Diabetologia (1983) 24: 399–403

Diabetologia

Review Articles

Insulin Antibody Determination: Theoretical and Practical Considerations

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Key words: Insulin, insulin antibody, immunogenicity, immunoglobulins, radio-labelled insulin.

Allergic reactions to insulin were described soon after the introduction of insulin for the treatment of diabetes [1]. Ten years later an anti-insulin factor was identified in a serum globulin fraction [2] despite doubts that insulin would be immunogenic in unmodified form [3]. It was not until 1956 that Berson et al. [4] demonstrated that most diabetic patients receiving heterologous insulin (i. e. from another species) develop antibody within a few weeks of starting insulin therapy. However, unlike most antigen: antibody systems studied at that time, complexes of insulin with its antibody failed to produce precipitin lines in standard immuno-precipitation techniques, and Berson and Yalow [5] confirmed that these complexes were soluble over a wide range of antigen: antibody ratios. Thus, from the outset, the detection and analysis of insulin antibodies has made special demands on methodology and it is not surprising that diverse approaches have been tried [6].

Insulin is of considerable interest to immunologists as well as diabetologists [7]. Its primary and tertiary structure is well documented; a number of useful analogues have been prepared; species variants are available; and it can be obtained in high purity. Insulin is the only protein antigen with such properties that is frequently injected into man. Animal studies using heterologous insulin as test antigen have focussed on the importance of immune response (Ir) genes linked to the major histocompatibility complex (MHC) in determining the degree of responsiveness in different inbred strains [8–11]. The association of insulin antibody production with HLA phenotype is now well-established [12–14] and further study may provide important information concerning the nature of Ir genes in man.

Several insulin formulations have been developed for clinical use [15] and, with at least three different species variants available (i. e. bovine, porcine and human), various factors may contribute to the immunogenicity of an individual preparation [16]. The production of insulin antibody is not only of academic interest for it does have a role in insulin allergy, injection site lipoatrophy [17] and some types of insulin resistance as well as having more subtle effects on the dose requirement, and on the pharmacokinetics of injected insulin [18–21]. Insulin-antibody complexes may have a deleterious effect in patients with vascular disease but there are insufficient data to be sure.

These considerations underline the importance of using precise methods for insulin antibody determination, especially at a time when rival claims are being made about the relative immunogenicity of different therapeutic preparations in current use.

Heterogeneity of Antibody

Variations in the structure of immunoglobulins are responsible for their subdivision into classes, sub-classes and light chain types. In conjunction with heritable allotypic variations, this gives rise to about 100 molecular possibilities even without reference to the antigen combining site [22] (Table 1). In addition, the immunoglobulins of an individual animal contain an immense repertoire of antigenic specificities. This major source of diversity is located in the variable regions of the molecules [23]. Chemical variation in this region creates the individual nature of the antigen-combining site (or idiotype) of a particular molecule and each clone of antibody-producing cells produces antibody of consistent idiotype. Many different clones of cells are stimulated to produce antibody in response to the simplest of antigens – hence the term polyclonal for all normal immune responses.

All antibody molecules consist of a four-chain unit structure containing two antigen combining sites. IgM and secretory IgA are polymeric molecules but most serum immunoglobulins have only two antigen binding sites. As most antigens contain multiple antigenic determinants, there is considerable variation in the size and nature of complexes formed at different antigen:

Table 1. Heterogeneity of immunoglobulin molecules

Category	Variants	Number	Total[a]
Classes	G, A, M, D, E	5	5
Subclasses	γ_1, γ_2, γ_3, γ_4, α_1, α_2	6	9
Light chains	κ or λ	2	18
Allotypes	Gm/Am/Km	>8	100 (approx.)
Idiotypes	variable regions	10^6–10^7	10^8–10^9

[a] The contribution of each category of variation is incorporated into a cumulative total

