Hypoglycaemia due to insulin autoimmune syndrome: report of two cases with characterisation of HLA alleles and insulin autoantibodies

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Abstract

Objective: Insulin autoimmune syndrome (IAS) has been reported mainly in Japan and so far only 27 IAS cases have been described from outside Asia. We describe two unrelated Portuguese patients with IAS and characterise their insulin autoantibodies and HLA alleles.

Patients: Patient 1, a 24-year-old white female suffered an episode of unconsciousness in the late postprandial state and blood glucose was found to be 33 mg/dl with serum insulin levels of >3980 μIU/ml (normal range 0–30 μIU/ml). She was receiving monthly injections of penicillin G for the prophylaxis of recurrent tonsillitis. Patient 2, was a 19-year-old white female, with episodes of sweating, hand tremor, weakness and hunger occurring in the postprandial state and blood glucose levels during the attacks of 28–56 mg/dl. Very high insulin levels (602–708 μIU/ml) were present.

Methods and Results: Anti-insulin antibodies, determined by a semi-quantitative method, were strongly positive in both patients (91.7% in patient 1 and 88.6% in patient 2; normal range ≤7%). Sephadex G-100 chromatography of the sera showed most of insulin immunoreactivity present in the void volume which was retained by an affinity column with anti-human-immunoglobulin G antibodies (87% and 95% from patients 1 and 2 respectively). Scatchard plot analysis and molecular typing of the DRB1 gene revealed a polyclonal antibody and DRB1*0406 in patient 1, and a monoclonal antibody and DRB1*0403 in patient 2.

Conclusions: These two Portuguese patients with IAS had different HLA-DR4 subtypes and insulin autoantibodies: DRB1*0406 and a polyclonal antibody in one case and a monoclonal antibody in the other.
hospital where blood glucose was found to be 33 mg/dl. During a 36 h fast, blood glucose levels fell to 30 mg/dl at 24 h and rose to 60 mg/dl at 28 h and 64 mg/dl at 36 h. There was no loss of consciousness during the test. Blood glucose levels were associated with very high insulin levels (>3980 μIU/ml; normal range 0–30 μIU/ml) and elevated C-peptide levels (range 3.6–8.8 ng/ml; normal range 0.8–4 ng/ml). Attempts at tumour localisation (abdominal echography, abdominal computed tomography scan, abdominal nuclear magnetic resonance and celiac angiography) failed. She was then referred to the Division of Endocrinology of the Portuguese Institute of Oncology. She had no personal history of insulin or hypoglycaemic agent usage, and was not employed in a health-related profession. She was receiving monthly injections of penicillin G for the prophylaxis of recurrent tonsillitis. Her father and paternal relatives suffered from vitiligo. Her physical examination was unrevealing; weight 61.2 kg, height 157 cm and blood pressure 125/80 mmHg. Haemoglobin and leukocytes, thrombocyte count, erythrocyte sedimentation rate and liver, renal, thyroid and adrenal functions were all normal. Serologic examination was unremarkable; tests for C-reactive protein, rheumatoid factor, antinuclear antibodies, anti-DNA antibodies and anti-thyroid antibodies were all negative. Serum immunoglobulin G (IgG), A and M levels were also within the normal ranges. Serologic examination was unremarkable; tests for C-reactive protein, rheumatoid factor, antinuclear antibodies, anti-DNA antibodies and anti-thyroid antibodies were all negative. Serum immunoglobulin G (IgG), A and M levels were also within the normal ranges. Islet cell antibody tests were negative but high titres of insulin antibodies (91.7%; normal range ≤7%) were found. An oral glucose tolerance test performed for 240 min showed a basal glucose value of 79 mg/dl, with a peak of 143 mg/dl at 60 min and a nadir of 54 mg/dl at 210 min. Plasma insulin during the test ranged from a basal level of 203 μIU/ml to a peak level of 325 μIU/ml at 60 min (normal range 0–30 μIU/ml). Basal C-peptide levels were 1.4 ng/ml (normal range 0.8–4 ng/ml). The diagnosis of IAS was made. Serum insulin levels and titres of anti-insulin antibodies, after discontinuing treatment with penicillin G, are shown in Fig. 1.

