Diabetologia (1988) 31: 670-674

Diabetologia

Insulin autoantibody polymorphisms with greater discrimination for diabetes in humans

T. Wilkin, I. Mirza, M. Armitage, C. Casey and L. Scott-Morgan

Endocrine Section, Department of Medicine II, General Hospital, Southampton, UK

Summary. Insulin autoantibodies, like islet cell antibodies, are found not only in the sera of newly diagnosed Type 1 (insulin-dependent) diabetic patients and their relatives, but also in patients with other autoimmunities who do not develop diabetes. Insulin autoantibodies are oligo/monoclonal and frequently binding-site restricted. As determinant selection is genetically determined, we questioned whether certain polymorphisms of insulin autoantibodies, identified by their binding site on the insulin molecule, could better discriminate for Type 1 diabetes, which is also HLA determined. First, we raised monoclonal antibodies to human insulin by classic fusion methods in order to determine the range of antibody polymorphism, and identified five distinct types by their binding profiles to a panel of insulin variants, using an enzyme-linked immunosorbent assay. Two of these polymorphisms, type A and type B, were subsequently found in insulin autoantibody positive human sera using the same panel of insulin variants, and successfully distinguished diabetes-related from diabetes-unrelated individuals. Thus, the type B polymorphism was responsible for binding in 60% of 41 insulin autoantibody positive individuals with polyautoimmune disease but no personal or family history of diabetes (diabetes unrelated), but in only 2% of a group which comprised 17 newly-diagnosed insulin autoantibody positive Type 1 diabetic patients, 19 insulin autoantibody positive discordant twins of Type 1 diabetes and six insulin autoantibody positive healthy siblings of Type 1 diabetic patients (diabetes related) ($p < 0.01$). Isolation of the type A polymorphism alone reduced the proportion of false negatives in the insulin autoantibody test for diabetes relatedness from 49% to 20% without diminishing its specificity. Thus, insulin autoantibody polymorphisms are more discriminating than the 'nominal' antibody, due possibly to linkage between immune response genes determining response to the type A epitope on the one hand, and susceptibility to Type 1 diabetes on the other.

Key words: Insulin autoantibodies, Type 1 (insulin-dependent) diabetes, antibody markers, autoimmunity.

Insulin autoantibodies (IAA) have been described in at least three different clinical contexts. They were first reported from Japan in association with the insulin autoimmune syndrome [1], which is characterised by normal fasting blood glucose, reactive hypoglycaemia, B-cell hypertrophy and absence of insulitis. More recently, IAA have been described in newly-diagnosed diabetic patients [2] and their relatives [3, 4], and in patients with apparently unrelated autoimmune disease [5].

We have shown previously that IAA are oligo/monoclonal and binding site restricted [6]. As determinant selection is genetically determined [7], we questioned whether particular polymorphisms of IAA, identified by their binding site on the insulin molecule, could better discriminate for Type 1 (insulin-dependent) diabetes, which is also genetically determined [8]. Accordingly, we raised monoclonal antibodies to human insulin in order to determine the range of IAA polymorphisms, and examined the IAA positive sera from different patient groups to establish whether IAA subsets segregated clinically.

Materials and methods

Immunisation protocol

Ten female BALB/c mice were immunised with 20 μg human or porcine monocomponent insulins (Novo, Bagsvaerd, DK) in complete Freund's adjuvant (0.1 ml) distributed equally between the rear footpads. On day 14, the draining lymph nodes (popliteal and inguinal) were removed and pooled. Serum was retained for determination of IAA.

