Originals

Influence of affinity of antibodies upon their detection by liquid phase radiobinding assay and solid phase enzyme linked immunosorbent assay. Demonstration using monoclonal antibodies raised against rDNA human proinsulin

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Summary. Hybridomas producing proinsulin antibodies were cloned by limiting dilution of cell cultures obtained by fusion of splenocytes of immunized mice with immortal myeloma cells. Some proinsulin monoclonal antibodies crossreacted with labelled insulin but none did with labelled C-peptide indicating that the involved epitopes were at one of the insulin/C-peptide junctions or included in the insulin moiety. Hybridoma supernatants were assayed for IgG concentration by a solid phase assay and for ligand binding by a radiobinding assay and an enzyme linked immunosorbent assay. The half-life of immune complexes formed with radioligand was measured and, as expected, correlated with affinity as measured by the method of Scatchard. Antibody titres determined by enzyme linked immunosorbent assay did not correlate to those measured by radiobinding assay.

IgG concentration correlated to enzyme linked immunosorbent assay titres but not to radiobinding assay titres. Finally, a significant correlation was found between radiobinding assay titre and the product of enzyme linked immunosorbent assay titre by the period of immune complexes. It is concluded that, except for very low affinity antibodies, enzyme linked immunosorbent assay is a capacity assay whereas radiobinding assay is influenced by both antibody concentration and affinity. The former assay is thus best suited to detecting low affinity antibodies whereas the latter is more efficient in the presence of low levels of high affinity antibodies.

Key words: Proinsulin monoclonal antibodies, affinity, solid-liquid phase assay, radiobinding assay.

Insulin antibodies which circulate in diabetic patients treated with insulin are easily detectable by radiobinding assay (RBA) as well as by solid phase assay (ELISA). Yet, even in these circumstances where the level of antibodies is usually high, agreement between the measurements of insulin antibodies by the two techniques is rated differently, some authors finding a good correlation [1], others finding no correlation [2]. Several explanations for these discrepancies have been proposed. Firstly, in the solid phase assay, insulin molecules may be systematically adsorbed by the same surface which therefore becomes unavailable for antibody binding. By extension, coating of insulin may partially denature the molecule and prevent some populations of antibodies from binding to distorted epitopes [3]. In the hands of others, the hypothesis of hidden or distorted epitopes proved an unsatisfactory explanation for all the discrepancies between RBA and ELISA results [2, 4]. Secondly, the presence of radioactive iodine on insulin alters the surface of the molecule and may modify the binding affinity of complementary molecules, be they insulin receptors [5, 6] or insulin antibodies [2]. Although it does occur, this phenomenon does not explain all the dif-

ferences between the two assay systems. Thirdly, the influence of affinity of the antibody for its ligand may be different in the two types of assay [7, 8].

If both assays give positive signals in diseases characterized by a high level of plasma antibodies such as insulin-treated diabetes mellitus [9], insulin autoimmune syndrome [10–15] and certain drug reactions [16–18], they may respond in the opposite manner in cases where the level of circulating antibodies are low such as in the normal population [19–21], in Type 1 (insulin-dependent) diabetes at onset [22–27] or in relatives of Type 1 diabetic patients, either unaffected twins [28] or siblings [29]. It therefore becomes important to understand better the basic mechanisms of both assays in order to select the most appropriate for the purpose. This type of study is not feasible on polyclonal serum containing several populations of antibodies of different affinity and epitope specificity. We therefore raised a panel of monoclonal antibodies against proinsulin, characterized them regarding immunoglobulin concentration and affinity for their ligand and compared their behaviour in the two assay systems.

Materials and methods

Preparation of monoclonal antibodies against human proinsulin

Hybridomas were produced from spleen lymphocytes of mice immunized with rDNA human proinsulin. Briefly, BALB/c or Biozzi mice were injected i. p. with 20 µg proinsulin formulated in complete Freund adjuvant. The mice received additional injections at 2–3 week intervals of the same amount of proinsulin emulsified in incomplete Freund adjuvant. They were screened by RBA for proinsulin antibodies 10 days later and the animals with the highest titres were selected for fusion. One to two months after the third injection, they received a booster of 20 µg proinsulin without adjuvant and, 4 days later, their spleens were aseptically dissected out. After collection in RPMI medium, the splenocytes were fused with SP2/0 myeloma cells as previously described [30]. Fused cells were then transferred to 96-well plates containing naïve mouse peritoneal macrophages as feeders in hypoxanthine-aminopterin-thymidine (HAT) medium supplemented with 10% fetal calf serum (FCS). After 1 week of culture, the HAT medium was replaced by hypoxanthine-thymidine (HT) medium containing 10% FCS. After cell proliferation, the supernatant of the cell cultures was screened for antibodies and the antibody producing hybridomas were cloned by limiting dilutions in HT medium supplemented with 10% FCS and 5% human umbilical cord serum [31]. Monoclonal antibodies were tested with ¹²⁵I-labelled insulin and C-peptide in order to define the molecule moiety containing the epitope. Some fully crossreacted with insulin, none crossreacted with C-peptide. Involved epitopes were therefore either included in the insulin moiety for the insulin crossreacting antibodies or located at one of the insulin-C-peptide junction for the non-crossreacting antibodies.

