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Complete laboratory diagnosis of Insulin Autoimmune Syndrome

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ABSTRACT

The definition of Insulin autoimmune syndrome includes the presence of high levels of blood insulin and insulin autoantibodies. We encountered a 45-years-old white man with a high insulin serum value that do not fit with the C-peptide result. To discard or to confirm an analytical interference and diagnose a possible Insulin Autoimmune Syndrome we performed the following investigations: dilution linearity test, heterophilic antibody blocking, polyethylene glycol precipitation, measurements with alternative assays, and gel filtration chromatography by size exclusion. The latter technique confirmed that most of the insulin was complexed with a 150-kDa protein, corresponding to immunoglobulin G, identified as insulin autoantibodies. These antibodies were responsible for hypoglycemia attacks in the patient, who had a previous autoimmune disease. This case highlights the importance of carefully analyzing the results and ruling out possible interferences, as well as considering all kinds of pathologies, even if they are infrequent.

1. Introduction

Insulin is widely known as the principal anabolic hormone of human body. It is secreted by beta cells of the pancreatic islets to regulate mainly carbohydrate metabolism promoting the absorption of glucose from the blood to the liver, skeletal muscle and adipose tissue [1]. The interaction of genetic predisposition with environmental triggers could lead in some people to the production of insulin autoantibodies that can cause clinical alterations [2]. This condition is known as Insulin Autoimmune Syndrome (IAS), which is characterized by spontaneous crises of hypoglycemia and high levels of blood insulin and anti-insulin antibodies, in a context of no previous exogenous insulin exposure and no pathological alterations of the pancreatic islets [3]. In 1972, Yukimasa Hirata described the very first case of IAS in a 47-year-old Japanese man with severe spontaneous hypoglycemia, so this syndrome can be also known as Hirata's disease [4]. IAS is especially common in East Asian countries (more than 90% of published cases up to 2009 occurred in the Japanese population), although the incidence among Caucasian people is increasing [2]. To some authors, this uneven geographical distribution is partially explained by the more prevalent immunogenic determinants of IAS (HLA-DR4, specifically DRB1*0406) in Asian population [5]. Other triggers have been described as inductors of IAS: medication (specially drugs containing sulfhydryl groups), viral infections and hematological and autoimmune diseases [6]. Due to their high linkage capacity, insulin autoantibodies (IAA) bind to secreted insulin forming large antigens-antibodies complexes and insulin becomes unable to exert its physiological effects, resulting in transient hyperglycemia. This hyperglycemia continues to stimulate the release of more insulin by pancreatic cells reaching a point where endogenous antibodies binding capacity is exceeded, so there will be free active insulin. In addition, after a while, the low affinity of IAA for insulin causes a spontaneously dissociation of the complexes and an excess of unbound insulin, which

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evokes hypoglycemia of varying severity [7]. This hypoglycemia is the responsible of the main clinical characteristics of IAS, which manifests with autonomic and neuroglycopenic symptoms that can be fatal for the patient if not treated in time [8]. Diagnosis of this condition requires careful analysis of clinical findings and laboratory test results. We present below the procedure followed by our clinical laboratory in a case of a patient with hypoglycemic attacks of unknown origin.

2. Case presentation

A 45-years-old Spanish man was referred to the Endocrinology Service complaining of dizziness and tremors that stop with the intake of carbohydrates. Capillary glucose concentrations measured during some of these crises were reported as being between 44 and 46 mg/dL. There was no history of diabetes mellitus, previous exposure to insulin or other reasons that could lower blood glucose such as alcoholism, liver disease and intake of drugs. The patient's Body Mass Index (BMI) was 31 kg/m^2. In 2005, he was diagnosed with immune thrombocytopenia that responds adequately to corticosteroid treatment and since then, he had suffered a few controlled relapses. His only medication at the time of the study was acetaminophen and ibuprofen. No other relevant medical history was found.

