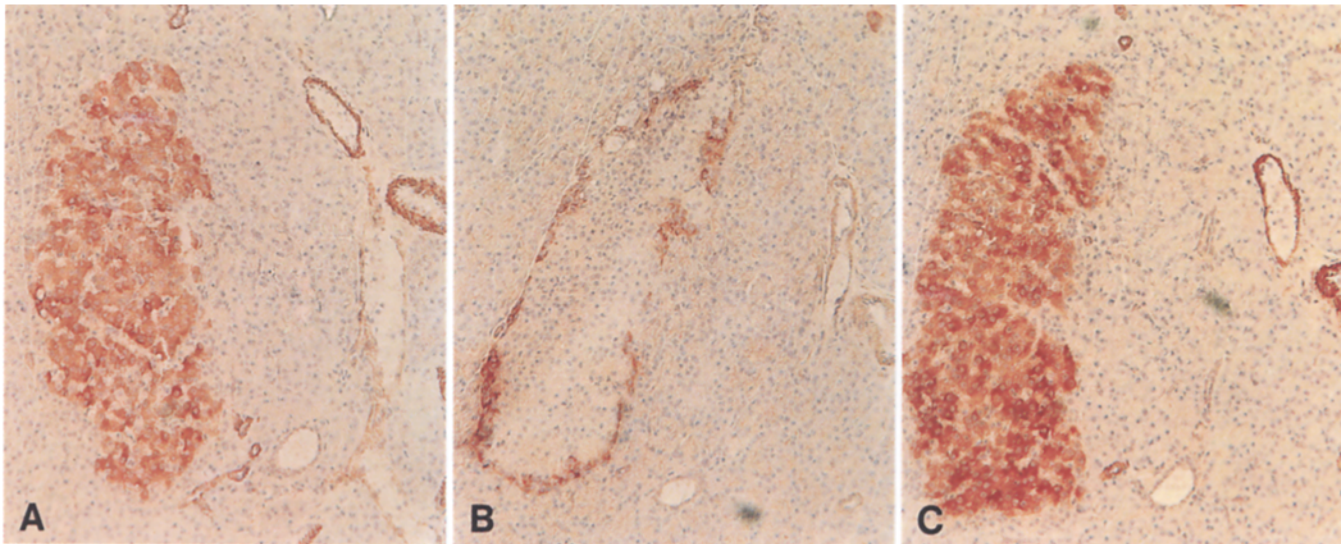

Fig. 2. Immunoperoxidase staining of sequential frozen sections of rat pancreas. The GAD antiserum NIMH-1440 (**A**), glucagon antiserum (**B**), and serum from APS I patient # 3 (**C**) were used. The pattern of staining with the patient serum is similar to that observed with GAD antiserum NIMH-1440, suggesting a beta-cell restricted expression for the target antigen

(PMSF) (homogenising buffer), using a syringe and a 20 gauge needle. Debris were removed by spinning the homogenates at 1,000 × g during 15 min at + 4 °C and the supernatants kept at − 70 °C for further use in Western blots.

Recombinant GAD

Recombinant rat GAD-65 and -67 were transiently expressed in a eukaryotic system (COS cells) as previously described [17], and used either in immunoprecipitation experiments after labelling with [^{35}S]-methionine, or in Western blot experiments.

Immunoprecipitations and Western blots

Gradient (10–15 %) sodium dodecylsulphate polyacrylamide gels (SDS-PAGE) were assembled as previously described [25]. For immunoprecipitations, sera of patients, healthy blood donors and specific antisera were incubated with aliquots of the [^{35}S]-methionine labelled lysates and the immune complexes formed were collected as described elsewhere [22]. Following electrophoresis, fixation of the gels was performed in a solution containing 10 % acetic acid and 30 % methanol in water (v/v) during 1 h. The gels were then incubated for 45 min in Enhance (DuPont, NEN Research Products, Boston, Mass., USA) and washed for 10 min in 2 % glycerol in water (v/v). Gels were finally dried in a vacuum dryer at + 65 °C for 90 min and exposed for fluorography with Fuji RX films at − 70 °C.

For Western blots, SDS-PAGE were prepared following the same procedures [25]. Lysates of either islet cells, RINm 5F cells, COS cells or homogenates of the different rat tissues were quantitated for their total protein concentration using the BCA (bicinchoninic acid) protein assay method (Pierce Chemical Company, Rockford, Ill., USA). Samples containing similar amounts of total protein were suspended in a sample buffer consisting of 0.1 mol/l Tris/Cl, pH 8.8, 3 % SDS, 10 mmol/l dithiothreitol, 10 % sucrose and trace amounts of bromophenol blue and were heated for 3 min at + 95 °C. After a short centrifugation at 10,000 × g, iodoacetamide was added to a final concentration of 50 mmol/l. An incubation of 15 min at room temperature preceded the loading onto the gel. For Western blots, proteins were transferred to a nitrocellulose membrane (Amersham International) after electrophoresis using a 200 mA current for 24 h, and the reactivity of sera examined as described [26].