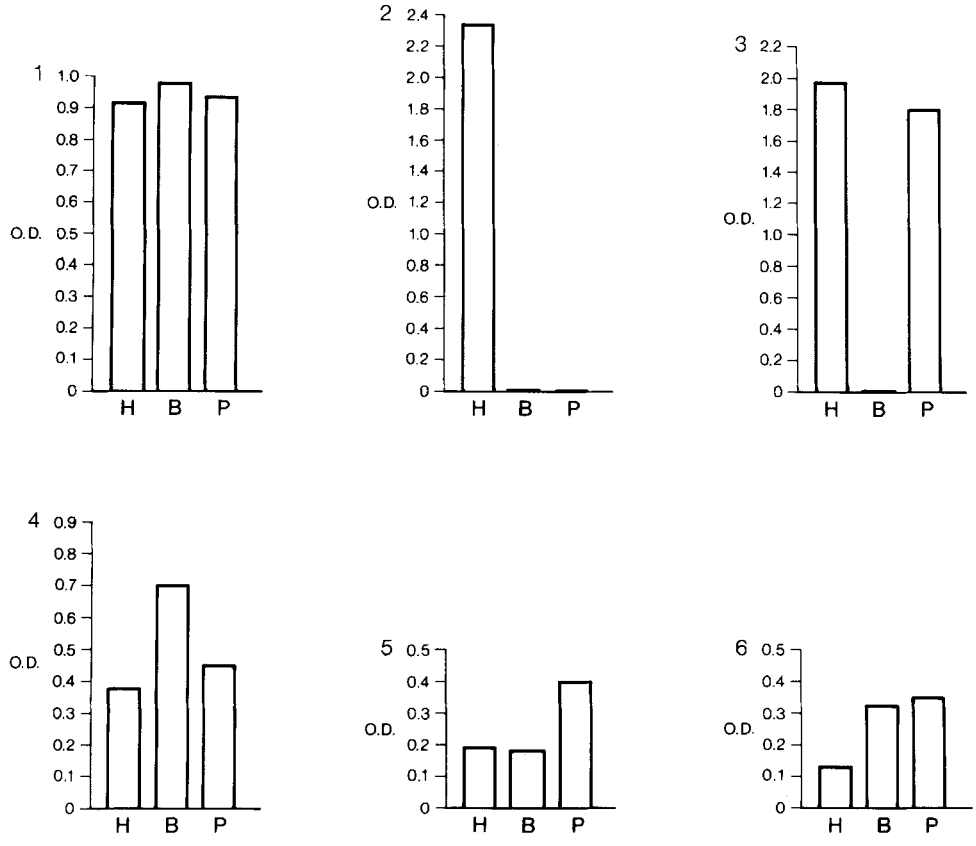

Fig. 1. Spectrum of monoclonal antibody specificities isolated by lymph node cell fusions, as measured by binding to human (H), bovine (B) and procine (P) insulins and expressed as optical density units (O.D.) in an ELISA. Six different monoclonals (1-6) are shown

Cell hybridisation and cloning of hybrid cell lines

The myeloma cell line NS-0 was used for cell hybridisation. The methods used have been detailed previously [9]. Single cell suspensions were prepared by teasing spleen or pooled lymph nodes through 100 gauge stainless steel mesh. Cells were washed in serum free RPMI 1640 supplemented with penicillin and streptomycin and mixed with myeloma cells in a ratio of 2:1. Cells were spun down to form a tight pellet and all medium was removed. Fusion was initiated by the addition of 50% polyethylene glycol (PEG) 6000 over 1 min. After a 90 s incubation at 37°C, the PEG was diluted out by the dropwise addition of serum free RPMI and cells gently spun down. Supernatant was aspirated, the cells resuspended in hypoxanthine-aminopterin-thymidine (HAT) selection medium and plated out onto four 96-well tissue culture plates with a macrophage feeder layer. Cells were fed with fresh HAT medium seven days after fusion and at day 12, cell culture supernatants were removed and screened for insulin antibodies. Positive hybridoma colonies were cloned by limiting dilution and after growth, single colonies were rescreened. Positive single colonies were then expanded in 2 ml 24-well plates and screened for antibody specificity against human, bovine and porcine insulins.

Screening assay

A solid phase enzyme linked immunosorbent assay (ELISA) was used in screening for anti-insulin antibodies [10]. Briefly the wells of an ELISA plate (Alpha-Labs, Eastleigh, Hants, UK) were coated with highly purified human, bovine and porcine insulin and other insulin variants (Eli Lilly, Indianapolis, Ind, USA) at 2.5 g/ml in carbonate/bicarbonate buffer pH 9.6 at 4°C overnight. Wells were washed 3 times with phosphate saline buffer +0.05% Tween-20 (PBST) and refilled with 200 µl of cell culture supernatant. Following a 2 h incubation at room temperature and further washing, rabbit anti-mouse IgG (polyclonal) conjugated to horseradish peroxidase (Sigma, Poole, Dorset, UK) was added at 1:1000 in phosphate-buffered saline +1% bovine serum albumin (Sigma)+1% normal rabbit serum and left at 4°C overnight. The assay was developed by the addition of phenylenediamine 340 g/ml in 0.15 mmol/l citrate phosphate buffer pH5. The colourimetric reaction was stopped with 34% H_2SO4 and the colour intensity measured in a spectrophotometer (Kontron SLT-60) at 490 nm.

Further expansion of hybridoma colonies

Hybridoma colonies were recloned twice by limiting dilution to ensure monoclonality and genetic stability before further expansion in 25 ml flasks using HAT medium.

