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Autoantibodies to the insulin receptor are infrequent findings in Type 1 (insulin-dependent) diabetes mellitus of recent onset

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Summary. To determine whether autoantibodies to the insulin receptor may represent markers of Type 1 (insulin-dependent) diabetes, the prevalence of such antibodies was investigated in sera of 60 newly diagnosed untreated Type 1 diabetic patients. A sensitive assay, based on enzyme linked immunosorbent assay has been set up which detects antibodies to the insulin receptor irrespective of their potentially inhibiting effect on insulin binding. Moreover, this method allows easy determination of the immunoglobulin class involved in the anti-receptor activity. Among the 60 sera examined, only one was found to contain anti-insulin receptor autoantibodies (IgG class). In view of our data, we conclude that autoantibodies to the insulin receptor are infrequent findings in Type 1 diabetes of recent onset.

Key words: Autoantibodies, insulin receptor antibody, Type 1 (insulin-dependent) diabetes, diabetes autoimmunity, ELISA

Several findings have pointed to autoimmune mechanisms in the pathogenesis of Type 1 (insulin-dependent) diabetes mellitus [1–3]. Thus, the clinical onset of Type 1 diabetes is associated with a high prevalence of autoantibodies [4]. The search for potential markers of ongoing islet cell destruction deserves increasing attention since immunosuppressive therapeutic regimens could be considered when there are still a substantial amount of beta cells to be saved. Among those antibodies, the most representative and specific are islet cell antibodies (ICA) and anti-insulin antibodies (AIA) [5, 6]. Autoantibodies to the insulin receptor were occasionally found to be part of such autoimmune constellations [7–9]. In such an autoimmune phenomenon, it is likely that the titre of antibodies would be low. This is a different situation from that observed in the rare syndromes of extreme insulin resistance (type B) where high titres of anti-insulin receptor antibodies have been found [10, 11]. More recently, such autoantibodies were also reported in autoimmune hypoglycaemia [12–18]. In these disease states, autoantibodies to the insulin receptor were readily detected by their capacity to inhibit the binding of insulin to its receptor [19–22]. But this technique could not detect antibodies not interfering with insulin binding as those recently described in man [23].

To examine the prevalence of antibodies to the insulin receptor in Type 1 diabetes, we have investigated 60 newly diagnosed diabetic patients at the clinical onset of the disease before any specific treatment. We have developed a test, based on the ELISA technique which appears more sensitive than previously used methods (inhibition of insulin binding to its receptor; insulin-like activity of antibodies; immunoprecipitation of labelled receptors). This assay allows for the detection of various types of antibodies irrespective of their ability to interfere with insulin binding to its receptor.

Subjects and methods

Materials

$Na^{125}I$ was purchased from C. E. A. (Saclay, France). Wheat germ agglutinin agarose was from Sigma (St. Louis, Mo., USA). All other reagents were of the best grade commercially available and were from Sigma or Serva (Heidelberg, FRG). Peroxidase labelled anti-human antibodies were from Dakopak (Glostrup, Denmark).

Subjects

Sixty newly diagnosed Type 1 diabetic subjects (aged 3–67 years) were included in this study. Samples of serum were obtained before any treatment, particularly before insulin administration. The diagnosis of Type 1 diabetes was based on clinical history (polyuria, polydipsia, massive weight loss) and biological features (fasting blood glucose > 11.1 mmol/l, constant glycosuria and ketonuria). These patients were investigated in three different centres as shown in

Table 1. Characteristics of Type 1 (insulin-dependent) diabetic patients

Centre	*n*	sex ratio male/female	mean age (years) (range)	(% positive patients)	
				ICA	AIA
Paris	18	8/10	8.6 (3–12)	75	71.4
Marseille	12	7/5	19.3 (6–35)	77	33.3
Nice	30	23/7	27.7 (9–67)	61	6.6

ICA: islet cell antibodies; AIA: anti-insulin antibodies

Table 1 which also displays some clinical and immunological features of the patients.

Negative control sera were obtained from children and adults (aged 1–45 years), either healthy volunteers or patients being investigated for problems unrelated to immunology or diabetes. Positive control sera were from two types of patients: (1) Sera from patients with insulin resistance (type B) due to autoantibodies to the insulin receptor: B2, B3, B4, B7, and B10 were kindly provided by Dr. P. Gorden (National Institutes of Health, Bethesda, Md., USA); (2) Serum PS, studied in our laboratory, was from a child presenting with autoimmune hypoglycaemia in relation to autoantibodies to the insulin receptor [18]. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Preparation and purification of human insulin receptors

Insulin receptors were prepared from rat embryo fibroblasts transfected with an expression plasmid encoding the human insulin receptor (expressing 10^6 human receptors per cell) (HIR cells). Non-transfected cells were used as controls. Cells were cultured as previously described [24]. Receptors were solubilised in 1% Triton X-100 and partially purified by chromatography on wheat germ agglutinin column [25]. Preparations obtained from HIR cells bound 500 (femtomol) of insulin per ml. Processing of the same number of cells, transfected or not, was performed in parallel on identical columns. Eluates from these columns were used as "Insulin Receptor Coating" and "Non-Specific Coating", respectively; they could be stored, without degradation, at −80°C for a one year period.