Patient 2, a 19-year-old white female, was admitted to our institution in February 1994, because of episodes of sweating, hand tremor, weakness and hunger that had been occurring since November 1993. The symptoms occurred during the postprandial state and were promptly relieved by food ingestion. Blood glucose levels during the attacks were 28–56 mg/dl. During a 24 h fast, blood glucose was 50 mg/dl at baseline, 67 mg/dl at 2 h, 75 mg/dl at 6 h, 74 mg/dl at 12 h, 76 mg/dl at 18 h and 61 mg/dl at 24 h. Very high insulin levels (602–708 μIU/ml; normal range is 0–30 μIU/ml) and normal C-peptide levels (range 0.4–0.6 ng/ml; normal range is 0.8–4 ng/ml) were present during the test. Abdominal echography, abdominal computed tomography scan, abdominal nuclear magnetic resonance and coeliac angiography showed normal findings. She had no personal history of insulin usage, intake of oral hypoglycaemic agents or employment in a health-related profession. Her father suffered from a thoracic rhabdomyosarcoma. Her physical examination revealed no abnormalities; weight 65.5 kg, height 160 cm and blood pressure 118/72 mmHg. Routine laboratory tests were normal as were liver, renal, thyroid and adrenal functions. Tests for rheumatoid factor, antinuclear antibodies, anti-DNA antibodies, islet cell and anti-glutamic acid decarboxylase II antibodies were negative. Levels of insulin antibodies were strongly positive (88.6%; normal range ≤7%). She was advised to fractionate her meals and the symptoms gradually disappeared within a few weeks. Serum insulin levels and titres of anti-insulin antibodies during follow-up are represented in Fig. 1.

Insulin and C-peptide assays

Insulin was measured by radioimmunoassay (RIA) with Coat-A-Count Insulin (DPC, Los Angeles, CA,
USA) which uses an anti-human insulin antibody immobilised to the wall of a polypropylene tube. This assay has a sensitivity of 1.2 mIU/ml and intra- and interassay coefficients of variation of less than 12.5% for concentrations between 2.5 and 400 mIU/ml. The normal range of serum insulin is 0–30 mIU/ml.

C-peptide was measured by RIA with Double Antibody C-Peptide (DPC). This assay employs two antibodies: a rabbit anti-human C-peptide antibody and a goat anti-rabbit antibody. Sensitivity is 0.05 ng/ml and intra- and interassay coefficients of variation are less than 10% for concentrations between 0.89 and 15.38 ng/ml. The normal range of C-peptide in serum is 0.8–4 ng/ml.

**Determination of serum insulin auto-antibodies**

Circulating anti-insulin antibodies were estimated on a semi-quantitative basis by the determination of $^{125}$I-Tyr-A14-insulin binding to a sample fraction precipitated by the polyethylene glycol (Semi-quantitative Determination of Free Anti-insulin Antibodies, CIS, Gif-sur-yvette, France). The binding percentage of $^{125}$I-human insulin (DPC) to Tyr-A14-insulin is calculated according to the following formula: Bound/Total % (B/T%) = Radioactive counts of sample/Total counts×100. Intra- and interassay coefficients of variation are less than 20% for a range of B/T% between 3.2 and 60. Binding percentages higher than 7 indicate the presence of circulating anti-insulin antibodies.

**Gel filtration**

Gel filtration was performed in a 100×1.6 cm column of Sephadex G-100 Superfine (Pharmacia, Uppsala, Sweden), equilibrated at 4 °C with 50 mM phosphate-buffered saline at pH 7.5. The column was calibrated with blue dextran, $^{125}$I-human insulin (DPC) and $^{125}$I-iodine ($^{125}$I, Amersham, Bucks, UK). Serum samples of 8.0 ml (case 4) and 3.0 ml (case 2) were applied to the column, and fractions of 3.25 ml were collected for the insulin and C-peptide assays. Both sera and fractions were aliquoted to minimise the number of freeze–thaw cycles. All samples were stored at −20 °C for less than 4 months.