Sera from patients

The sera from two groups of IAA positive patients were studied, group A with one or more additional autoantibodies from a panel of 15, but no personal or family history of diabetes, and group B who were "diabetes-related". Group A comprised 15 males and 26 females, mean age 44 years. Sixteen out of 41 had endocrine autoantibodies (not islet cell antibody) and 25 non-organ specific autoantibodies. In group B there were siblings of Type 1 diabetic patients (five females, one male; mean age 5.7 years), 15 newly diagnosed but non-insulin-treated Type 1 diabetic patients (8 females, 9 males; mean age 13.7 years) and 19 identical twins discordant for Type 1 diabetes (10 females, 8 males; mean age 27.0 years). There was insufficient serum from most subjects in group B to carry out tests for further autoantibodies.

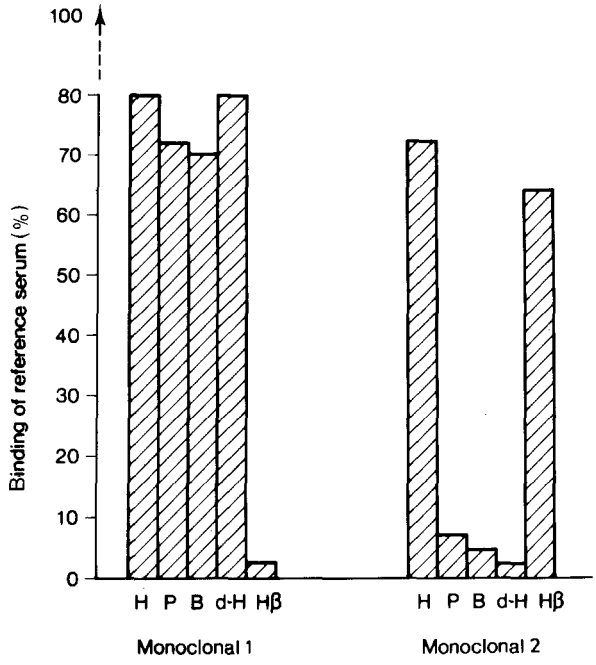

Fig. 2. Direct binding of monoclonals 1 and 2 to human (H), porcine (P), bovine (B), dethreoninated human (d-H) insulins and isolated human β chain (H-β)

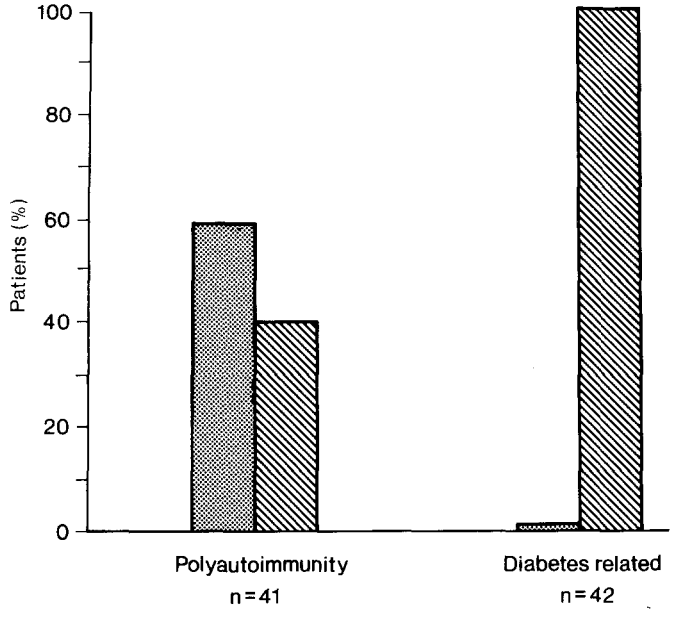

Fig. 3. The distribution of type A (cross-reactive) and type B (human insulin specific) insulin autoantibody subsets according to clinical circumstance. ▨ Sera which bound only human insulin; ▧ Sera which bound human, porcine and bovine insulins equally. All data corrected for non-specific binding. The columns express the proportion (percent) of patients in each clinical group showing either the type A or type B polymorphism

Absorption studies on human sera

Apparently human insulin specific IAA positive sera were incubated with serial quantities of human or porcine insulin coupled to a constant mass of Sepharose 4B beads prior to measurement in the ELISA [11], in order to confirm their specificity for human insulin. All data are expressed as the difference between the raw ELISA binding signal and the signal remaining after prior absorption with human insulin coupled to sepharose. The interassay coefficient of variation of the ELISA used here is < 10% at low, medium and high levels of binding.