Insulin receptors meant for iodination were prepared from transfected cells solubilised in 10 mmol/l (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS), purified by wheat germ agglutinin chromatography and immunoaffinity chromatography using a monoclonal antibody specific for human insulin receptor cross-linked to a CNBr-activated Sepharose matrix (Pharmacia, Uppsala, Sweden). The procedure was performed as previously described [26] except that 1% Triton X-100 was replaced by 10 mmol/l CHAPS.

ELISA test

The ELISA test was performed as follows: microtitre plates (Microtest III plates, Falcon, Becton Dickinson, Lincoln Park, NJ, USA) were coated overnight at 4°C with 100 µl of the receptor preparations (diluted to have 3 fmol insulin binding capacity) in 50 mmol/l carbonate coating buffer (pH 9.6). Wells were then saturated for 2 h at room temperature with phosphate-buffered saline (140 mmol/l NaCl, 3 mmol/l KCl, 6.5 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4) containing bovine serum albumin (20 mg/ml) (blocking buffer). All subsequent incubations were carried out at 15°C and plates were washed five times with saline buffer containing 0.1% Tween 20 after each of the following incubations. Serum samples (100 µl of a 1/100 dilution in blocking buffer) were added to the plates for 1 h. Receptor-bound antibodies were then detected with horseradish peroxidase-conjugated rabbit anti-human IgG (dilution 10^{-4}) or IgM (dilution 10^{-3}), followed by incubation with the substrate solution for 30 min in the dark (0.1 mol/l phosphate, 44 mmol/l citrate buffer pH 5.5 containing 0.02% H_2O_2 and 16 mmol/l O-phenylenediamine dihydrochloride). Finally, the reaction was stopped with 100 µl of H_2SO_4 (2 mol/l) and absorbances were measured at 490 nm with a microplate reader. Within each microplate, each serum was tested twice with insulin receptor coating and once with non-specific coating. Background linked to the immunoglobulin content in the sera was taken into account given the use of non-specific coating. The specific signal was calculated as the difference between these two readings of absorbance. For negative control sera this difference was about zero. Background signal due to peroxidase labelled antibody was negligible (0.037 ± 0.001). Furthermore, in each plate test, serum B7 and serum B7 diluted (1/100) in normal serum were used as internal positive controls. The later control was made to reproduce artificially the conditions of a serum containing low titres of antibodies to the insulin receptor in a normal immunoglobulin environment.

Intraassay ($n = 18$) and interassay ($n = 8$) coefficients of variation of the ELISA test were 2.33% and 5.5%, respectively.

Immunoprecipitation of iodinated receptors

Iodination of affinity purified insulin receptors was carried out with IODO-GEN iodination reaction (Pierce, Oud-Beijerland, The Netherlands). Unreacted iodine was separated from labelled receptors by Sephadex G-25 chromatography. Five µl of iodinated receptors was incubated overnight at 4°C with various dilutions of serum, in a final volume of 100 µl. Protein A Sepharose was then added for 1 h under constant shaking. Immune complexes were collected by centrifugation, washed three times with Hepes buffer 30 mmol/l, pH 7.4, NaCl 30 mmol/l, CHAPS 10 mmol/l, and radioactivity was measured.

Islet cell cytoplasmic antibodies (ICA) and anti-insulin antibodies (AIA)

Cytoplasmic type ICA were detected by immunofluorescence on a frozen section of human pancreas as recently recommended [27]. AIA were detected by the liquid phase radiobinding method described by Palmer [28].

Insulin binding inhibition assay

The human lymphoblastoid cell line (IM-9) was used for the insulin binding inhibition test. Cells were maintained in RPMI 1640 medium supplemented with 10% dialysed fetal calf serum. Inhibition of insulin binding on IM-9 cells was measured as previously described [22].

Statistical analysis

The upper limit of the normal range was defined as the mean + 3 SD of the values obtained with negative control sera tested in the same experiment at the same dilution.