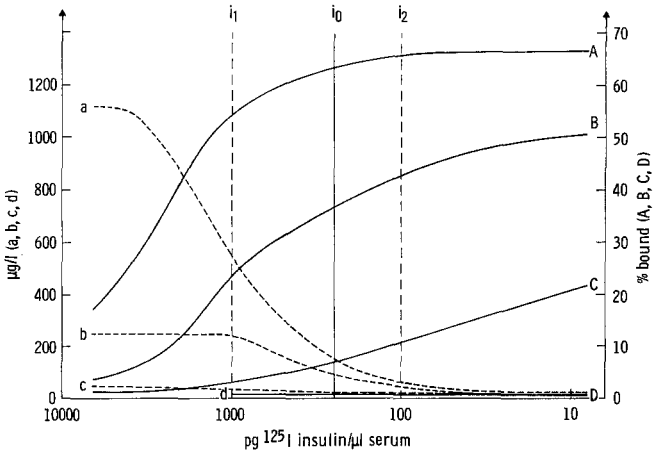

Fig. 1. Effect of variation in concentration of ^{125}I insulin used to document the binding of three sera containing insulin antibody, expressed as percentage of insulin bound *(A, B* and *C)* or μg insulin bound per litre of serum *(a, b* and *c)*. d-D indicates the binding observed with normal human serum. i_1 and i_2 indicate the outer limits of iodinated insulin concentration for use in a routine assay, i_0 being an optimal concentration (see text). Adapted from Reeves and Kelly [30]

antibody ratios, i.e. the combination is not stoichiometric. However, the relatively small size of insulin (mol.wt. 5,600) and the modest size of insulin-containing immune complexes present in human sera [24] suggest that only one or at most two antibody molecules are able to combine with a single insulin molecule in these circumstances. Larger, precipitating complexes can be produced when bovine insulin is injected into guinea pigs [25] and the insulins of these two species have 18 sequence differences. Although insulins used in the treatment of diabetes show up to three sequence differences, the antibody produced in response to their injection is not usually specific for these variant residues but reacts with determinants shared by the endogenous human insulin molecule [16]. Similar findings have been demonstrated in other species [26, 27]. Very little is known about the topography of these antigenic determinants on the surface of the molecule but it is likely that they occur in sufficient proximity to each other to cause steric hindrance between their respective antibodies.

Assays in Current Use

Most assays in current use belong to three main categories. Quantitative radioimmunoelectrophoresis was developed by Christiansen [28] and has been widely used to document the binding of IgG antibody to radiolabelled insulin by rocket immunoelectrophoresis into anti-IgG-containing agarose. Fluid phase methods account for most other assays using radio-labelled insulin, the separation of 'bound' from 'free' insulin being achieved by coated charcoal, cellulose, gel filtration, ultracentrifugation or precipitation with salt, ethanol, polyethyleneglycol (PEG), protein A or a second antibody, e.g. anti-IgG. Generally, methods using non-specific methods of phase separation show higher levels of background binding. PEG and anti-IgG are probably the most widely used phase separants in current use [29, 30]. Some workers have favoured competitive binding, e.g. between antibody and both labelled and unlabelled insulin [31] or between labelled insulin and a mixture of a standard guinea pig antibody and the human antibody under examination [32].

Solid-phase immobilisation of insulin to plastic tubes [33], paper discs [34] or sepharose particles (as in the radioallergosorbent test – RAST) [35, 36], or immobilisation of second antibody (as in the paper radioimmunosorbent test – PRIST) [37], have been used chiefly to document the much smaller amounts of IgE antibody in the circulation of insulin-treated patients. However, different immunoglobulin classes may compete for binding to immobilised antigen [38] and immobilisation of second antibody is associated with a loss of sensitivity in some instances [39]. With either approach great care has to be taken to maintain minimal and consistent levels of non-specific protein binding.

Analysis of Binding Curves

Whichever assay is selected it is important to determine the binding characteristics of high, medium and low-binding sera. Figure 1 demonstrates the increasing percentage binding for three sera containing insulin antibody (A, B and C) in association with decreasing concentrations of labelled insulin used as ligand. Conversely, if the binding is expressed in absolute units, e.g. μg insulin bound per litre of serum, then the actual amount bound decreases with decreasing dose of ligand (a, b and c). In optimising the amount of labelled insulin to be used routinely one wishes to avoid saturating conditions for almost all antibody-containing sera (seen with serum b at 1,000 pg insulin/μl serum, i.e. i_1) and yet it is useful to use a concentration which will give maximum discrimination between different levels of insulin antibody in terms of percentage binding. This progressively reduces at concentrations less than 100 pg insulin/μl serum, i.e. beyond i_2. Thus, an intermediate but arbitrary concentration of 125 pg insulin/μl serum, i.e. i_0, is a useful optimal concentration to use. Whether results are expressed in terms of percentage binding or in absolute units (e.g. μg/l or ng/ml), both can be changed dramatically by shifting the dose of antigen used in the as-