Ion-exchange chromatography

A DEAE-Sepharose (Pharmacia, Uppsala, Sweden) column (1 cm × 0.5 cm) was equilibrated with 10 volumes of 0.1 mol/l Tris/Cl, pH 8.2 (equilibration buffer 1) followed by 10 volumes of 20 mmol/l Tris/Cl pH 8.2, plus 0.1 % Triton X-100 (Eastman Kodak Co., Rochester, NY, USA), 20 mmol/l PMSF and 1 % Trasylol (Bayer, Leverkusen, Germany) (equilibration buffer 2). [^{35}S]-methionine lysates of islet or RINm 5F cells were applied to columns in aliquots of 0.2 column volumes. Washing was performed with 10 volumes of equilibration buffer 2. Three different elution buffers were used sequentially, the first containing 20 mmol/l NaCl, the second containing 0.2 mol/l NaCl and the third containing 0.5 mol/l NaCl, each prepared with the equilibration buffer 2. Fractions of 0.2 volumes were collected from the flow-through and from the different stepwise eluates and pooled according to the radioactive peaks counted in a LKB β-counter (LKB Scintillation Products, Bromma, Sweden). Pools were used in subsequent immunoprecipitation experiments.

Lentil lectin chromatography

A Lentil lectin-Sepharose 4B (Pharmacia) column (2 cm × 0.5 cm) was equilibrated with 10 volumes of 20 mmol/l Tris/Cl pH 8.0, 150 mmol/l NaCl, 0.02 % NaN_3 and 0.2 % Triton X-100 (equilibration buffer 3). A 0.2 volume lysate of [^{35}S]-methionine labelled lysate of RINm 5F cells was loaded onto the column which was then washed with equilibration buffer 3. Elution of glycosylated proteins was performed with 150 mmol/l α-methyl-D-mannoside (Sigma) in equilibration buffer 3. The radioactive peaks of the flow-through and the eluate were pooled separately and used in immunoprecipitation experiments.

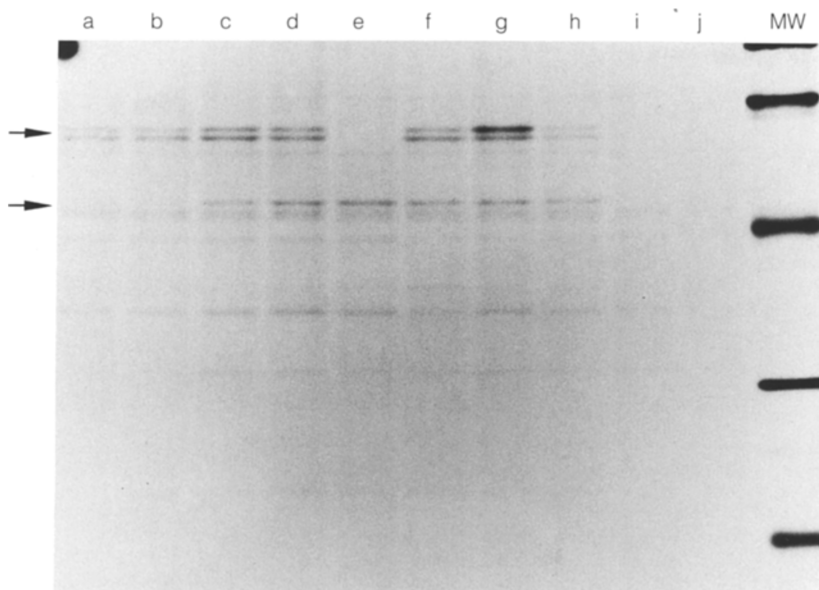

Fig. 3. Fluorography of an SDS-PAGE of immunoprecipitates from rat islet lysates labelled with [^{35}S]-methionine. GAD antiserum NIMH-1440 (lane a), serum from a patient with recent-onset Type 1 diabetes (lane b), sera from six patients with APS I (lanes c to h), and sera from two healthy blood donors (lanes i and j) were used. Molecular weight markers (MW) corresponding to 97.4 kDa, 69 kDa, 46 kDa, 30 kDa and 21.5 kDa appear in the right margin. In the left margin, arrows indicate the position of GAD (upper arrow) and of the 51 kDa antigen (lower arrow). Among the APS I patients only patient # 3 (lane e) did not precipitate GAD. The 51 kDa antigen was precipitated by all six APS I patients

