ELSEVIER

Journal of Autoimmunity 26 (2006) 127–132

journal of autoimmunity

www.elsevier.com/locate/issn/08968411

A sensitive radioimmunoassay of insulin autoantibody: Reduction of non-specific binding of [^{125}I]insulin

Hiroshi Murayama [a,*], Nobuo Matsuura [b], Tomoyuki Kawamura [c], Taro Maruyama [d], Nobuyuki Kikuchi [e], Tetsuro Kobayashi [f], Fumi Nishibe [a], Atsuo Nagata [a]

[a] *Immunology Laboratory, Diagnostics Department, YAMASA Corporation, 2-10-1 Araoi-cho, Choshi, Chiba 288-0056, Japan*
[b] *Department of Early Childhood Education, Seitoku University, Chiba 271-8555, Japan*
[c] *Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan*
[d] *Department of Internal Medicine, Saitama Social Insurance Hospital, Saitama 330-0074, Japan*
[e] *Department of Pediatrics, Yokohama City University Medical Center, Kanagawa 236-0004, Japan*
[f] *Third Department of Internal Medicine, Yamanashi University School of Medicine, Yamanashi 409-3898, Japan*

Received 12 August 2005; revised 19 November 2005; accepted 21 November 2005

Abstract

There has been a lack of consensus among results of assays for insulin autoantibody (IAA) carried out by different laboratories, despite a reduction in the non-specific effect using a cold insulin competitor in radioimmunoassays (RIAs) for IAA in type I diabetes. We speculated that the discrepancies are partly a result of the non-specific binding (NSB) of [^{125}I]insulin to unidentified molecules in serum on polyethylene glycol separation, and tried to improve IAA RIAs. The molecular weight of a candidate for the factor causing NSB was estimated to be about 700 kDa by gel filtration analysis, resembling that of alpha 2-macroglobulin (a2M). Further, the addition of purified a2M to the assay resulted in an increase in NSB. Screening revealed that heterocyclic compounds, such as isothiazolinone derivatives (ProClin300), were greatly effective at reducing NSB in control subjects from 2.904 ± 0.909% to 1.347 ± 0.254% ($n = 283$, mean ± SD, $p < 0.0001$). Using our newly developed IAA RIA with ProClin300, the sensitivity for newly diagnosed type I diabetes patients ($n = 55$) was 32.7% and 30.9% with or without insulin competition, respectively, whereas that of the former assay without ProClin300 was only 20.0%.

Keywords: Insulin autoantibodies; Isothiazolinone; Non-specific binding; Radioimmunoassay

1. Introduction

Anti-islet autoantibodies are often observed in newly diagnosed type I diabetes patients [1–3]. Insulin autoantibody (IAA), one of these autoantibodies, was reported to be useful for the prediction and diagnosis of the disease [4–7]. Several methods have been reported for the detection of IAA in serum, most of which include the elimination of non-specific binding (NSB) by subtracting the signal obtained with an excess amount of unlabeled insulin from the signal without unlabeled insulin [5–9]. Nevertheless, standardization of the assay is more difficult than for assays of other autoantibodies such as glutamic acid decarboxylase autoantibodies or tyrosine phosphatase-like protein autoantibodies [10,11].

The difficulty with the radioimmunoassay (RIA) of IAA may be partly caused by fluctuating NSB of the [^{125}I]insulin tracer to unknown molecules in serum samples and subsequent precipitation of the NSB by polyethylene glycol separation. Few reports are available on NSB in IAA RIAs. On the other hand, enzyme-linked immunosorbent assays (ELISA) have been evaluated in some laboratories, but the results were shown to be less related to insulin-dependent diabetes mellitus than those of IAA RIA [12,13].

Abbreviations: IAA, insulin autoantibody; NSB, non-specific binding; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; a2M, alpha 2-macroglobulin; SRID, single radial immunodiffusion.

* Corresponding author. Tel.: +81 479 229840; fax: +81 479 229845.
E-mail address: murayama@yamasa.com (H. Murayama).

0896-8411/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jaut.2005.11.003

The purpose of this study was to investigate the causes of the NSB of [^{125}I]insulin and to develop a reliable IAA RIA.

2. Materials and methods

2.1. Materials

Recombinant human Insulin was purchased from Intergen Inc. (USA). A RIA grade of bovine serum albumin was obtained from Sigma (UK). Lactoperoxidase was obtained from Calbiochem (Los Angeles, CA, USA). Purified human alpha 2-macroglobulin (a2M) was provided by ICN Pharmaceuticals Inc. (Ohio, USA). Sheep anti-a2M was purchased from The Binding Site Ltd. (Birmingham, UK). Na^{125}I (IMS300) was obtained from Amersham Bioscience Co. (Piscataway, NJ, USA). A 3:1 mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (ProClin300) was purchased from SPELCO (USA). Peptides were obtained from Sawady Technology (Tokyo, Japan). All other materials of analytical or HPLC grade were purchased from Nakarai-Tesque Co. (Kyoto, Japan).