Measurement of IgG concentration in mouse hybridoma culture supernatants

IgG concentration in the mouse hybridoma culture supernatants was measured by a solid phase assay [32]. Briefly, immunoplates were coated overnight at 4°C with 100 µl of a solution of purified rat monoclonal antibody directed against mouse IgG K light chain (25 µg/ml LO-MK-2, Experimental Immunology Unit, UCL, Louvain La Neuve, Belgium) in 0.1 mol/l bicarbonate buffer, pH 9.6. After washing with distilled water, the remaining sites were saturated with 250 µl phosphate buffer containing 0.5% bovine serum albumin and 0.1% Tween 20. Fifty microliters of serial two-fold dilutions of the mouse hybridoma culture supernatants (starting dilution 1/1000) and mouse immunoglobulin reference serum (starting dilution 1/5000; ICN immunobiologicals, Lisle, Ill., USA), were added to the coated wells and incubated for 2 h at 37°C. After washing with distilled water, 50 µl rat monoclonal antibody against mouse K light chain conjugated to peroxidase (LO-MK-1 peroxidase, Experimental Immunology Unit) was added to the wells and incubated for 2 h at 37°C. After washing with distilled water, 50 µl of the H₂O₂-chromogen solution was added (5 mg/ml O-phenylene diamine (Abbot Laboratories, Wiesbaden, FRG), 0.003% H₂O₂ in 0.1 mol/l citrate-phosphate buffer, pH 5.5). The reaction was stopped 15 min later by addition of 100 µl 2N H₂SO₄ and optical density at 492 nm was measured. A blank was included in the first well of each row to quantify non-specific binding of the immunoconjugate.

Enzyme linked immunosorbent assay for proinsulin antibodies

Titres of anti proinsulin monoclonal antibodies were measured using a modification of a previously described method [33]. Briefly, 75 µl of 13.3 µg/µl proinsulin in carbonate-bicarbonate coating buffer pH 9.6

were aliquoted in the wells of microtitre plates (NUNC, n° 4-39454, Gibco, Paisley, UK). After overnight incubation at 4°C, the plates were washed three times with phosphate buffered saline (PBS), 0.5% Tween 20, 0.2% bovine serum albumin pH 7.2 and allowed to stand for 30 min at room temperature in order to saturate the remaining polystyrene reactive sites. The wells were then refilled with serial two-fold dilutions of the monoclonal antibodies, the first well of each row receiving buffer alone and serving as a blank. After 2 h incubation at 37°C and three successive washes, all the wells were refilled with 50 µl of a 1/250 dilution of rabbit anti-mouse immunoglobulin serum conjugated to peroxidase (HRP-RAM-Ig, P260, Dakopatts, Glostrup, Denmark). After 1 h incubation at 37°C followed by four washes with tap water, the assay was developed by adding 50 µl of ABTS solution (2,2' - azino-di-3 ethylbenzthiazolinosulfat 6, 75 mg/100 ml, Boehringer, Mannheim, FRG) containing H₂O₂ (0.004% volume/volume). The reaction was finally stopped 30 min later by the addition of 150 µl 2 N H₂SO₄ and absorbance was read at 405 nm using a multi-channel spectrophotometer (LP-200, Institut Pasteur, Paris, France).

Radiobinding assay (RBA) for proinsulin antibodies

RBA was performed using reverse phase HPLC purified Tyr A14 monoiodinated [¹²⁵I] proinsulin of specific activity 200–233 µCi/µg or, for the 100% crossreacting antibodies, Tyr A14 monoiodinated [¹²⁵I] insulin of specific activity 300–350 µCi/µg [34–35]. Briefly, a constant amount of radioligand (20,000 cpm in 100 µl) was mixed with duplicate 100 µl samples of several two-fold monoclonal antibody dilutions in serum pre-treated with dextran-coated charcoal. After an overnight incubation at 4°C, 1 ml of 18% (weight/volume, w/v) polyethylene glycol (PEG) 6000 (Fluka, Buchs, Switzerland) was added to each tube. After 15 min at room temperature, the tubes were centrifuged, supernatants were discarded, precipitates were washed with 1 ml PEG 15% (w/v) and their radioactivity was measured using a multi-well gamma counter (Berthold LB 2101, Wildbad, FRG). The percentage of antibody-bound radioligand was calculated as the percentage of radioactivity of the precipitate. Blank values obtained by substituting serum free for monoclonal antibody solution were subtracted from sample values.