Results

Immunohistochemistry

In initial experiments sera from all six APS I patients were used in immunoperoxidase staining of frozen sections of pancreas and cerebellum. Serum from a patient with Type 1 diabetes known to be positive in islet staining and with strong reactivity against GAD was used in parallel. Also, the NIMH-1440 GAD antiserum and sera from two healthy blood donors were used. All six APS I sera stained strongly the islets of Langerhans, and all but one serum (# 3) showed strong staining of the Purkinje and granular cells in the cerebellum (Fig. 1). Comparative staining with a glucagon antiserum and the NIMH-1440 GAD antiserum were performed using sequential sections of frozen pancreas. The pattern of reactivity of sera from all APS I patients was similar to that observed with the NIMH-1440 GAD antiserum, i. e. restricted to the beta cells (Fig. 2). These observations were further confirmed by immunofluorescence double staining. The APS I patient sera reacted in a pattern similar to that observed with the insulin antiserum, but did not stain the glucagon containing cells (not shown). The lack of cerebellar staining with serum from patient # 3 indicated reactivity against an antigen different from GAD.

Immunoprecipitation of rat islet lysates

Immunoprecipitations using sera of six APS I patients were performed in parallel with the NIMH-1440 GAD antiserum, serum from a Type 1 diabetic patient known to react against GAD, and two healthy blood donors. Except for patient # 3, all sera from APS I patients precipitated a doublet in the 65–67 kDa range which co-migrated with GAD precipitated by the NIMH-1440 antiserum and by the serum of a patient with Type 1 diabetes (Fig. 3). Moreover, sera from all six APS I patients precipitated a band of 51 kDa which was not detectable in the immunoprecipitates of the other control sera (Fig. 3).

Western blots with tissue homogenates and cell lysates

To investigate the tissue distribution of the 51 kDa antigen, Western blot experiments were performed using homogenates from spleen, testes, adrenals, brain, exocrine pancreas and small intestine from Wistar-Furth rats. Also, lysates from a rat insulinoma cell line RINm 5F, which does not express GAD, and an SV-40 transformed, simian renal cell line COS, were used. The homogenates and cell line lysates were run in similar protein concentrations and in parallel with a rat islet lysate. Analysis of reactivity was performed using sera from all APS I patients and two healthy blood donors. All APS I patient sera recognized a 51 kDa band in islets of Langerhans and in RINm 5F cells, but not in any of the tissues tested or in the COS cells. In adrenals, reactivity against a protein migrating at 53 kDa and identical to the cytochrome P450 side-chain cleavage enzyme was observed [27]. In addition, all but one (patient # 3) of the APS I patients reacted against a protein of 64 kDa (supposedly GAD) in the islet lysate and in the brain homogenate (data not shown).

Patients with other autoimmune endocrine diseases

The reactivity against the 51 kDa islet antigen was tested using sera from patients with autoimmune endocrine diseases and lysates of [^{35}S]-methionine la-