Looking for an impairment of glucose metabolism due to the hypoglycemic crises, a biochemical blood test was requested to the laboratory. It included the measurement of fasting glucose, glycated hemoglobin (Hb A1c), insulin, C-peptide and some islet autoantibodies: anti-insulin antibodies, antibodies against the 65-kD isoform of Glutamic Acid Decarboxylase (GAD65) and again the Islet Antigen 2 (IA2). The main result of this patient's examination was the elevated concentration of serum Insulin: 201 μU/mL (2.6–24.9), which did not fit to C-peptide value: 2.02 ng/mL (1.1–4.4). The possibilities of insulin injection or abnormal excess of insulin secretion were ruled out. Besides, a high titer of anti-insulin antibodies was found, suggesting the presence of an autoimmune syndrome as they are common markers of type 1 diabetes mellitus. Serum glucose (74 mg/dL) and Hb A1c (5.3%) result within expected values as the patient did not have diabetes mellitus. The screening for Anti GAD65 and Anti IA2 antibodies was negative, so autoimmune diabetic disorders or other endocrine disorders were ruled out. This discrepancy between the high serum insulin and normal C-peptide levels, in a context of normal fasting blood glucose level, and the presence of IAA, led to the performance of complementary tests to discard or to confirm an analytical interference and diagnose a possible Insulin Autoimmune Syndrome (IAS). We followed the logical protocol to perform when analytical interference is suspected, which includes: the repetition of the analysis to ensure the result, exclude pre-analytical problems, dilution studies, the treatment of the sample with Scantibodies, polyethylene glycol (PEG) precipitation, the repetition of the analysis on another instrument from a different manufacturer and specialized investigations in a specialist laboratory if necessary [9].

For insulin measurement, we used the Elecsys Insulin Immunoassay "ECLIA" (EleCtrochemiLuminescence ImmunoAssay) for cobas e 801 analyzers (Roche Diagnostics), which employs two monoclonal antibodies according to the Sandwich principle. Insulin was measured in pure plasma and then following a serial dilution scheme, a very simple way to detect possible interferers. The determination of islet autoantibodies, anti-insulin antibodies included, was carried out on the fully-auto chemiluminescence immunoassay (CLIA) analyzer MAGLUMI (Maglumi 1000, Snibe Diasnogtic) by a competition sandwich immunoluminometric assay. To eliminate the possible heterophilic antibody interference, a heterophilic blocking tube (HBT) was used. The HBT, which represents a sample pretreatment, contains a blocking reagent composed of specific binders (scantibodies) targeted specifically against heterophilic antibodies. It is necessary to transfer 500 μl of the patient sample to the HBT, mixing and incubating for 1 hour at room temperature [10]. Polyethylene glycol (PEG) precipitation allows the determination of certain proteins, such as immunoglobulins, that can interfere with the results of the analysis [11]. With this purpose, 500 μl of patient serum and 500 μl of PEG solution were put together in a vial and mixed in a vortex. The mixture was centrifuged at 3500 rpm for 30 minutes at 5 °C. 500 μl of the supernatant, free of antibodies, that have precipitated, were mixed with 500 μl of physiological serum and this sample was measured in the analyzer. This procedure involves a 1/4 dilution that must be considered when obtaining the result. To confirm the result we measured insulin with two alternative immunoassays on other instruments from different manufacturers, Atellica Solution (Siemens Healthineers) and Alinity (Abbot Laboratories) (see Table 1). Finally, all the results above expected values were confirmed in an independent analysis by the company Roche Diagnostics in a CIR lab in Penzberg, Germany. There, the sample was fractionated by Size Exclusion Chromatography (SEC) and insulin was measured in all fractions using Elecsys Insulin sales lot to evaluate the size of the proteins that show reactivity in the test. 500 μL of the serum sample were applied to a Superdex 200 Increase 10/300 GL (24 mL) prepacked column. This column permits the analysis and characterization of proteins with high molecular weights such as antibodies. The eluent used was a phosphate buffer with a pH of 7,4 and a flowrate of 0,75 mL/min.

The patient was asked for a new sample to confirm the results, which were very similar to the previous ones: Insulin: 218 μU/mL; C-peptide: 2.21 ng/mL. Serial dilutions supported the idea of the presence of an interfering in the sample as the linearity of the

Table 1
Main laboratory patient's findings.