**Affinity chromatography with anti-human IgG agarose column**

In each case, a pool of three to six chromatographic fractions, that contained most of the insulin immunoreactivity present in the void volume, was applied to a column (30×1.5 cm) of goat anti-human IgG covalently bound to agarose (Sigma Quimica, Madrid, Spain). The binding capacity of the resin was at least 1.5 mg of human immunoglobulin G (hIgG) from human serum per ml of agarose and the amount of IgG in the pool applied to the column was always lower than the total binding capacity of the agarose. The column was washed with 105 ml 10 mM sodium phosphate at pH 7.4 (unretained material). IgG was eluted with 105 ml 0.1 M glycine at pH 2.5, into tubes containing 1 M Tris at pH 8.0 (retained material). Both the retained and unretained fractions were assayed for insulin by RIA (DPC), for anti-insulin antibodies by a semi-quantitative assay (CIS) and for IgG by radial immunodiffusion (The Binding Site, Birmingham, UK).

**Scatchard analysis of insulin antibodies**

Serum, with insulin removed by dextran-coated charcoal (4), was used for the Scatchard analysis (5). The serum was incubated with 100 μl of $^{125}$I-human insulin $(1.2×10^4$ c.p.m. per 30–40 pg of human insulin per tube; Amersham) in the presence of 100 μl of serial concentrations of human insulin solution. After precipitation with poly(ethylene glycol) 6000, the radioactivity of the pellets was counted with an automatic gamma counter (ARC-950M; ALOKA, Tokyo, Japan).

**HLA typing**

Patients were typed for polymorphic HLA-A, -B, -C, -DR and -DQ alleles. Lymphocyte isolation was performed using immunomagnetic particles coupled to monoclonal antibodies specific for either T or B lymphocytes. HLA typing was performed by a microlymphocytotoxicity assay (6), using Lambda Monoclonal Typing Trays First Class I and II (One Lambda, Canoga Park, CA, USA) with 74 specificities for HLA-A and -B loci, 10 specificities for HLA-C and 38 specificities for HLA-DR and -DQ loci.

Molecular typing was focused on class II molecules because of the primary involvement of DRB1 molecule in IAS patients (7). DNA was extracted from peripheral white blood cells and typing was performed by PCR-MPH (microtitre plate hybridisation) (8).

**Results**

**Gel filtration**

The results of the distribution of insulin and C-peptide after Sephadex G-100 chromatography of the sera from the two patients are illustrated in Fig. 2. Gel-filtration profiles of both cases showed that most of the insulin immunoreactivity was present in the void volume. C-peptide immunoreactivity in patient 2 was also present in the void volume, but in patient 1, only 59% of C-peptide immunoreactivity was detected in this first peak, with the remaining percentage appearing in fractions where proinsulin would be expected to elute.
Affinity chromatography with an anti-hIgG agarose column

As shown in Fig. 3, approximately 87% and 95% of insulin from patients 1 and 2 respectively, were retained by the affinity column. The insulin peaks were coincident with the peaks of anti-insulin antibodies and IgG thus confirming the presence of an insulin autoantibody.

Scatchard analysis of insulin autoantibodies

Patient 1 had polyclonal insulin autoantibody with $K_1$ of $0.489 \times 10^8 \text{ M}^{-1}$ and $b_1$ of $0.19 \times 10^{-8} \text{ M}$ (Fig. 4).

HLA typing

Serological typing of HLA alleles revealed the allelic combination A23(9)/68, B44/55, Cw2/9(3.1), DR4/13,
DQ1/4 in patient 1 and A23/66, B49/51, Cw2/7, DR4/7, DQ2/7(3) in patient 2. Analysis of the DRB1 gene sequence showed DRB1*0406 in patient 1 and DRB1*0403 in patient 2.

Discussion
IAS was suspected in our patients because hypoglycaemia was present in association with extremely high levels of serum insulin. This probably results from the dissociation of insulin from its antibodies, several hours after meals, when no further absorption of glucose is occurring (9). As a consequence, in both patients, hypoglycaemia occurred in the late postprandial period and not during fasting.