Table 2. Important sources of variation in assay technique

Pre-treatment of sera to remove insulin
Species and dose of ligand
Iodination; method and characterisation
Incubation pH and ionic strength
Electrophoretic effects
Phase separation: is the binding moiety antibody?
Excess versus optimum second antibody
Washing precipitate versus volume marker (^{22}Na)
Normal serum binding (? subtraction)
Number of replicates
Quality control
Data format
Intra-batch versus inter-batch data (coefficient of variation)

say and thus "absolute units" have little meaning when compared between assays that have not been standardised.

Determination of Binding Constants

The classical studies of Berson and Yalow [5] favoured heterogeneity of antibody combining sites rather than multivalency of insulin to explain the heterogeneity of insulin-antibody complexes. Their results were close to a theoretical curve derived from a model of univalent insulin reacting with antigen-combining sites on two different antibody molecules. Their most striking data came from dissociation rate studies but they pointed out that the K values measured represented average values for groups of different antibody molecules. Since then various workers have endeavoured to fit asymptotes to non-linear Scatchard plots to determine the slope – and hence the binding affinity – of two idealised antibody populations: Ab_1 of high affinity and Ab_2 of low affinity [40, 41]. However, the shape of such curves varies with incubation time, presence of free insulin, range and frequency of labelled insulin concentrations used, as well as the method of phase separation [27, 40]. For the reasons discussed above, it seems unlikely that there should be only two antigenic determinants on the insulin molecule capable of combining in a standard manner with two molecular varieties of insulin antibody. Doubts concerning the validity of analysing curvilinear Scatchard plots in terms of two stoichiometric reactions are borne out by recent studies on monoclonal, i.e. homogenous, antibodies against IgG in which significant deviations from linearity were both predicted and observed experimentally in Scatchard plots [42]. Preliminary studies with monoclonal antibodies to insulin demonstrate the existence of several antigenic determinants recognised by the murine antibody response to bovine insulin [43]. Measurements of affinity constants for polyclonal sera are fraught with theoretical and practical problems [44] and the nearest one should probably endeavour to go is to determine average constants for association, dissociation or overall avidity [5, 19, 45].

Sources of Variation in Assay Results

Even when assays are confined to the determination of percentage binding (or related units), there are a number of reasons why data may vary between different laboratories (Table 2). The presence of unlabelled insulin, endogenous or exogenous, in the test serum prior to assay inhibits antibody binding to labelled insulin. Various methods have been used to remove free insulin, e.g. dialysis, gel filtration or adsorption onto coated charcoal at acid pH [6]. It is possible that electrophoretic and solid-phase methods are less prone to such interference. However, comparative data are required before this source of variation can be adequately assessed. It is not sufficient to assay sera taken from insulin-treated patients 'fasting' or more than 12 h after the last injection of insulin, since the half life of circulating insulin is considerably prolonged in the presence of insulin antibody [18, 19]. The dose of ligand has been considered above and should, ideally, be standardised in molar terms. For most sera the species of insulin used, i.e. bovine, porcine or human, is not of great importance in that they show comparable binding for all three ligands [16, 46]. Occasional sera do, however, show preferential binding, e.g. for bovine insulin, and depending on the nature of the study more than one species of ligand may be used in parallel.

A major source of difficulty and variation in insulin antibody determination is the iodinated insulin used in the assay. Some commercial sources of ^{125}I insulin cannot be relied upon to provide consistent material for use in insulin antibody determination. Most current methods of iodination, e.g. chloramine t, iodate, lactoperoxidase or iodogen, yield material labelled in the A14 and A19 positions. The ratio of these two labelled products present in the final material is not as important as the degree of contamination with protein damaged by the iodination process. Labelled products can be purified and separated into mono-iodinated A14 and A19 insulins by ion exchange chromatography, high voltage electrophoresis or high pressure liquid chromatography [47–50]. These refinements are usually unnecessary for routine purposes if gentle and standard iodination conditions are used.