Assay	Result	Reference value	Reference
Insulin	201 μU/mL	2.6–24.9	Elecsys Insulin "ECLIA" – Cobas e 801, Roche Diagnostics
C-Peptide	2.02 ng/mL	1.1–4.4	Elecsys C-peptide "ECLIA" – Cobas e 801, Roche Diagnostics
Anti-insulin antibodies	56.8 U/mL	0.0–20.0	"CLIA" – Maglumi 1000, Snibe Diagnostics
Insulin	29.42 μU/mL	5.0–27.0	"CLIA" – Atellica IM, Siemens
Insulin	105.6 μU/mL	7.0–24.0	"CMIA" – Alinity i, Abbott
PEG'ed serum Insulin	10.76 μU/mL	2.6–24.9	Elecsys Insulin "ECLIA" – Cobas e 801, Roche Diagnostics

consecutive results (after multiplication by dilution factor) was lost: Insulin ½: 187 μU/mL; Insulin ¼: 115 μU/mL; Insulin 1/10: 56 μU/mL. After the HBT treatment using Scantibodies, the results were like the prior ones: Insulin: 211 μU/mL; C-peptide: 2.03 ng/mL. This ruled out the hypothesis of an interference by heterophile antibodies. Precipitation with PEG served to demonstrate the presence of immunoglobulins in the sample, as the results change considerably after the precipitation: Insulin: 10.76 μU/mL; C-Peptide: 2.25 ng/mL. The insulin result provided by Abbot was 105.6 μU/mL (7.0–24.0), while the one provided by Siemens, whose antibodies bind to different insulin epitopes promoting the capture of free insulin, was 29.42 μU/mL (5.0–27.0).

The measurements done by Roche in the CIR laboratory confirmed the reported altered insulin result and the normal insulin result after PEG precipitation. They also fractionated the sample by SEC and used two standards as reference. Fig. 1 shows these standards and the peaks are signed with molecule and molecular weight.

Fig. 2 is the representation of the overlay of the chromatogram and the Insulin Elecsys results in each fraction. As can be seen, the reactivity with the Insulin kit was mainly in fractions with proteins of high molecular weight. In these fractions, proteins of ~150 kDa like immunoglobulins as IgG are expected. Minimal reactivity was found in fractions where free Insulin (molecular weight of 5.8 kDa) is expected indicating the low presence of unbound insulin. The tailing of the IgG corresponding peak towards fractions with lower molecular weight might be due to partially dissociation of the immune complex during SEC.

3. Discussion

The definition of IAS includes the presence of high levels of blood insulin and IAA. This analysis began with the detection of a mismatched analytical result between insulin and C-peptide. C-peptide is a polypeptide originating from proinsulin after its cleavage in the pancreas. It is released into the circulation in equimolar amounts together with insulin, the other cleavage product, thus the insulin concentration should correlate with the C-peptide concentration [12]. The elevated concentration of insulin with an anti-insulin antibodies positive result, almost confirms the diagnosis [6]. However, it is recommended to exclude an interference or a false-positive assay when IAA is positive through gel filtration chromatography (GFC), which is the gold standard technique for the detection of immunoglobulin-bound macro-analytes [13]. Following this recommendation, we have confirmed by SEC that most of the insulin was complexed with a 150 kDa protein, corresponding to immunoglobulin G. Besides, the screening for Anti GAD65 and Anti IA2 was negative, as it is described for IAS [14]. These IgG IAA are the cause of the hypoglycemic crises in our patient. The reason for our patient to release these autoantibodies could be his pre-existing autoimmune disorder (immune thrombocytopenia), indicating that his immune system does not work properly. The association between IAS and autoimmune pathologies is well documented by several case reports [15] but to our knowledge, this is the first reported case of IAS related to immune thrombocytopenia. When approaching a patient suffering from hypoglycemia, the differential diagnosis is essential and, although infrequent, IAS should be suspected. Most physicians do not usually include it in the differential diagnosis because of the general unawareness of this disease [6]. This leads the patient to go through unnecessary and expensive biochemical and imaging examinations and/or even invasive surgical procedures and drug therapy [16]. So to prevent IAS from being identified late or misdiagnosed, the clinical practice guidelines by the American Endocrine Society already include the measurement of IAA among the first-line tests to perform in non-diabetic and non-acutely ill individuals with an unexplained cause of hypoglycemia [17]. IAA have high linkage capacity but weak affinity for insulin, inducing an episode of hyperglycemia immediately after glucose uptake and then, hypoglycemia occurs 2–3 hours later. These antibodies, therefore, interfere with the bioactivity of the insulin causing a clinical alteration. But besides, IAA (macrocomplexes) interfere with the measurement of the hormone by immunoassay and the obtained result is falsely elevated [2].