Dissociation of immune complexes formed at the solid-liquid phase interface

Four different monoclonal antibodies were tested on proinsulin coated plates. The wells received 50 µl of serial two-fold dilutions of each monoclonal and after 2 h incubation at 37°C and three washes, were refilled with 50 µl PBS with or without a large excess (400 µg/ml) proinsulin. The plates were incubated at 4°C for 24, 48 or 96 h. After aspiration of the liquid phase and three washes, the wells successively received, as described above, rabbit anti-mouse immunoglobulin serum conjugated with peroxidase followed by ABTS-H₂O₂ and stopping solution and absorbance at 405 nm was recorded.

In order to differentiate true dissociation of immune complexes from desorption of coated ligand, the desorption rate of radio-labelled ¹²⁵I-proinsulin was estimated. Microplates were coated as described above except that in addition to native proinsulin, the coating solution also contained Tyr A14 monoiodo [¹²⁵I] proinsulin. The wells were filled with 50 µl buffer and left at 4 or 37°C. The liquid phase was withdrawn 0, 1, 4, 24 and 48 h later and the radioactivity counted and expressed as a percentage of the initial coated activity (14% of the radioactivity added in the coating solution).

Dissociation of immune complexes formed by radioligand in liquid phase

Immune complexes were formed by an overnight incubation of monoclonal antibodies with ¹²⁵I radioligand (20,000 cpm). At the start of the dissociation experiment, an excess of native ligand (10 µg

Fig. 1. Correlation between titres of hybridoma culture supernatants determined by radiobinding assay (RBA) and enzyme linked immunosorbent assay (ELISA). Eleven culture supernatants corresponding to at least five different monoclonal antibodies originating from four different fusions were studied: $\blacksquare$ = F65 ($n = 1$), $\blacktriangle$ = S1 ($n = 2$), $\bullet$ = S2 (species 100% cross-reacting with insulin and proinsulin, $n = 2$), $\circ$ = S2 (proinsulin specific species, $n = 4$), $\blacksquare$ = S3 ($n = 2$)

Fig. 2. Dissociation rate of immune complexes in homogeneous liquid phase. $\blacktriangle$ = S1, $\bullet$ = S2 (proinsulin specific species) and $\blacksquare$ = S3. The equation $A_t = A_0 e^{-\lambda t}$ where A_t and A_0 are the amounts of immune complexes at times 0 and t , λ , is the dissociation rate and t , the time, was used to calculate the half-life (T) of the immune complexes

in 1 ml) was added, duplicate aliquot samples were drawn at selected times and immediately precipitated by 1 ml PEG 18% w/v. After centrifugation and elimination of the supernatant, the radioactivity of the pellet was measured and expressed as a percentage of the total.

Measurement of affinity constant

Affinity constants of the various monoclonal antibodies was measured using the method of Scatchard [36]. Each monoclonal was diluted so as to bind 60% of the radioactive tracer (20,000 cpm) after

a 4 day incubation at 4°C in the absence of added native ligand. Duplicate samples of this monoclonal antibody dilution received a constant amount of radioligand (20,000 cpm in 100 μ l) and 100 μ l buffer alone or containing increasing concentrations of competitor (from 2.07×10^{-11} to 1.4×10^{-7} mol/l). After 4 days incubation at 4°C, immune complexes were precipitated by PEG and their radioactivity measured. After subtraction of the non-specific binding measured on samples containing a large excess of native ligand (10 μ g/ml), results were plotted as bound/free antigen ratio vs bound antigen.

Results

For each of the 11 monoclonal antibodies, titres of culture supernatants were determined by solid and liquid phase assays. The reciprocal of the monoclonal antibody dilution corresponding to 1.0 U of optical density was taken as the ELISA titre and that corresponding to 50% radioligand binding was taken as the RBA titre. As illustrated in Figure 1, there was no correlation between the two sets of titres ($r^2 = 0.019$, $p > 0.3$).

