Clonally restricted insulin autoantibodies in a cohort of 2200 healthy blood donors

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Summary. Serum samples of 2200 blood donors were screened for anti-insulin IgG by enzyme-linked immunosorbent assay. Specificity of detected antibodies was verified by competition with an excess of insulin and observation that saturated anti-insulin IgG were no longer measurable. The upper limit of measured signal in the population was defined as the mean plus three SD. In the direct assay, 32 sera were positive. Among these, 22 (1%) contained saturable insulin antibodies (true positive) and 10 were non-saturable and considered as non-insulin-specific. The positive blood donors were requested to answer a questionnaire and according to their answers, none had ever received insulin, was a first degree relative of a Type 1 (insulin-dependent) diabetic patient or had experienced a hypoglycaemic episode. None of

the 22 true positive sera detected by enzyme-immunosorbent assay bound ¹²⁵I-insulin to any significant extent. The nine sera yielding the highest signal were further characterized with regard to heavy and light chains as well as species specificity of ligand. Anti-insulin IgG from healthy blood donors contained only one heavy (γ 1 2/9; γ 3 7/9) and one light (κ 8/9; λ 1/9) chain. Three sera were human insulin specific; two were non-species-specific; the other four bound insulin in the order human = porcine > bovine. These results indicate that low affinity clonally restricted antibodies against insulin are present in unselected blood donors with a prevalence of 1%.

Key words: Insulin autoantibodies, IgG isotype, light chain, species specificity, blood donors, affinity.

First described as an immune reaction against therapeutically administered insulin [1, 2], insulin antibodies have recently been described in patients with autoimmune hypoglycaemia [3–6] or juvenile diabetes before any administration of insulin [7]. More recently, in the latter patients, autoantibodies specific for human proinsulin have also been found [8].

In Type 1 (insulin-dependent) diabetes, emergence of antibodies against insulin or proinsulin, as that against islet cells (ICA), has been considered as a potential marker of the ongoing process of autoimmune Beta-cell destruction. Insulin autoantibodies are present in about one third of subjects with newly diagnosed Type 1 diabetes and prevalence of insulin autoantibodies is inversely associated with age [9–16]. Insulin autoantibodies are also detectable in non-diabetic relatives of diabetic probands, for instance, in monozygotic twins discordant for diabetes [9, 10].

Type 1 diabetes is often associated with manifestations of autoimmunity other than against the Beta cell. Markers of organ specific autoimmunity may also be encountered in relatives of diabetic probands, with a particularly high frequency when they are present in the probands themselves [17].

Detection of insulin antibodies is limited by the sensitivity and the specificity of the assay technique. Further-

more, as recently discussed, currently available assay techniques do not always yield concordant results [18].

The purpose of the present work is to establish the detection limits of insulin antibody enzymatic immunoassay (EIA) using a large number of plasma samples collected from a population of healthy blood donors. In order to increase assay specificity, all the samples were analysed with and without preincubation with a large excess of native insulin [19]. Of the sera tested 1% contained insulin antibodies which were further characterized. Other autoantibodies were searched for and the individuals were typed for HLA A, B, C and DR.

Subjects and methods

Subjects

A series of 2200 sera were collected from healthy blood donors (Blood Transfusion Centre, Liège, Belgium). They were selected according to guidelines laid out by the Council of Europe (Strasbourg 1986). Briefly, the donors' age ranges from 18 to 65. To be eligible, they should be of normal appearance and among a list of exclusion criteria, they should be free of chronic allergic diseases such as asthma, autoimmune disease and diabetes.