Results

Validation of ELISA to detect antibodies to insulin receptor

In order to validate our technique, ELISA results were compared with those obtained by immunoprecipitation of iodinated receptors and inhibition of insulin binding for positive and negative control sera. Figure 1 shows the de-

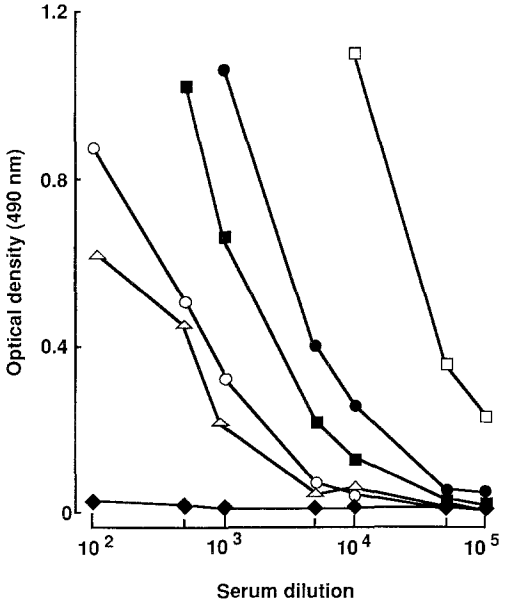

Fig. 1. Detection of autoantibodies to the insulin receptor by ELISA. Insulin receptors were prepared from rat embryo fibroblasts transfected with an expression plasmid encoding the human insulin receptor or from non-transfected cells. They were coated on microplates and ELISA was carried out with increasing dilutions of five sera from patients with type B insulin resistance: B2 (□), B3 (△), B4 (■), B7 (●), B10 (○), and compared to the mean values of four normal sera (◆). Anti-receptor antibodies were revealed with peroxidase-conjugated rabbit anti-human IgG. Specific absorbance was calculated by difference, for each dilution, between total signal and non-specific signal as described in Methods. Shown are the means of three experiments

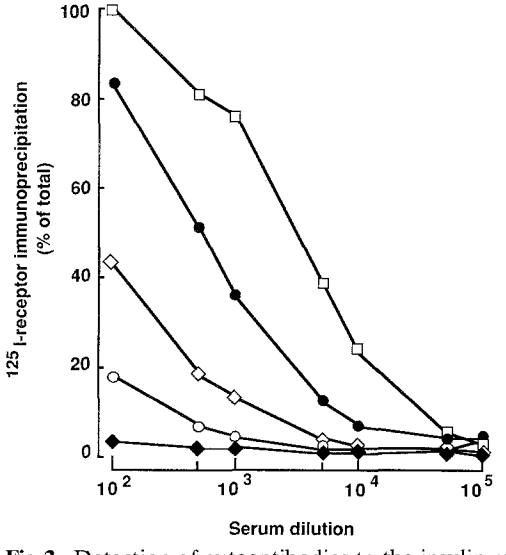

Fig. 2. Detection of autoantibodies to the insulin receptor by iodinated receptor immunoprecipitation. Iodinated receptors were incubated overnight at 4°C, with various dilutions of sera from patients with type B insulin resistance: B2 (□), B7 (●), B10 (○), PS (◇), or from negative control normal sera (◆). Immune complexes were precipitated with protein A, and radioactivity measured as described. Results are expressed as a percentage of total immunoprecipitation, defined as the maximum immunoprecipitation achieved at 1/20 (and below) dilution with the most potent serum tested (B2)

tection of antibodies to insulin receptor (IgG class) in positive control sera from five patients (B2, B3, B4, B7, B10) with the type B syndrome of extreme insulin resistance [10, 11]. Their sera were analysed at varying dilutions and compared to four negative control sera. Antibodies to the insulin receptor were detected in a concentration-dependent manner, whereas the specific signal with negative controls was almost zero. All five positive control sera were detected at dilutions as high as $1/10^4$ (Fig. 1), and three of them were still positive at $1/10^5$. The sensitivity of immunoprecipitation of iodinated receptors was analysed using various sera in a similar fashion (Fig. 2). As was observed in the ELISA, insulin receptor immunoprecipitation increased in a concentration-dependent manner for positive control sera and was negligible for the negative controls. The same rank order of potency was observed with the three positive control sera (B2, B7, B10) tested in the two assay systems (Fig. 1 and 2). However, the ELISA was distinctly more sensitive than the immunoprecipitation assay. Thus, sera B2, B7 and B10 were still positive in the ELISA at dilutions that were about one order of magnitude greater than those allowing detection by immunoprecipitation (Fig. 1 and 2).