2.2. Serum samples

Serum samples were collected from 55 type I diabetes patients (25 males and 30 females, 9.9 ± 5.1 years old) before insulin treatment or within 10 days after disease onset. All patients with type I diabetes were less than 20 years old at the time of the diagnosis. Serum samples of control subjects were collected from patients who visited hospitals with diseases other than diabetes (153 males and 135 females, 8.6 ± 5.6 years old) or from healthy adult donors (61 males and 60 females, 38.0 ± 11.0 years old). Informed consent was obtained from all donors, or their parents, before the collection of blood samples. Sera were stored at −40 °C prior to use.

2.3. Preparation of [^{125}I]insulin

[^{125}I]Monoiodo-Tyr$_{14}$-human insulin was prepared basically according to the procedure of Frank et al. [14]. Briefly, 100 μl of recombinant human insulin (1 mg/ml) dissolved in 2 mM HCl was added to 37 MBq (ca. 2 μl) of Na^{125}I (IMS300) together with 10 μl of 0.06 M potassium phosphate buffer (pH 7.0). Then, iodination was started by adding 20 μl of a 25 U/ml lactoperoxidase solution in phosphate buffer and 10 μl of 0.003% hydrogen peroxide. The mixture was kept for 15 min at room temperature and applied to a HPLC column (Wakosil II 5C18HG, 0.46 × 25 cm, Wako Pure Chemicals Co., Japan) equilibrated with 24% acetonitrile in 0.3 M ammonium sulfate. At a flow-rate of 1 ml/min, the 6th radioactive peak of the fraction was collected and diluted with assay buffer (80 mM phosphate buffer containing 0.5% bovine serum albumin, 0.9% sodium chloride and 0.05% sodium azide). The specific activity of purified ^{125}I-labeled insulin was no less than 13 MBq/μg (350 mCi/μg).

2.4. IAA RIA

Serum levels of IAA were determined by the method of Vardi et al. with some modification [4,5,15]. Briefly, 50 μl of serum was pipetted to a tube followed by the same amount of assay buffer with or without 250 mU of unlabeled recombinant human insulin. The mixture was incubated for 30 min at room temperature. Next, 100 μl of ^{125}I-labeled human insulin was added to each tube and the mixture was incubated for 24 h at 23 °C. The binding complex was precipitated by adding 2 ml of ice-cold 16.2% polyethylene glycol in barbital buffer with 0.1% Tween-20, incubated for 30 min at 4 °C and centrifuged at 3000 rpm ($2000 \times g$) for 30 min at 4 °C. The supernatant was discarded and 1 ml of the polyethylene glycol solution was added again. After centrifugation and removal of the supernatant, the radioactivity of the tubes was measured with a gamma counter for 2 min.

Percent binding of serum samples without unlabeled insulin was evaluated as the non-specific assay. The difference in percent binding with or without an excess amount of unlabeled insulin was evaluated as the specific assay.

Values above the 99th percentile of controls in respective assays were considered positive. The cut-off value of 0.716 percent binding in the specific assay with ProClin300 was equivalent to 220 nU/ml of the radioactive ligand ([^{125}I]Insulin), when the concentration of IAA was expressed as the concentration of insulin with which IAA can bind [5]. The 99th percentile of healthy adult donors ($n = 121$) was 0.327 percent binding and 105 nU/ml in the specific assay with ProClin300. Inter- and intra-assay coefficients of variation (CV) of the specific assay with ProClin300 around the cut-off value were 3.9% and 5.1%, respectively.

2.5. Gel filtration analysis

The precipitated pellet with high NSB in the non-specific assay was dissolved in phosphate-buffered saline and filtered. The filtrate was applied to Sepharose 6B (10 × 480 mm) equilibrated with phosphate-buffered saline and fractions of 1 ml were collected. The radioactivity of the fractions was measured with a gamma counter. The molecular mass of the candidate was determined using a standard curve generated with a MW calibration kit (Amersham Biosciences, UK).

2.6. Measurement of a2M

Serum levels of a2M were measured by single radial immunodiffusion (SRID). Two microliters of sample was added to 2-mm diameter holes on a 1-mm thick agarose (1.2%) plate with 1% sheep anti-human a2M antibody in 100 mM Tris–borate buffer (pH 8.4), 2 mM EDTA, and 3% polyethylene glycol #6000, and kept for 72 h at room temperature. The diameter of each immune precipitate was measured and the concentration of each sample was calculated from a standard curve made with purified a2M.