Fig. 1. Histogram of frequencies of optical density in the direct assay ($n = 2,200$)

Fig. 2. Histogram of frequencies of differences of optical density between direct and competitive assay ($n = 2,200$)

Assay of anti-insulin IgG by EIA

EIA of insulin IgG was performed as previously described [20]. Polystyrene microplates (NUNC, n° 4-39454, Gibco, Paisley, Scotland) were coated overnight at 4°C with 75 µl of a solution containing 13 µg/ml of human crystallized insulin (Novo Industry AS, Copenhagen, Denmark). Blood donors sera were diluted 1/5 in phosphate buffered saline (PBS, NaH₂PO₄ · 2H₂O: 0.52 g, Na₂HPO₄ · 3H₂O: 2.87 g; NaCl: 8.76 g; H₂O ad 1 l, pH 7.4) containing bovine serum albumin: 2 g; Tween 20: 0.5 ml (PBS-BSA-Tween). Each serum

sample was mixed with an equal volume of PBS containing 200 µg/ml human insulin and left overnight at 4°C. For the assay, 50 µl of each sample (preincubated and not preincubated) were transferred onto the coated plates and left for 2 h at 37°C. After three washes with PBS-BSA-Tween, a mouse monoclonal antihuman IgG (MH001, Gamma S.A., Liège, Belgium) was added. After 2 h at 37°C and three washes with PBS-BSA-Tween, rabbit immunoglobulins anti mouse IgG conjugated to peroxidase (P-260, Dakopatts, Glostrup, Denmark) was allowed to react for 1 h at 37°C. After four washes with tap water, 2,2'-azino-di-3 ethylbenzthiazolino sulfonate 6 (ABTS, Boehringer, Mannheim, FRG) was added. Oxidized chromogen was measured at 405 nm. Blank values were obtained from wells which received all reagents but human serum.

Species specificity of the insulin antibodies was tested using plates coated with bovine, porcine or human insulin. Animal insulins were also generously donated by Novo Industry AS.

Reproducibility was assessed at different levels of optical densities (OD). It was found that the coefficients of variation respectively were: 86%, 19% and 6% for intraassay and 133%, 22% and 15% for interassay at OD levels of 0.05, 1 and 2 units.

Characterization of heavy and light chains of insulin autoantibodies

Subunits composition of insulin autoantibodies was studied by replacing the γ chain specific monoclonal antibody used as detector by monoclonal antibodies specific for each IgG isotype and type of light chain. The following monoclonal antibodies were used. Anti γ1: M15015; anti γ2: M10015; anti γ4: M16013; anti kappa light chain M03010 and anti lambda light chain M04018 (Unipath Laboratories, Bedford, UK); anti γ3: MH013 (Gamma S.A., Liège, Belgium). The assay had previously been standardised using myeloma proteins.

Radiobinding assay of insulin antibodies

Radiobinding assay (RBA) was performed by mixing a constant amount of HPLC purified ¹²⁵I-Tyr A14 bovine, porcine or human insulin (20,000 cpm in 100 µl) with 100 µl of serum diluted 1/2 in phosphate buffer (NaH₂PO₄ · 2H₂O: 2.07 g; NaCl: 9 g H₂O ad 1 l, pH 7.4) containing 3.5 g of crystallized bovine serum albumin with or without insulin 1 µg/ml. After overnight at 4°C, 0.4 ml of dextran coated charcoal (1 volume of charcoal suspension: 50 mg/ml buffer and 1 volume Dextran T70: 5 mg/ml in PBS) and 1 ml of PBS-BSA were added to the samples and vigorously mixed. After 10 min at 4°C, the tubes were spun at 3,000 × g for 10 min in a refrigerated centrifuge and radioactivity of precipitates and supernatants was measured. Blank values were obtained by substituting serum free of antibodies for test serum. In normal individuals, in absence of added insulin, the percentage of radioactivity in the precipitate was 0.4–0.8% (average ± SD; $n = 112$).

HLA A, B, C, DR typing

HLA typing was performed using the microlymphocytotoxic technique described by Terasaki [21]. The HLA-DR technique was carried out by microlymphocytotoxicity method coupled with 2 colour fluorescence staining according to van Leeuwen [22].

Determination of other autoantibodies

Islet-cell antibodies (ICA) were assayed by indirect fluorescence on sections of blood group 0 cryofixed human pancreas employing the method of Bottazzo et al. [23–25]. Other autoantibodies directed against smooth, myocardial and striated muscle, thyroid peroxidase,

Fig. 3a, b. Histogram of frequency of individuals with (filled box) and without (open box) insulin autoantibodies according to age in male subjects (**b**) and female subjects (**a**)

thyroglobulin, nuclei, mitochondria, adrenal and gastric H^+ , K^+ -ATPase were searched for using commercially available kits.