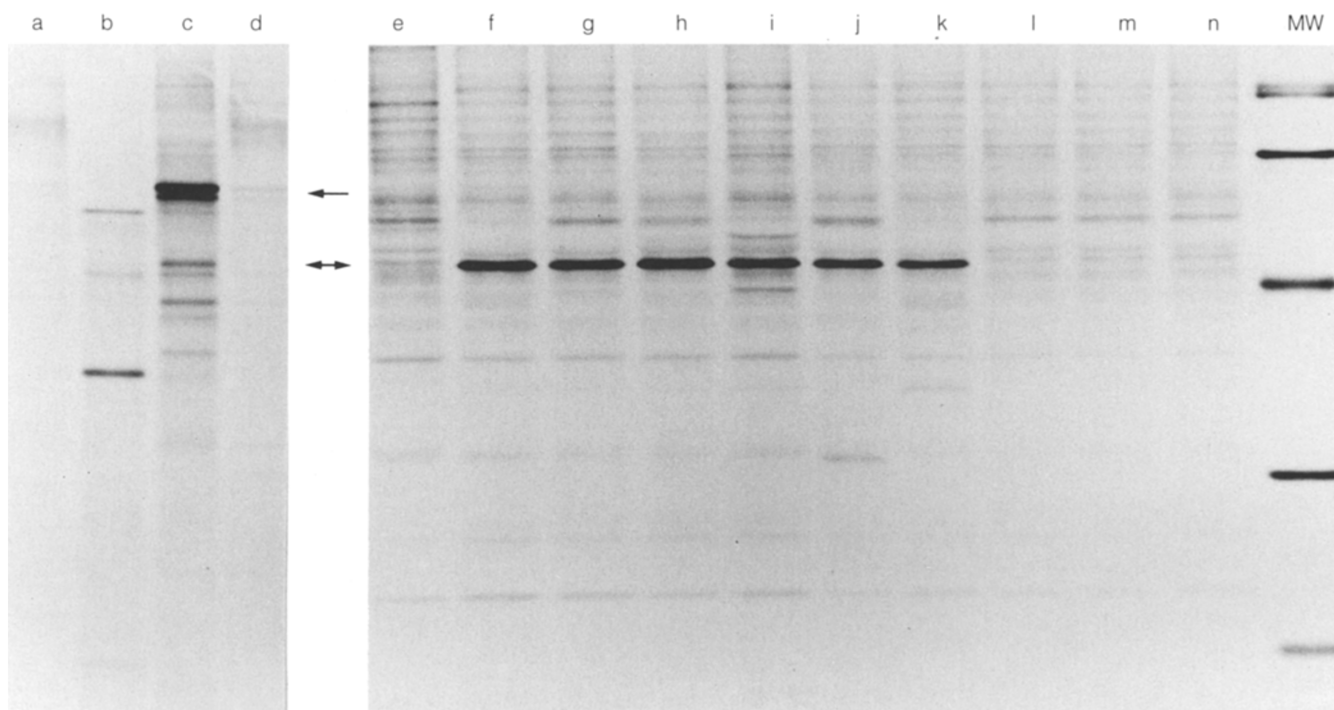

Fig. 4. Fluorography of an SDS-PAGE of immunoprecipitates from rat islets (lanes a to d) and RINm 5F cells (lanes e to n) lysates labelled with [^{35}S]-methionine. Different fractions from the partial purification of the 51 kDa antigen in rat islets, performed on a DEAE-Sepharose ion-exchange chromatography, are shown in lanes a to d. Pools from the flow-through (lane a), the fraction eluted with 0.02 mol/l NaCl (lane b), the fraction eluted with 0.2 mol/l NaCl (lane c) and the fraction eluted with 0.5 mol/l NaCl (lane d), were immunoprecipitated by serum from APS I patient # 1. A partial purification of the 51 kDa as well as GAD was obtained in the fraction eluted with 0.2 mol/l NaCl (lane c). The arrows between lanes d and e indicate the positions of GAD (upper arrow) and the 51 kDa antigen (lower arrow). RINm 5F lysates were submitted to the same ion-exchange purification procedure and the fraction eluted with 0.2 mol/l NaCl was used for immunoprecipitations with sera from one patient with recent-onset Type 1 diabetes (lane e), six APS I patients (lanes f to k), two patients with stiff-man syndrome (lanes l and m), and a healthy blood donor (lane n). Molecular weight markers (MW) corresponding to 97.4 kDa, 69 kDa, 46 kDa, 30 kDa and 21.5 kDa are on the right margin. An arrow indicating the position of the 51 kDa antigen is between lanes d and e (lower arrow)

belled RINm 5F cells. Sera from seven patients with Addison's disease, five patients with Graves' disease, nine patients with Type 1 diabetes and four patients with autoimmune gastritis were tested in parallel with sera from six healthy blood donors and two APS I patients (# 1 and # 3). Except for the APS I patient sera, no other serum showed reactivity against the 51 kDa antigen when the immunoprecipitates were analysed by SDS-PAGE and fluorography (not shown).

Biochemical characterization of the 51 kDa antigen

Upon DEAE-Sepharose chromatography at pH 8,2, the 51 kDa protein was recovered in a fraction eluted with 0.2 mol/l of NaCl (Fig. 4). The antigen did not appear to be glycated since it was not retarded on Lentil lectin-Sepharose 4B (data not shown). A 0.2 mol/l NaCl eluate from DEAE-Sepharose chromatography of a [^{35}S]-methionine labelled RINm 5F lysate was employed in immunoprecipitations with sera from the six APS I patients. A band of 51 kDa was regularly found whereas sera of patients with stiff-man syndrome or Type 1 diabetes did not precipitate such a protein (Fig. 4). Immunoprecipitations of [^{35}S]-methionine labelled COS cell lysates with the six APS I patient sera did not identify any 51 kDa protein (data not shown).