Statistical analysis

The frequencies with which IAA polymorphisms occurred in the two study populations were compared by the Chi squared test.

Results

At day 14 after inoculation the sera from all ten mice carried high titre IgG IAA wholly cross-reactive with human, porcine and bovine insulins. Lymph node cell fusions were characterised by high frequencies of hybridomas producing anti-insulin antibodies. These were cloned by limiting dilution and further tested for insulin specificity by screening against human, bovine and porcine insulins. A spectrum of specificities was observed - (1) equal binding to human, bovine and porcine insulins (2) human specific (3) human and porcine specific (4) bovine predominant (5) porcine predominant (6) porcine and bovine predominant. The different binding profiles are shown in Figure 1. All data were confirmed by prior absorption with all three insulins. The majority of the lymph node derived hybridomas secreted monoclonal antibodies of type 1. Monoclonals of types 4, 5 and 6 were interesting because they were heteroclitic, i.e. they showed greater binding to cross-reacting antigens (bovine or porcine insulins) than to the inducing antigen, human insulin. Monoclonal 2 appeared on direct binding to be exclusively human insulin specific and absorption tests similar to those described for the sera under "Materials and methods", in which quantities ranging from 10^{-6} to 10^{1} mg/ml supernatant of human, dethrenoninated human, porcine and bovine insulin coupled covalently to sepharose 4B were preincubated with the supernatant, confirmed this (not shown).

Specificities 1 and 2 were also found in IAA positive human sera and studies using isolated human insulin β chain and dethrenoninated human insulin showed that specificity 2 was indeed restricted to the β chain of (human) insulin and specificity 1 to the α chain (Fig. 2). Thus, specificities 1 and 2 were restricted to mutually exclusive binding sites on human insulin and of the six specificities defined by the monoclonals, were the most distinct from each other.

The binding reactions with human, porcine and bovine insulins of sera from the 83 IAA positive subjects are shown in Figure 3, segregated according to clinical circumstance. Human insulin specificity was seen in 26 individuals, representing 25 of the 41 with polyautoimmunity but only one of the 42 who were diabetes-related ($X^2 = 33.1$, $p < 0.001$). The remaining sera were cross-reactive with human, porcine and bovine insulins. The absorption data on a typical human-

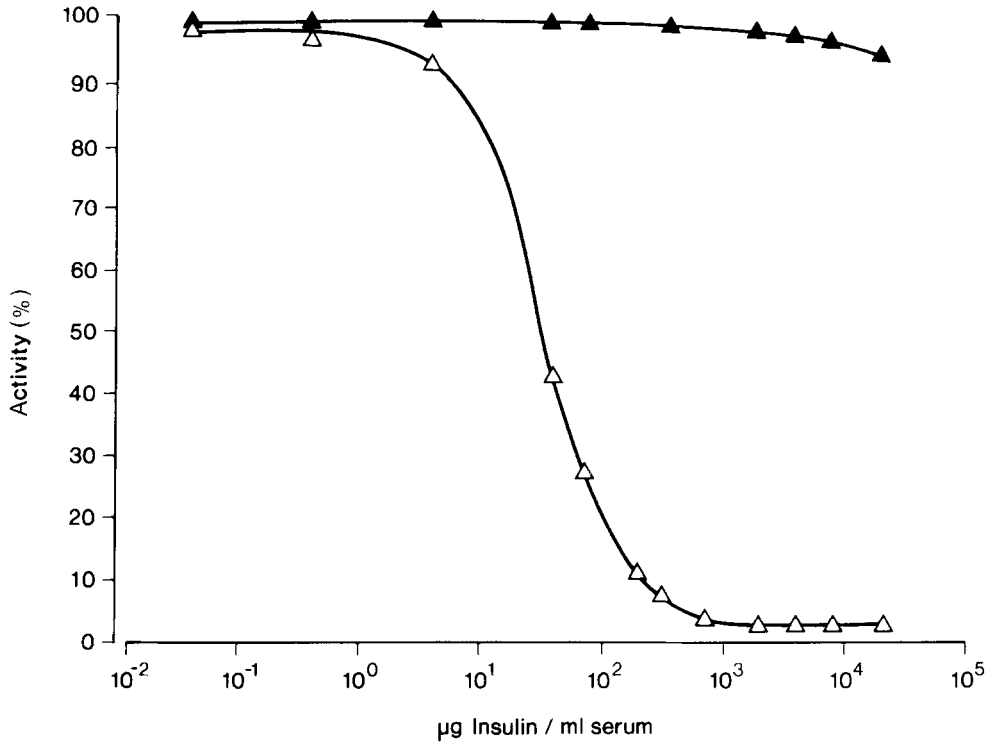

Fig. 4. Absorption of a high titre human insulin specific IAA serum seen in this study by incremental quantities of porcine (▲) and human (△) insulin covalently coupled to sepharose 4B beads. Binding reaction against human insulin

insulin specific serum (Fig. 4) shows how the restriction to human insulin was essentially complete.