Statistical analysis [26]

A statistical analysis was applied to the data of both direct and indirect EIA, with two aims: firstly, to determine the type of distribution (gaussian or not) and secondly, to calculate a threshold of OD value above which one can conclude that the sample tested contains anti-insulin antibodies.

As is the case for most biological parameters, the histograms of OD values (in direct and indirect assay) exhibited an asymmetry to the right. Therefore, the H spread (spread between centiles 25 and 75) and sH (standard deviation estimated by using the H spread) were calculated. These two parameters are useful when the distribution is tailing. Indeed, they are not affected by extreme values at either end of the population.

Results

Directly and after competition with an excess of insulin 2200 samples were assayed by EIA and statistical analyses were performed on three sets of values: OD of the direct EIA, OD of the competition EIA and difference between these two OD values. Results are summarised in Table 1.

For the three sets of values, the coefficients of symmetry (G1) and of curtosity (G2) were greater than zero indicating that none of the three distributions was normal. Examination of Figures 1 and 2 confirms the asymmetry of the cohort of OD values after direct assay and that of differences. Indeed, due to the presence of insulin autoantibodies, both populations are tailing to the right.

Per definition, identification of insulin-specific antibodies requires that two criteria be satisfied; firstly a signal significantly different from background must be observed in the direct assay and secondly, a significant inhibitory effect of an excess of insulin must be seen in the competitive assay. Sera satisfying the first but not the second criterion were considered as non-insulin-specific or "false positive". The mean + 3 SD was taken as upper limit of normal ($p = 0.0013$). To avoid overestimation of SD due to tailing of the distribution to the right, an SD estimate (sH), was also used.

As shown in Table 2, using a threshold based on SD, 32 sera were positive in the direct assay and 22 contained insulin-specific autoantibodies. Substituting sH for SD, 47 sera were positive in the direct assay and of these, 37 were insulin specific. Thus, depending upon the chosen cut off point, the prevalence of insulin autoantibodies in the population of blood donors was 1 to 1.7%.

As illustrated in Figure 3, prevalence of insulin autoantibodies was equal in both sexes. Positive subjects were found in age classes containing the largest number of individuals, particularly between 30 and 50 years. There is no clustering at either side of the age distribution. The 22 sera found positive in EIA were tested by radiobinding assay and all were found negative. The nine sera yielding the highest signal in EIA were further characterized regarding IgG isotype and light chain composition.

Figure 4 illustrates the titration curve of the positive blood donor sera with the various mouse monoclonal antibodies. In the direct assay, each one of the nine sera contained anti-insulin IgG made of one single heavy chain and of one single light chain. In the competitive assay, the OD signal was reduced to blank level. As summarized in Table 3, IgG isotype was 1 in two cases and 3 in the other seven. Gamma 2 or 4 heavy chains were not observed. Light chain was Kappa in eight cases and Lambda in one.

Table 1. Statistical parameters of the studied population

	OD after direct assay (1)	OD after competition assay (2)	Difference between (1) and (2)
Mean	0.090	0.084	0.006
Standard deviation	0.109	0.078	0.105
sH	0.059	0.067	0.045
Range	-0.090 to 1.890	-0.110 to 0.880	-0.300 to 1.860
Symmetry ^a	2.66	1.44	2.85
Curtosity ^b	84.86	13.47	111.08

^a g1. This coefficient is zero in a gaussian population and positive in a right tail distribution;

^b g2. This coefficient is zero in a gaussian population and positive in a "sharp" distribution;

OD = optical density; sH = standard deviation estimated using the H spread

Table 2. Number and percentage of sera yielding a signal above threshold in direct and inhibition EIA

Statistical criterion	cutt-off (OD)	direct EIA	inhibition EIA
mean + 3 SD	0.417	32 (1.5%)	22 (1.0%)
mean + 3 sH	0.267	47 (2.2%)	37 (1.7%)

Fig. 4. Titration curves of the positive sera using mouse monoclonal antibodies against IgG (■), $\gamma 1$ (★) and $\gamma 3$ (●) heavy chain and K (▲) and lambda (○) light chain. No reaction occurred with mouse monoclonal antibodies against $\gamma 2$ or $\gamma 4$ isotypes. OD signal was abolished in the competitive assay

Patterns of species specificity are illustrated in Figure 5 and summarised in Table 3. Three of the nine sera displayed an exclusive specificity for human insulin. Four bound equally human and porcine insulin and displayed little or no affinity for bovine insulin. Two were non-species specific.