Sequential immunoprecipitations for characterization of the 51 kDa antigen

Carboxypeptidase-H, a 52 kDa enzyme involved in the processing of pro-insulin to insulin, present in the beta-cell secretory granule and reported to be a potential autoantigen in Type 1 diabetes [28], was examined as a potential candidate for the novel 51 kDa antigen. [^{35}S]-methionine labelled rat islet lysates were used for sequential immunoprecipitation experiments in incubations with the polyclonal antisera against carboxypeptidase-H or GAD, followed by immunoprecipitations of the supernatant with the serum from APS I patient # 1. The immunoprecipitation of lysates with the carboxypeptidase-H antiserum did not suppress the capacity of the APS I patient serum to identify the 51 kDa protein. When the pre-precipitation was per-

formed using the NIMH-1440 GAD antiserum, the 51 kDa protein was unchanged and a weak band possibly corresponding to the 67 kDa isoform of GAD, appeared with reduced strength in the APS I immunoprecipitate (Fig. 5). Thus, depletion of the GAD protein from the islet lysate did not affect the amount of 51 kDa antigen, demonstrating that the latter is unrelated to GAD.

APS I reactivity against the different isoforms of GAD

Reactivity against recombinant GAD-65 could be detected by immunoprecipitation and Western blot using sera from all but one (patient #3) of the APS I patients (Fig. 6). Patients #1, #2 and #5 also recognized recombinant GAD-67 but with lower capacity. Serum from a patient with stiff-man syndrome was analysed in parallel and reacted against both isoforms of GAD either by immunoprecipitation or by Western blot (Fig. 6).

Intravenous glucose tolerance test

First phase insulin release to a 0.5 g/kg glucose load was investigated in all APS I patients. As shown in Table 1 the insulin responses were in the normal range [29] in all subjects.

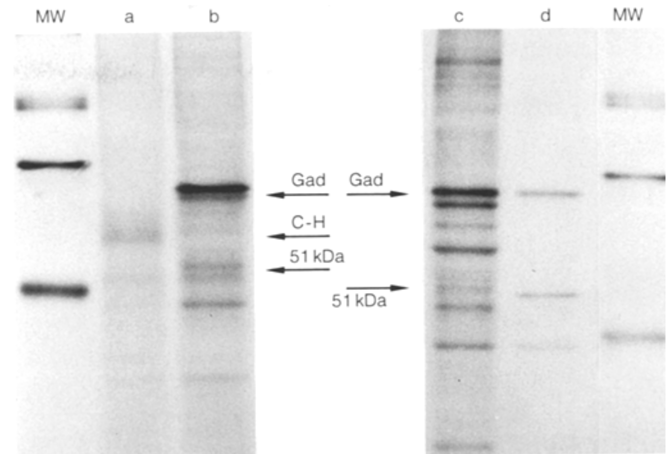

Fig. 5. Fluorography of an SDS-PAGE of immunoprecipitates from rat islet lysates labelled with [^{35}S]-methionine. Sequential immunoprecipitations were performed using specific antisera against carboxypeptidase-H (lanes a and b) and GAD (NIMH-1440) (lanes c and d). The labelled lysates were first immunoprecipitated with an excess of the specific antisera (lanes a and c) and the resulting supernatant was then immunoprecipitated with the APS I patient (patient #1) serum (lanes b and d). Molecular weight markers (MW) corresponding to 97.4 kDa, 69 kDa and 46 kDa are on the margins. The arrows between lanes b and c indicate the positions of GAD, carboxypeptidase-H (C-H) which appears as a wide band in lane a, and the 51 kDa antigen

Discussion

In the present study, using sera from patients with APS I, we found a strong reactivity against GAD in five out of six sera examined, and discovered a novel islet autoantigen, a non-glycated protein of 51 kDa. Sera from patients with APS I reacted with GAD similarly to sera from patients with stiff-man syndrome, i. e. recognising the denatured and reduced protein in Western blots, while sera of diabetic patients do not [4]. Also, APS I sera stain islets in frozen sections of pancreas, or the Purkinje and granular cells in sections of cerebellum even at a dilution of more than 1:10,000, while sera from diabetic patients seldom stain at dilutions up to 1:2,000 (data not shown). Furthermore, most of the APS I sera react with both the 67 kDa and 65 kDa isoforms of GAD, albeit preferentially against GAD-65, whereas only few sera of diabetic patients recognize the 67 kDa isoform [17].

Sera from all six APS I patients showed strong reactivity against a novel 51 kDa antigen when analysed both by immunoprecipitations and Western blots. Christie et al. [30] have described reactivity against a 50 kDa tryptic fragment of GAD as marker for development of diabetes in a study of identical twins. Using the NIMH-1440 GAD antiserum and performing sequential immunoprecipitation experiments, no relationship between the 51 kDa antigen recognized by the APS I patients and GAD was observed.