Table 2. Detection of insulin receptor autoantibodies in positive control serum (serum PS). Comparison of three different assays

Serum PS dilutions	% inhibition of insulin binding to IM9 cells	% immunoprecipitation of iodinated receptor	ELISA absorbance
100	33.6[a]	43.75[a]	1.664[a]
500	7.4	18.48[a]	nd
1000	nd	13.09[a]	0.480[a]
10000	nd	2.05	0.067[a]

Various dilutions of serum PS were assayed using three different techniques. Inhibition of insulin binding to IM-9 cells as described previously [22]. Immunoprecipitation of iodinated insulin receptors was performed as follows: iodinated receptors were incubated overnight at 4°C with various dilutions of serum PS, immune complexes were precipitated with protein A and radioactivity measured. Results are expressed as a percentage of total immunoprecipitation, defined as the maximum immunoprecipitation achieved at 1/20 dilution with the most potent serum tested (B2). ELISA was performed as follows. Insulin receptors from transfected or from non-transfected cells were coated on microplates and ELISA was carried out with increasing dilutions of serum from PS. Anti-receptor antibodies were revealed with peroxidase-conjugated rabbit anti-human IgG. Specific absorbance was calculated by difference, for each dilution, between total signal and non-specific signal as described in Methods.
[a] Positive results were defined as exceeding the mean + 3 SD of values obtained with negative control sera at the same dilutions; nd = not determined

The validity of ELISA was further assessed by comparing its results with those obtained by the insulin binding inhibition assay. Table 2 displays the signal obtained with a positive control serum (PS) [18] using the three screening assays. ELISA appeared to be 10 times more sensitive than the immunoprecipitation technique and at least 100 times more sensitive than inhibition of insulin binding (Table 2).

Screening for antibodies to insulin receptor in Type 1 diabetes of recent onset

Using this ELISA technique, we examined the sera of 60 Type 1 diabetic subjects at the onset of their disease,

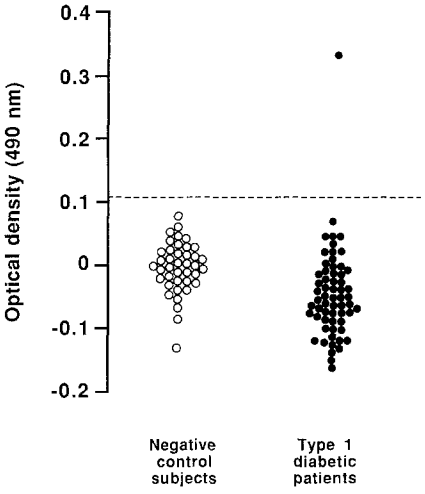

Fig. 3. Screening of IgG autoantibodies to the insulin receptor in diabetic patients. ELISA was carried out with sera (dilution 1/100) from Type 1 (insulin-dependent) diabetic patients ($n = 60$) and from negative control sera ($n = 40$) as described in Figure 1. Anti-insulin receptor antibodies were revealed with horseradish peroxidase-conjugated rabbit anti-human IgG. Specific absorbance is presented as in Figure 1. The dashed line indicates the value of the mean + 3 SD of control sera, and defines the threshold of positivity

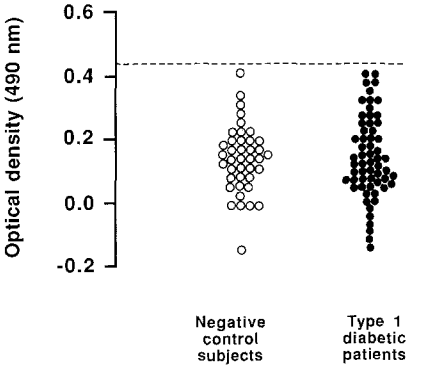

Fig. 4. Screening of IgM autoantibodies to the insulin receptor in Type 1 (insulin-dependent) diabetic patients. Sera were assayed at 1/50 dilution as described in Figures 1 and 3, and autoantibodies were revealed with a peroxidase-conjugated rabbit anti-human IgM. Specific absorbance is presented as in Figure 1

and compared them to 40 control sera. In order to screen both IgG and IgM type autoantibodies, the assays were performed with either peroxidase-conjugated anti-human IgG or anti-human IgM rabbit antibodies as the revealing antibody. As illustrated in Figure 3, only one serum (SR) was positive for autoantibodies to the insulin receptor of the IgG class. The positive serum gave absorbance values markedly higher than the upper limit of the normal range defined as the mean + 3 SD of the values obtained with negative control sera. These results were obtained in three different assays performed on different days. No serum out of the 60 diabetic patients contained antibodies to insulin receptors of the IgM class (Fig. 4). Serum SR was also able to immunoprecipitate iodinated insulin receptor since it precipitated 52% and 37% of total iodinated receptors at 1/20 and 1/100 dilutions, respectively.