The same nine sera were screened for other organ and non-organ-specific autoantibodies. It was found that subject LAL was rated + for ICA (< 5 JDF units), subject DEL was weakly positive for thyroid peroxidase anti-

bodies and rated + for striated muscle antibodies. A doubtful reaction for striated muscle antibodies was also observed for subject PLE. Other autoantibodies against nuclei, mitochondria, smooth muscle, myocardium, salivary gland, H^+ , K^+ -ATPase, adrenal, thyroglobulin and TSH receptor on thyrocytes were negative.

As also shown in Table 3, haplotypes positively associated with Type 1 diabetes, and in particular DR3 DR4, were not clustered in our positive donors.

Discussion

In a population of 2200 healthy blood donors, using a cut off point of 3 SD above the mean, 22 subjects were positive for insulin antibodies by EIA. None of the EIA positive sera bound 125 -I human insulin. Discrepancy between

Table 3. Properties of insulin autoantibodies and characteristics of the donors

Donor	Sex	Age	IgG H,L ^a	Species specificity ^b	HLA				Other autoantibodies ^c
					A	B	C	DR	
Tai	M	37	1, K	H = P = B	11	15, 35	w3, w4	1, 4	—
Eng	F	28	3, L	H = P > B	1, 2	8, 40	2	5, 7	—
Lal	M	38	3, K	H	3, 9	5, 35		5, 6	ICA + / -
Mar	M	41	3, K	H	1, 9	8, 12	5	4	—
Del	M	48	3, K	H = P > B	3	17, 35		6	StMA + / -, TPA +
Gil	F	57	3, K	H = P = B	2	7, 70	7	2, 7	—
Bau	M	41	3, K	H = P	3, 33	7, 14		1, 7	—
Ple	M	43	1, K	H = P > B	1	8, 17	6, 7	3, 7	StMA + / -
Vra	M	35	3, K	H	2	18, 27	1	8, 7	—

^a IgG isotype and light chain composition;

^b Insulin species: H (human), P (porcine) and B (bovine);

^c Islet cell antibodies (ICA), striated muscle antibodies (StMA), smooth muscle antibodies, myocardial muscle antibodies, thyroid peroxidase antibodies (TPA), thyroglobulin antibodies, nuclei antibodies, mitochondrial antibodies, adrenal antibodies and gastric H^+ , K^+ -ATPase antibodies

Fig. 5. Plates coated with human (●), porcine (▲) and bovine (■) insulin were used to titrate the nine positive sera. In the competitive assay, the OD signal was abolished

the two assays results may, at least in part, be explained by the fact that radiobinding assay of insulin antibodies follows the law of mass action and is highly sensitive to the affinity of the antigen-antibody reaction whereas the solid phase assay follows different rules. Intensity of EIA signal is affinity dependent for low affinity antibodies, allowing one to demonstrate species specificity of low affinity autoantibodies. By contrast, in the presence of high affinity antibodies, EIA signal is maximum and independent of small differences of affinity. As already discussed, EIA is not appropriate to study species specificity of (high) affinity antibodies circulating in insulin-treated diabetic patients [18]. Direct evidence for differential sensitivity of the two assay systems to avidity of the antigen-antibody reaction was recently obtained using monoclonal insulin antibodies raised in mice (unpublished data).

The nine sera exhibiting the highest OD values were further analysed with regard to molecular structure. In each case, the anti-insulin IgG appeared to be made of one single type of heavy (7 γ 3, 2 γ 1) and light (8 κ 1, 1 λ) chain, a finding consistent with marked clonal restriction. These insulin autoantibodies are thus structurally different from insulin antibodies produced by insulin treatment of diabetic patients in whom the sole or most frequently predominant heavy chain is γ 1 [20] and oligo/polyclonality is evidenced by the coexistence of the 2 light chains [27].