Carboxypeptidase-H, a glycated protein of the beta-cell insulin secretory granule, has been reported to be an autoantigen recognized by antibodies in sera of pre-diabetic subjects [28]. Classically, carboxypeptidase-H is regarded as a 52 kDa protein [28] but a soluble form of 50 kDa [31] and an immature form of 57 kDa [19] have also been reported. With the use of a specific antiserum, Guest et al. [19] found by Western blots analysis of insulin granule preparations, a wide band spreading from 57 kDa to 53 kDa, probably corresponding to intermediary products between the immature and the soluble forms. Using the same antiserum and lysates of rat islets we performed sequential immunoprecipitation experiments with serum from an APS I patient. Although carboxypeptidase-H was seen as a wide band between 57 kDa and 53 kDa, serum against this enzyme did not suppress the following immunoprecipitation of the 51 kDa antigen with the APS I patient serum.

Karounos and Thomas [32] found reactivity against an RINm 5F cell membrane antigen of 52 kDa when analysing sera from non-obese diabetic (NOD) mice. Using a similar procedure, we tested sera from 10 pre-diabetic female NOD mice in parallel with sera from two APS I patients (#1 and #3). Although two of the ten NOD mice sera reacted weakly against a 52 kDa protein, it migrated slower in the SDS-PAGE than the 51 kDa antigen recognized by the APS I patients (data not shown).

Recent evidence suggests that a considerable fraction of APS I patients eventually develop Type 1