Sephadex chromatography of patients’ sera demonstrated that insulin immunoreactivity was of very high molecular weight co-eluting with blue dextran (molecular weight of approximately 2000 kDa), suggesting the binding of insulin to serum protein(s). Affinity chromatography with anti-IgG agarose confirmed that the abnormal insulin-binding proteins were IgGs. Moreover, insulin autoantibodies tested strongly positive by a semi-quantitative method.

Gel-filtration chromatography showed that 59% and 100% of C-peptide immunoreactivity, from patients 1 and 2 respectively, eluted in the void volume. Since patients with IAS do not have C-peptide antibodies (10), this finding is most probably due to the increased concentrations of proinsulin (bound to circulating anti-insulin antibodies) which, according to the manufacturer, presents a 20% crossreactivity with the C-peptide antibody in the C-peptide assay.

Long-term follow-ups showed that the insulin autoantibodies spontaneously declined in a few months but persisted above normal in both patients. Serum insulin levels eventually normalised in patient 2 and were slightly elevated for several years after diagnosis in patient 1. Interestingly, during the follow-up of patient 1, in the beginning of 1991, insulin levels appeared to increase after an episode of tonsillitis which was treated with penicillin G. For several years before IAS was diagnosed, she had been treated with penicillin G injections given on a monthly basis for prophylaxis of recurrent tonsillitis. Like penicillamine, a degradation product of penicillin which has been associated with IAS (2), a beta-lactam antibiotic penicillin G, may also provide a sulphhydryl group and may have contributed to the IAS in this patient. Imipenem, a beta-lactam antibiotic which does generate a sulphhydryl group, has been recently implicated in the pathogenesis of a case of IAS (11). However, to the best of our knowledge, the association of penicillin with IAS has not been reported previously. Scatchard analysis revealed that insulin-binding antibodies in this patient (patient 1) were polyclonal. Polyclonal anti-insulin autoantibodies are the rule in East-Asians with IAS, whereas monoclonal IAS is more prevalent in Caucasians. A polyclonal antibody pattern was observed in 50 of 51 Japanese and in 9 of 10 non-Japanese East Asians with IAS. Insulin autoantibodies in our patient 2, and in 6 other non-Asian IAS patients so far studied (2), were found to be monoclonal.

East-Asian and Caucasian patients with IAS also differ in terms of HLA alleles (2). In fact, Japanese IAS patients are DR4 positive in 96% (49 out of 51) of cases, possessing either DRB1*0406 (42 cases), DRB1*0403 (5 cases) or DRB1*0407 (1 case) in the polyclonal type, and DRB1*0405 in the monoclonal case. On the other hand, a DR4 phenotype was present only in four of the seven cases of Caucasians.
(DRB1*0407, polyclonal; DRB1*0401, DRB1*0402 and DRB1*0404, each monoclonal). The remaining three Caucasians with IAS had non-DR4 phenotypes, either DRB1*0101, DRB1*1501 or DRB1*0701. All IAS polyclonal-type patients had Glu74 on the DRB1 chain while Ala74 was shared by the six monoclonal responders, suggesting that the type of amino acid at position 74 is essential for determining the clonality of the insulin autoantibodies. Our patient 1, who has a polyclonal insulin autoantibody, is DRB1*0406 like the vast majority of Asian IAS patients. Patient 2, a monoclonal responder, has DRB1*0403. Curiously, this phenotype had been so far reported only in Japanese patients with polyclonal IAS and this is the first time that it has been detected in a Caucasian.

In summary, we have reported the cases of two Portuguese patients with IAS who had different HLA-DR4 subtypes and insulin autoantibodies: DRB1*0406 and a polyclonal antibody in a patient treated with penicillin, and DRB1*0403 and a monoclonal antibody in a patient with ‘idiopathic’ IAS.

References
11 Lidar M, Rachman R, Half E & Ravid M. Insulin autoimmune syndrome after therapy with imipenem. Diabetes Care 1999 22 524-