Further evidence for clonal restriction was provided by identification of the binding region of the insulin molecule. Three of the nine tested sera exclusively bound human insulin, pointing to one single epitope containing

threonine B30. Four sera equally bound human and porcine insulin but exhibited little or no affinity for bovine insulin, pointing to an epitope containing the A chain loop in its human/porcine configuration. The last two sera appeared non-species specific. Clonal restriction of insulin autoantibodies in 11 patients with various medical and genetic backgrounds has similarly been underlined by Nell et al. [27].

Recently, Wilkin et al. [28] compared the epitopes of insulin autoantibodies in patients with polyautoimmune disease but no personal or family history of diabetes (diabetes unrelated group) and in newly diagnosed Type 1 diabetic patients, discordant twins or normal siblings of Type 1 diabetic patients (diabetes related group). Specificity for human insulin (epitope including B30 area) was found in only 1 of 42 subjects of the diabetes related group and in 61% of individuals with diabetes unrelated polyautoimmunity. Crossreaction with human, porcine and bovine insulin was found in 41 of 42 diabetes related subjects and in only 39% of the diabetes unrelated subjects. Similar findings were obtained in our laboratory. As will be reported in more detail elsewhere, insulin autoantibodies were of lower affinity in blood donors than in Type 1 diabetic patients before the initiation of insulin therapy.

Additional indications that blood donors insulin autoantibodies are not related to diabetes are: (1) lack of diabetes in the personal and familial history of the probands; (2) absence of diabetes prone HLA phenotype such as DR3, DR4 and, on the contrary frequent identification of DR7, a phenotype negatively associated with Type 1 diabetes; [29] (3) absence of ICA [30].

Insulin-autoantibodies in blood donors probably more closely resemble those described in patients affected with other autoimmune diseases [31, 32] but it should be

stressed that our blood donors were free of such disease on a clinical basis (see exclusion criteria for blood donors) and according to laboratory tests (absence of other autoantibodies).

It is interesting to note that in two patients suffering from a non-drug related autoimmune hypoglycaemic syndrome [33, 34], very high plasma concentrations of insulin autoantibodies qualitatively similar to those found in blood donors were identified. In both instances, the anti-insulin IgG were of low affinity and structurally homogeneous ($\gamma 3$ K in one case and $\gamma 1$ K in the other one). In one case, the autoantibody was highly specific for human insulin, whereas, in the other, it was of the cross reacting type. It is therefore tempting to hypothesize that "clonally restricted" insulin autoantibodies different from those found in Type 1 diabetes are identifiable in healthy individuals (low insulin autoantibody plasma concentration and low prevalence), in patients suffering from autoimmune diseases other than Type 1 diabetes (higher prevalence) and in some patients with autoimmune hypoglycaemic syndrome (extremely low prevalence but very high insulin autoantibody plasma concentration).

In view of their low plasma concentration and of their low avidity for insulin, the insulin autoantibodies of blood donors should not alter the bioavailability of insulin and consequently not cause hypoglycaemic episodes. Actually, as expected, analysis of answers to a questionnaire did not support the contention that the probands were at high risk of hypoglycaemia. Because of beforehand agreement with the Blood Transfusion Department that the donors would not be informed of the results of the study and would not undergo any additional testing, we were unable to verify this statement by a glucose tolerance test. From a laboratory stand point, because they did not bind radioiodinated insulin to any detectable extent, these insulin autoantibodies should not interfere with plasma insulin assay.

In conclusion, insulin autoantibodies are detectable by EIA in at least 1% of healthy adult subjects. Structural homogeneity and epitope restriction strongly suggest that the autoantibodies are "monoclonal". Available evidence differentiates them from those found in Type 1 diabetic patients before the onset of insulin therapy. In view of their low plasma concentration and low affinity for insulin, they do not exert clinical effects and do not interfere with plasma insulin assay. It is speculated that they originate from incomplete suppression of a forbidden clone, that their prevalence is increased in some dysimmune states and that, when their plasma concentration is very high, hypoglycaemic episodes ensue.

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