The IAA in human sera could therefore be divided into two basic subsets. The one designated type B corresponded to the human insulin specific monoclonal and very seldom occurred in association with Type 1 diabetes. The type A sera may have corresponded to the monoclonal specificity 1 uniquely or may have been a heterogeneous mixture, incorporating a number of clones which together imbued the serum with cross-reactivity. Type 1 binding was found in all but one of the diabetic patients and in 16 (40%) of those with polyautoimmunity.

Use of the type A polymorphism as a marker for diabetes-relatedness among the 83 IAA positive subjects studied reduced the proportion of "false negatives" from 49% (41/83) to 20% (17/83) without materially affecting specificity.

Discussion

Murine monoclonal antibodies raised to human insulin showed considerable polymorphism with respect to binding epitope recognised. Patterns similar to those we describe have been reported previously [12]. Two binding patterns, designated 1 and 2, showed mutually exclusive restriction and were also detected in human sera where they segregated clearly between patients who were IAA positive in a diabetic context (type A) and patients who had no personal or family history of diabetes (type B). A form of insulin-dependent diabetes designated 1b has been described in which the clinical onset is relatively slow [13], although immune markers are usually present to indicate the presence of insulitis. We cannot rule out the presence of insulitis in our patients with human insulin specific IAA, but none was positive for islet cell antibodies, and none subsequently became diabetic.

Although it is traditional to speak of antibodies to insulin, thyroglobulin, etc. as if the antibody recognised the whole molecule, antibodies recognise surface shapes or epitopes quite independently of the remaining structure [14]. Genetic control of the clonal response to each epitope is correspondingly independent, so that the epitopes on a molecule such as insulin recognised by one individual may be quite different from those recognised by another [5, 6, 15]. Such differences are not usually apparent in sera from insulin-treated diabetic patients which are polyclonal for insulin and recognise many epitopes shared among the species variants of insulin, although a human insulin-specific clone was clearly represented among the many which responded to the inoculation of mice with human insulin. Autoantibodies, on the other hand, are clonally restricted so that differences in clonal representation (restriction) become apparent in binding experiments.

We have demonstrated binding site polymorphism in IAA positive human sera, and found that a particular subset (designated here type B) was virtually never found in association with diabetes. This observation considerably improved the specificity of IAA as a marker for diabetes relatedness without compromising its sensitivity. This is of clinical importance because both insulin-dependent diabetes and IAA cluster in

patients and families with autoimmunity [5, 16]. IAA are present in 3% of patients referred to our open service for autoantibody measurement, and our most recent analysis (unpublished) suggests that IAA may be present in up to 30% of patients with clinical manifestation of two or more autoimmune endocrine disorders other than diabetes.

As both specific clonal responsiveness [17] and Type 1 diabetes risk are genetically determined, we would speculate on some linkage between the genes coding for type A response to insulin and the genes influencing susceptibility to diabetes. Antigen presentation by B lymphocytes may explain the distinct patterns of antibody binding observed, and the finding may have more general application where simple means are available to discriminate antibody subsets serologically. It is well established, for example, that thyroglobulin autoantibody sera are polymorphic, although the clinical significance is uncertain [18].

This study focussed on differences in binding site restriction of disease-specific autoantibodies in a highly organ-specific autoimmune disorder. Disease-specific cross-reactive idiotypes, not necessarily incorporating the antigen binding site of the antibody, have been described in non-organ specific disorders such as systemic lupus erythematosus [19]. These cross-reactive idiotypes are also clonally restricted and it is possible that they correspond to, or are analogous to, the binding site polymorphisms we describe here.

Acknowledgements. TJW is generously supported by the Wellcome Trust. We are grateful to Dr. R.D.G. Leslie for access to sera from discordant twins, to Drs. Betts, Brooks, Cove and Leatherdale for diabetic sera and to Mrs. W. Couper for preparing the manuscript.

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Received: 7 April 1988
and in revised form: 25 July 1988

Dr. T.J. Wilkin
Endocrine Section
Department of Medicine 2
Level D, South Block
General Hospital
Southampton S09 4XY
UK