Antigen : antibody reactions are greatly affected by variations in pH and ionic strength and labelled-antigen binding assays are no exception [51]. The pH and conductivity of the diluent buffer should be specified and checked regularly. Where an electrophoretic step is included, great care has to be taken to avoid heating effects and dissociation of insulin : antibody complexes formed during incubation.

Ideally, all antibody determinations should document the binding material as antibody. Although there is little direct evidence for the binding of insulin by other serum proteins, it is interesting that the background binding is generally higher when non-specific phase separants are used, e.g. PEG. A consequence of

Table 3. Inter and intra-batch variation in IgG insulin antibody binding levels for positive and normal control sera.

	Positive control serum		Pooled normal human sera	
	Inter-batch ($n=12$)	Intra-batch ($n=12$)	Inter-batch ($n=12$)	Intra-batch ($n=12$)
Mean ± SD	68.27 ± 5.07	71.17 ± 2.18	1.44 ± 1.27	1.29 ± 0.50
Coefficient of variation	7.42	3.06	87.81	36.88

Data derived using an immunochemical assay [30] and expressed in μg/l.

this is that there is less 'binding territory' left in which to discriminate between positive sera of different binding capacities. Second-antibody assays reduce this problem, the reagent being used either in standard excess [52] or at an optimal concentration for each individual serum [30]. The need to wash precipitates after phase separation introduces further error which can be obviated by incorporation of a volume marker, i.e. ^{22}Na. Allowance is made for the 'free' ^{125}I insulin which remains after removal of about 80% of the supernatant, by determining ^{22}Na and ^{125}I counts in the precipitate [30].

Laboratories vary widely in their approach to the binding levels of normal human sera. Some subtract a binding level for single or pooled normal human sera from all test data with the curious result that negative binding levels can occur. Others express their results without background subtraction; biologically this may be more meaningful in that sera from some patients treated with immunogenic insulins do have such low antibody levels as to be indistinguishable from normal human sera. These low levels of binding are commonly seen in association with the HLA B8/DR3 phenotype [12–14]. Most assays are conducted in duplicate, but if not then the number of replicates performed will affect precision. The incorporation of high, medium and low-binding positive control sera in addition to normal serum in each batch, as well as the cumulative recording of quality control data, are essential for consistent results and will soon indicate when a labelled insulin preparation is unsatisfactory. The way in which data are expressed would greatly benefit from standardisation as discussed above. It is also important to know the limitations of an individual technique in terms of the coefficient of variation at different binding levels (Table 3). Differences between inter-batch and intra-batch coefficients of variation are greater at lower binding levels and when longitudinal studies are being performed on individual patients it may be preferable to run all sera from an individual patient in the same batch.

Conclusion

The immunogenicity of insulin preparations is of both academic and clinical interest. The links between insulin antibodies and insulin allergy, some forms of insulin resistance and injection site lipoatrophy are well-established, but other more subtle metabolic effects require further examination. Contamination with impurities (e.g. proinsulin) has been a major factor in the immunogenicity of conventional bovine insulin preparations but the less frequent, although still detectable, immunogenicity of highly purified porcine and human preparations remains enigmatic. Further work is required to analyse the physico-chemical factors involved, while the genetic control of the immune response to insulin is of fundamental interest.

In order to facilitate comparative studies of different insulin preparations and data translation between different laboratories, it is essential that efforts be made to introduce some elements of standardisation in assay techniques, reporting of results and assessment of precision, accuracy and sensitivity. International collaborative laboratory studies have been successful in various other areas of clinical research relevant to diabetes, notably the series of HLA workshops [53] and comparisons of the radioimmunoassay and bioassay of insulin [54, 55] and the radioimmunoassay of C-peptide [56]. It is hoped that present efforts to achieve successful collaboration for insulin antibody determination will harmonise the diverse approaches to the problems which continue to surround the immunogenicity of insulin.

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