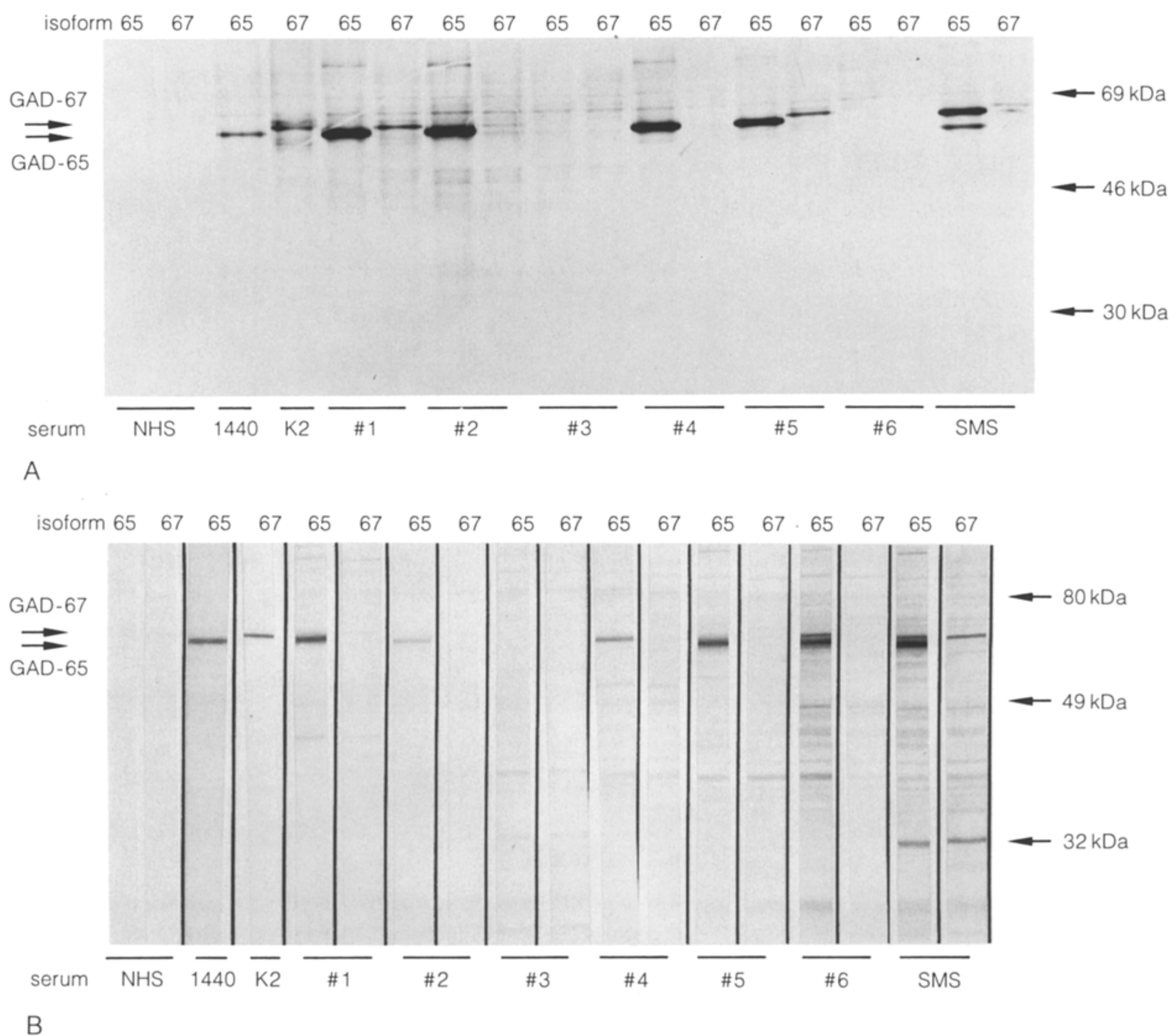

Fig. 6 (A, B). APS I reactivity against the different isoforms of GAD. **A:** Fluorography of an SDS-PAGE of immunoprecipitates from [^{35}S]-methionine labelled lysates of COS cells transfected with recombinant GAD-65 or GAD-67. Sera from all six APS I patients were analysed in parallel with specific antisera NIMH-1440 and K2, a stiff-man syndrome (SMS) patient serum and a healthy blood donor serum (NHS). APS I patients #1, #2, #4, #5, #6 reacted against GAD-65 as did the NIMH-1440 and the SMS patient serum. APS I patients #1, #2, and #5, as well as K2 antiserum and serum from the SMS patient, reacted against GAD-67. **B:** Western blot analysis of lysates of COS cells transfected with recombinant GAD-65 or GAD-67. Sera from all six APS I patients were analysed in parallel with the specific antisera NIMH-1440 and K2, and sera from a patient with SMS and a healthy blood donor (NHS). GAD-65 was recognized by APS I patients #1, #2, #4, #5 and #6, as well as by the NIMH-1440 antiserum and the SMS patient serum. GAD-67 was recognized by the sera of APS I patients #1 and #5 as well by the K2 antiserum and the SMS patient serum. Arrows in the left margin indicate approximate positions of GAD-67 and GAD-65. Molecular weight markers are indicated in the right margin

diabetes in the course of their disease [9, 15]. The pattern of evolution towards beta-cell failure however, differs from that observed in patients with classic Type 1 diabetes, and the courses are likely to reflect separate genetic as well as environmental influences on the susceptibility to islet cell damage. Possibly, longitudinal studies of APS I patients following the diagnosis of their syndrome can help to identify pathogenetic events preceding the onset of Type 1 diabetes. In this study we show that the reactivity of APS I sera against a major islet cell antigen, GAD, differ from that of sera from patients with classic Type 1 diabetes and that the APS I sera also react against a novel islet cell 51 kDa antigen. Since the majority of proteins recognized as antigens in autoimmune diseases are enzymes [33] with important roles in the physiology of the organs [4, 26, 27, 34], we suggest that the novel 51 kDa antigen might be involved in an important function of the islets of Langerhans.