As shown in Figure 2, preformed immune complexes made with monoclonal antibodies and radioligand dissociated in a semi-logarithmic fashion. The half-life (T) of the immune complexes was calculated (Table 1) and found to range from 30 to 570 min.

The affinity constant of 9 of the same 11 monoclonal antibodies was determined by Scatchard analysis. All Scatchard plots were linear. Individual values of affinity and the coefficients of correlation for linearity of the Scatchard plots are listed in Table 1. As expected, a highly significant correlation existed between the half-life of the immune complexes and affinity as measured by Scatchard analysis ($y = 4.92 \times 10^{-9} + 31$; $n = 9$; $r^2 = 0.930$; $p < 0.005$).

Before measuring the dissociation rate of immune complexes formed in solid phase between the coated ligand and the soluble monoclonal antibody, we estimated the desorption rate of the coated ligand. Assuming that the rate of desorption of radioligand is the same as that of the native one, we measured that, even after 2 days at 37°C, less than 2% of the coated ligand returned to the liquid phase (Table 2). This percentage was considered as negligible and no correction for spontaneous desorption was made for the dissociation experiments.

Table 1. Properties of 11 monoclonal antibodies against human proinsulin

Clone name	IgG isotype	IgG concentration, μ g/ml	Specificity ^a	RBA titre	ELISA titre	Half-life of immune complexes in liquid phase (T_1 min)	Affinity as determined by Scatchard analysis (l/mol)	r^2 ^b
F65	IgG1, K	6	Hi, HPi	1580	28	160	$1.7 \cdot 10^{10}$	0.781
S1 7B7F9	IgG1, K	1	Hi, HPi	20480	30	280	ND	
S1 7B7E2	IgG1, K	ND	Hi, HPi	40000	60	570	ND	
S2 2E2D12	IgG1, K	9	Hi, HPi	400	140	30	$2.6 \cdot 10^9$	0.890
S2 6A12G11	IgG1, K	17	Hi, HPi	320	160	42	$1.0 \cdot 10^9$	0.976
S2 6G4D3	IgG1, K	10	HPi	20000	225	165	$2.8 \cdot 10^{10}$	0.811
S2 7B8F8	IgG1, K	7	HPi	5000	70	170	$3.1 \cdot 10^{10}$	0.866
S2 7B8H10	IgG1, K	8	HPi	20000	270	175	$3.1 \cdot 10^{10}$	0.916
S2 6G4B11	IgG1, K	< 0.1	HPi	225	5	204	$3.4 \cdot 10^{10}$	0.847
S3 9D7F2	IgG1, K	11	Hi, HPi	400	50	61	$7.1 \cdot 10^9$	0.964
S3 9D7B6	IgG1, K	ND	Hi, HPi	2000	180	52	$6.3 \cdot 10^9$	0.984

^a Hi, HPi: 100% crossreactivity for Hi and HPi; HPi: no crossreactivity with either insulin or C-peptide; ^b Coefficient of correlation for linearity of Scatchard plot.

RBA = Radiobinding assay

Fig. 3. Dissociation rate of immune complexes in heterogeneous liquid-solid phase, in the absence (closed symbols, solid lines) or the presence (open symbols, dashed lines) of excess native ligand. $\blacktriangle$ = S1, $\bullet$ = S2 (proinsulin specific species), $\blacksquare$ = S3 and $\blacklozenge$ = S8. ELISA = enzyme linked immunosorbent assay

Fig. 4. Correlation between radiobinding assay (RBA) titres and the product of enzyme linked immunosorbent assay (ELISA) titres by half-life of immune complexes of the same 11 hybridoma culture supernatants as in Figure 1. Same symbols as in legend of Figure 1

As shown by Figure 3, immune complexes formed at the solid-liquid interface did not significantly dissociate after 4 days incubation in the absence of added native ligand. This held true even for clone S8 which, in liquid phase, formed very unstable immune complexes characterized by a dissociation period of less than 1 min. By contrast, in the presence of an excess of native ligand, dissociation became significant for clone S8. A mild dissociation was observed for clone S3 (T in liquid phase = 60 min) and no effect whatsoever was discernable for clones S1 and S2 (respective T in liquid phase = 570 min for S1 and 170 min for S2). As shown by Figure 4, when RBA titres were plotted against a parameter integrating antibody concentration and affinity such as the product of ELISA titre by the period of immune complexes, a highly significant correlation was found ($r^2 = 0.93$, $p < 0.005$).