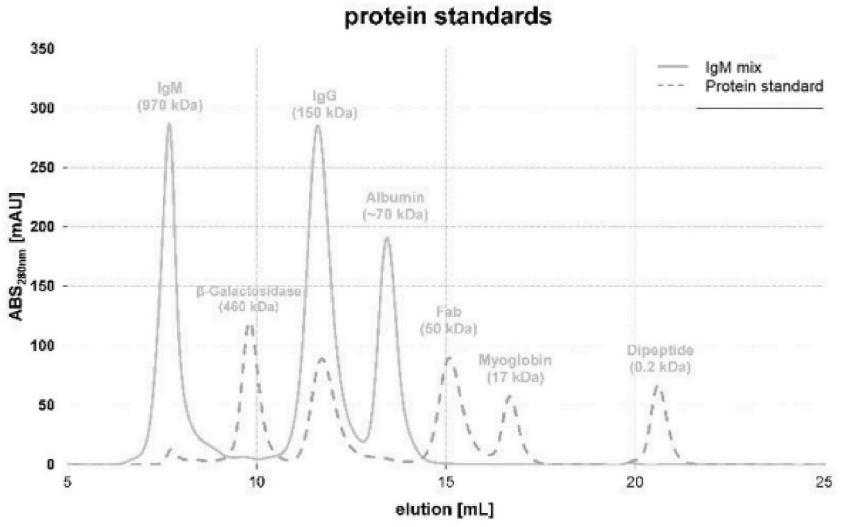

Fig. 1. Protein standard: β-Galactosidase (460 kDa), Fab (50 kDa), Myoglobin (17 kDa), Dipeptide (0.2 kDa); and IgM mix: IgM (970 kDa), IgG (150 kDa), Albumin (70 kDa) used in the SEC procedure as reference for the determination of insulin complex.

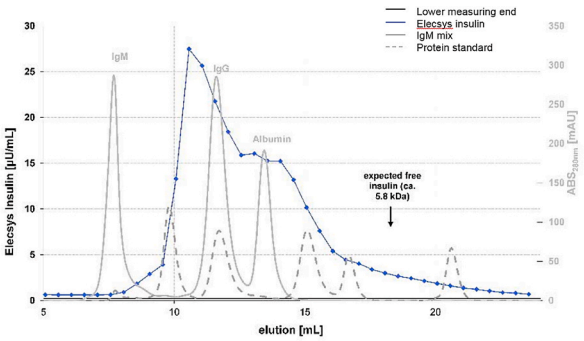

Fig. 2. Representation of the overlay of the chromatogram of the serum patient sample and Elecsys insulin results. The high insulin concentration is found in the fraction where immunoglobulin G is expected.

To avoid discrepancies, we recommended the physicians to assess the patient with the C-peptide measurement instead of insulin. Despite the complications, this autoimmune syndrome usually is self-limiting, another reason why it is difficult to calculate the exact incidence of the disease [6]. Our patient was only recommended to eat small and frequent meals low in carbohydrates. This is effective because postprandial hyperglycemia is reduced and consequently, the insulin release [18].

4. Conclusion

We have diagnose a new case of Insulin Autoimmune Syndrome in a white man thanks to an in-depth laboratory analysis. The procedure followed by our laboratory to confirm the results has avoided unnecessary diagnostic and therapeutic procedures in the patient. This highlights the importance of carefully analyzing the results and ruling out possible interferences, as well as considering all kinds of pathologies, even if they are infrequent.

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Patient consent

Informed consent has been obtained from the patient (or patient's guardian) for publication of the case report and accompanying images.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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