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References

1. Gepts W (1965) Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 14: 619–633
2. Bottazzo GF, Florin-Christiansen A, Doniach D (1974) Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet II: 1279–1282
3. Atkinson MA, Kaufman DL, Campbell L et al. (1992) Response of peripheral blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 339: 458–459
4. Baekkeskov S, Aanstoot H-J, Christgau S et al. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature (Lond) 347: 151–156
5. Baekkeskov S, Landin M, Kristensen JK et al. (1987) Antibodies to a 64,000 Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J Clin Invest 79: 926–934
6. Atkinson MA, Maclaren NK, Scharp DW, Lacy PE, Riley WJ (1990) 64,000 Mr autoantibodies as predictors of insulin-dependent diabetes. Lancet 335: 1357–1360
7. Ahonen P, Koskimies S, Lokki M-L, Tiilikainen A, Perheentupa J (1988) The expression of autoimmune polyglandular disease appears associated with several HLA-A antigens but not with HLA-DR. J Clin Endocrinol Metab 66: 1152–1157
8. Maclaren NK, Riley W (1986) Inherited susceptibility to autoimmune Addison's disease is linked to human leukocyte antigens-DR3 and/or DR4, except when associated with type I autoimmune polyglandular syndrome. J Clin Endocrinol Metab 62: 455–459
9. Ahonen P, Myllärniemi S, Sipilä I, Perheentupa J (1990) Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med 322: 1829–1836
10. Neufeld M, Maclaren N, Blizzard R (1980) Autoimmune polyglandular syndromes. Pediatr Ann 9: 154–162
11. Maclaren NK, Riley WJ (1988) Autoimmune polyendocrinopathies. In: Samter M, Talmage DW, Franck MM, Austen KF, Claman HN (eds) Immunological diseases. 4th edn. Little Brown, Co. Boston, pp 1737–1744
12. Blizzard RM, Chee D, Davis W (1966) The incidence of parathyroid and other antibodies in the sera of patients with idiopathic hypoparathyroidism. Clin Exp Immunol 1: 119–128
13. Goldstein DE, Drash A, Gibbs J, Blizzard RM (1970) Diabetes mellitus: the incidence of circulating antibodies against thyroid, gastric and adrenal tissue. J Pediat 77: 304–306
14. Irvine WJ, Clarke BF, Scarth L, Cullen DR, Duncan LJP (1970) Thyroid and gastric autoimmunity in patients with diabetes mellitus. Lancet II: 163–168
15. Eisenbarth GS, Jackson R (1992) The immunoendocrinopathy syndromes. In: Wilson JD, Forster DW (eds) Williams textbook of endocrinology. W. B. Saunders Company, Philadelphia, pp 1555–1566
16. Bingley PJ, Colman P, Eisenbarth GS et al. (1992) Standardization of IVGTT to predict IDDM. Diabetes Care 15: 1313–1316
17. Velloso LA, Kämpe O, Hallberg A, Christmanson L, Betsholtz C, Karlsson FA (1993) Demonstration of GAD-65 as the main immunogenic isoform of glutamate decarboxylase in type 1 diabetes and determination of autoantibodies using a radioligand produced by eukaryotic expression. J Clin Invest 91: 2084–2090
18. Oertel WH, Schmechel DE, Tappaz ML, Kopin IJ (1981) Production of a specific antiserum to rat brain glutamic acid decarboxylase by injection of an antigen-antibody complex. Neuroscience 6: 2689–2700
19. Guest PC, Ravazzola M, Davidson WH, Orci L, Hutton JC (1991) Molecular heterogenity and cellular localization of carboxypeptidase H in the islets of Langerhans. Endocrinology 129: 734–740
20. Kaufman DL, Houser CR, Tobin AJ (1991) Two forms of the gamma-amino butyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56: 720–723
21. Andersson A (1978) Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets. Diabetologia 14: 397–404
22. Kämpe O, Andersson A, Björk E, Hallberg A, Karlsson FA (1988) High glucose stimulation of 64,000-Mr islet cell autoantigen expression. Diabetes 38: 1326–1328
23. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir CG, Lauris V (1980) Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. Proc Natl Acad Sci USA 77: 3519–3523
24. Gluzman Y (1981) SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23: 175–182
25. Blobel G, Dobberstein B (1975) Transfer of proteins across membranes. J Cell Biol 67: 835–851
26. Winqvist O, Karlsson FA, Kämpe O (1992) 21-hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet 339: 1559–1562
27. Winqvist O, Gustafsson J, Rorsman F, Karlsson FA, Kämpe O (in press) Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. J Clin Invest
28. Castaño L, Russo E, Zhou I, Lipes MA, Eisenbarth GS (1991) Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type 1 diabetes. J Clin Endocrinol Metab 73: 1197–1201
29. Heinze E, Holl RW (1991) Tests for β-cell function in childhood and adolescence. In: Ranke MB (ed) Functional endocrinologic diagnostics in children and adolescents. J. & J. Verlag, Heidelberg, pp 188–190
30. Christie MR, Tun RYM, Lo SSS et al. (1992) Antibodies to GAD and tryptic fragments of islet 64K antigen as distinct markers for development of IDDM. Diabetes 41: 782–787
31. Fricker LD, Snyder SH (1983) Purification and characterization of enkephalin convertase, an enkephalin-synthesizing carboxypeptidase. J Biol Chem 258: 10950–10955
32. Karounos DG, Thomas JW (1990) Recognition of common islets antigen by autoantibodies from NOD mice and humans with IDDM. Diabetes 39: 1085–1090
33. Banga JP, McGregor AM (1991) Enzymes as targets for autoantibodies in human autoimmune disease: relevance to pathogenesis? Autoimmunity 9: 177–182
34. Karlsson FA, Burman P, Lööf L, Mårdh S (1988) Major parietal cell antigen in autoimmune gastritis with pernicious anemia is the acid-producing H^+, K^+-adenosine triphosphatase of the stomach. J Clin Invest 81: 475–479