Table 2. Percent of desorption of ^{125}I -Tyr A14 human insulin

	1 h	4 h	24 h	48 h
4°C	0.2	0.3	0.7	0.8
37°C	0.5	0.4	0.9	1.1

Discussion

As already mentioned, using polyclonal insulin antibodies developed in insulin-treated diabetic patients, we did not find any correlation between antibody titres determined by both assays [2]. This observation is now confirmed using monoclonal antibodies.

A major difference between the two assays lies in the fact that RBA follows the law of mass action and that, in view of the low concentration of ligand (0.06 ng/tube), formation of immune complexes is strongly dependent upon affinity of the antigen-antibody reaction. Although indirect evidence supported this contention, no direct proof could be provided because of the impossibility to measure affinity of a polyclonal immune serum, which yields curvilinear dissociation curves and Scatchard plots. By contrast, monoclonal antibodies which, in liquid phase, provided mono exponential dissociation curves and linear Scatchard plots allowed straightforward measurements of affinity. In liquid phase, the association rate depends upon physical parameters such as size of the ligand and thermal agitation whereas the dissociation rate rather reflects the structural complementarity between the epitope and the paratope. The relationship between affinity (K) and half-life of immune complexes (T) is shown by the equation:

$$K = \frac{K_a}{0.678} T$$

K = affinity, K_a = association constant, T = half-life of immune complexes. The association constant K_a may be defined by the equation $K_a = A e^{-E_a/RT}$ where A is a frequency factor, E_a is the energy of activation, R is the molar gas constant and T the absolute temperature [37].

The association constant directly depends on the diffusion rate and is comparable for all antigen-antibody reactions, ranging from 10^7 – 10^8 mol^{-1} for haptens to 10^5 – 10^6 mol^{-1} for protein antigens [38]. As expected from these thermodynamic considerations, a statistically significant correlation was found between the half-life of the immune complexes and the affinity constant measured by Scatchard analysis. Therefore, both parameters allow ranking of the antibodies according to affinity.

In the solid phase assay, a much greater amount of ligand (140 ng) is coated on the wall of the wells [33]. Therefore, in ELISA, proinsulin or insulin locally achieve extremely high densities thereby conferring to this assay a very low degree of sensitivity to affinity. This is indeed proved by the fact that monoclonal antibodies of extremely low affinity (T of immune complexes in liquid phase < 1 min) form stable immune complexes at the solid-liquid interface. Addition of an excess of native ligand had no apparent effect on the dissociation of coated ligand-monoclonal antibody immune complexes except

when the affinity of the antibody was low or very low. This is taken as evidence that dissociation does occur in the solid phase assay but to a negligible extent for antibodies of high affinity (10^8 – 10^{10}) and to increasing extent when affinity decreases. Thus, when antibodies of high affinity are assayed, ELISA is insensitive to affinity and the final optical density signal merely reflects antibody concentration (C). As seen above, the RBA signal is a biparametric variable depending not only upon antibody concentration (C) but also upon antibody affinity (K). It therefore comes as no surprise that no correlation exists between ELISA and RBA titres as there is no proportionality between C alone and the product CK whereas a significant correlation exists between the RBA titre and a parameter integrating antibody concentration and affinity such as the product of ELISA titre by the period of immune complexes. Furthermore, a significant correlation also existed between ELISA titres and IgG concentration ($r^2 = 0.73$, $p < 0.005$, $n = 9$) whereas RBA titres and IgG concentration did not correlate ($r^2 = 0.02$, $p > 0.20$, $n = 9$).

The hypothesis that the window in which ELISA is sensitive to affinity is much lower than that of RBA also accounts for the observation that autoantibodies with low affinity for insulin such as those found in the normal population are detectable by ELISA only and not at all by RBA [21]. It may further be implied that the ratio of RBA/ELISA titres is a function of affinity. Therefore, it becomes easily conceivable that a small amount of high affinity antibodies be detectable mostly or even exclusively by RBA. This could explain the much greater efficiency of RBA to detect insulin autoantibodies in Type 1 diabetic patients before onset of insulin therapy [39].

In conclusion, RBA and ELISA for insulin antibodies rely on markedly different thermodynamic reactions, the former being exquisitely sensitive to affinity, the latter being a capacity assay in the upper range of affinity and becoming sensitive to affinity only in the lower range of affinity. This hypothesis explains why both assays give a positive signal in the presence of a high concentration of antibodies such as in insulin-treated diabetes but that no correlation exists between the titres determined by the two assays. Very low affinity antibodies will be detectable by ELISA only whereas, on the contrary, small amounts of high affinity antibodies may be preferentially detected by RBA. As previously reported, because it is a capacity assay, ELISA may be standardised whereas, being a biparametric assay, RBA does not lend itself to standardisation using a reference serum [8].

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