Discussion

Given the low titre of antibodies that could be expected for an autoimmune marker and the polyclonal nature of these antibodies, it is necessary, when testing a serum, to use either a set of complementary assays or a test that can detect all types of antibodies reacting with the insulin receptors. Whatever approach is taken, the sensitivity of the assay is critical. In order to meet these requirements, we have developed a test based on the ELISA technique. We have found that our ELISA has several advantages: (1) Its sensitivity is greater than that of other assays currently available; (2) It is capable of detecting antibodies to the insulin receptor regardless of their functional properties which may vary among these antibodies. Thus, it can detect antibodies to the insulin receptor not interfering with insulin binding domains as well as antibodies failing to recognize insulin receptors in an immunoprecipitation assay [29]; (3) With the use of the class specific peroxidase-labelled anti-human antibodies it is possible to determine, without any purification step, which class of immunoglobulin is involved in the anti-receptor activity; (4) Anti-insulin antibodies cannot interfere with this assay, as they can do in the insulin binding inhibition system and with immunoprecipitation of receptors cross-linked to iodinated insulin. Given the high prevalence of spontaneous AIA in newly diagnosed Type 1 diabetic subjects, especially in children [30], the test used for the detection must be devoid of such drawbacks; (5) The ELISA methodology, with a storable source of human antigen, and no need for radioactive reagents, makes this test readily available.

Using this test we have searched for antibodies to the insulin receptor in the sera of 60 newly diagnosed Type 1 diabetic patients. Only one serum (SR) was positive for antibodies of the IgG class, and none of them for antibodies of the IgM class. The clinical history of this patient is unusual. Briefly, SR is a man, aged 24, whose glycaemic control was fairly well maintained for at least 20 months by an oral hypoglycaemic agent (glibenclamide, 10 mg/day), despite a poor Beta-cell residual function. Indeed, the C-peptide level was very low (190 pmol/l) when fasting and did not increase significantly (287 pmol/l, $p > 0.05$) after glucagon (1 mg i.v.).

Our results are not in agreement with three previously published studies [7–9]. The study by Maron et al. [7] was performed in 22 children before treatment with exogenous insulin. Antibodies were screened for their ability to inhibit insulin binding to rat adipocyte insulin receptors and by their capacity to stimulate lipogenesis. They were present in 45% of these children and were all of the IgM class. In addition to different methodological approaches, we cannot exclude the remote possibility that antibodies of the IgM class had undergone some degradation in our sera during prolonged storage, a classic phenomenon for IgM. However, it is also possible that Maron et al. detected antibodies against some rat species specific antigenic determinant. Ludwig et al. [8], reported three positive sera out of 29 newly diagnosed Type 1 diabetic patients, by measuring the inhibition of insulin binding to IM-9 cells. However, none of them would have been positive if they had used, as for their AIA assay, 3 SD instead of 2 SD

above the mean values of control sera. Finally, it is difficult to interpret the study by Boden et al. [9], since three out of the five positive patients (among 51 diabetic patients) presented with other manifestations of autoimmune disease, and since most of their diabetic patients had been treated with insulin for many years.

Circulating ICA are an important indicator of Type 1 diabetes. The prevalence of ICA positivity varies from 55 to 80% in newly diagnosed diabetes mellitus. Although the pathogenic role of ICA is unclear, it has emerged as a predictive immunomarker of beta-cell destruction [5]. Besides ICA, it has become increasingly clear that Type 1 diabetes is, at least, associated with a B-lymphocyte polyclonal activation that results in the production of autoantibodies that are directed against a wide variety of antigens [4]. This is characteristic of polyendocrinopathies and the latter antibodies may be considered as non-specific and innocent bystanders of the autoimmune process [31]. Antibodies to the insulin receptor could have been representative of such a phenomenon. However, our data suggest that such antibodies are rather unusual in recently diagnosed Type 1 diabetes. Alternatively, antibodies to insulin receptor could arise as anti-idiotypes from AIA [32–34]. Indeed, AIA are, after ICA, the most frequent antibodies found in this clinical situation since they have been found to be positive in 20 to 40% of patients [3, 6, 28, 35]. The idiotype of AIA could serve as an antigen for production of anti-idiotypes. This mechanism is unlikely, at least in our study, since no autoantibodies to insulin receptor were found in our paediatric subgroup (Paris, Table 1) despite a high percentage of positivity for AIA. Nevertheless, our ELISA is a sensitive assay to search for antibodies in clinical situations that can be linked to antibodies to the insulin receptor in a variety of autoimmune situations [31].

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