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# Antigenicity of the carboxyl terminus of insulin: isolation of human insulin-specific monoclonal antibodies

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## SUMMARY

Monoclonal technology was used to isolate antibodies binding the B30 (carboxy) terminal residue in the polyclonal-provoked immune response to human insulin. Although both spleen and lymph node cell fusions were carried out, only the latter were successful in isolating monoclonal antibodies that bound the carboxy terminal of human insulin. The binding of such antibodies was abolished or diminished by substitutions of the B30 residue. Studies with insulin species variants showed that the molecular binding between antibody and insulin may be critically determined by a subresidue feature, e.g. presence or absence of a single methyl group, as shown by the binding of the monoclonal antibody D10 to human insulin (threonine at B30) but not to rabbit insulin (serine at B30). Such studies are of interest in the study of the molecular basis of antibody–antigen interaction.


## INTRODUCTION

Study of the molecular basis of antibody–antigen binding has been facilitated by a group of well-defined proteins (Benjamin *et al.*, 1984). Among this group, insulin has been used to investigate the effect of amino acid substitutions on protein immunogenicity (Keck, 1975; Barcinsky & Rosenthal, 1977) and epitope antigenicity (Schroer *et al.*, 1983; Storch *et al.*, 1985; Rathjen & Underwood, 1986). Insulin is a globular protein of well-known primary and tertiary structure (Blundell *et al.*, 1972) and consists of two intertwined chains linked at two sites by disulphide bridges. Within the A chain, a loop region is formed by an intrachain disulphide bridge between the cysteines at residues 6 and 11. The A chain is further organized into two helical portions on either side of the A chain loop. The amino and carboxyl termini of the B chain are arranged into pleated sheets joined by a central helical region.

Considerable homology in amino acid sequence exists between the mammalian insulins (Fig. 1). For most of these, the variable residues are located at residues 8–10 on the A chain and residues 29–30 at the carboxy-terminal of the B chain. Species variants of insulin therefore allow the study of the effects of specific amino acid exchanges on the immunogenicity of insulin and study of epitope restriction in the insulin immune response. Antigenic determinants on the insulin molecule have been studied by a number of groups using monoclonal antibodies. Schroer *et al.* (1983) identified two antigenic determinants on insulin, one involving the residues 8–10 on the A chain loop and another involving residues from both chains. Other determinants on both conserved and variable regions of the insulin molecule have been found and monoclonal antibodies have been isolated which bind to isolated A and B chains or to epitopes made up of both chains (Rathjen & Underwood, 1986).

The B27–B30 region of the insulin molecule has been implicated recently as a further antigen site (Storch *et al.*, 1985). Evidence that this region may be immunogenic has come from sera containing human insulin-specific autoantibodies (Wilkin & Nicholson, 1984; Diaz & Wilkin, 1987). These sera are able to discriminate between human and porcine insulins which differ by a single amino acid residue at B30. The B27–B30 region of insulin is immunologically interesting because it represents the C terminal end of the B chain, and it is already known that the C and N termini of synthetic peptides generally show increased immunogenicity compared with the rest of the peptide (Thornton & Sibanda, 1983; Williams & Moore, 1985).

The aim of this study was to use monoclonal technology to seek antibodies binding the B30 terminal residues in the polyclonal provoked immune response to insulin as evidence for the antigenicity of the C terminal region of the insulin molecule. Human insulin was used to study the influence of the B30 C terminal end on the insulin antibody repertoire of BALB/c mice. Although both spleen cell and lymph node cell fusions were carried out, only the latter were successful in generating monoclonal antibodies that bound the C terminal region of human insulin. The binding of such antibodies was abolished or diminished by substitutions of the B30 residue. A novel finding was the isolation of a monoclonal antibody that was able to bind to human insulin but not to rabbit insulin. These two insulins differ by a single methyl group on the B30 residue. The factors contributing to the antigenicity of this region are discussed.

Abbreviations: BSA, bovine serum albumin; HAT, hypoxanthine, aminopterin, thymidine selection medium; PEG, polyethylene glycol.

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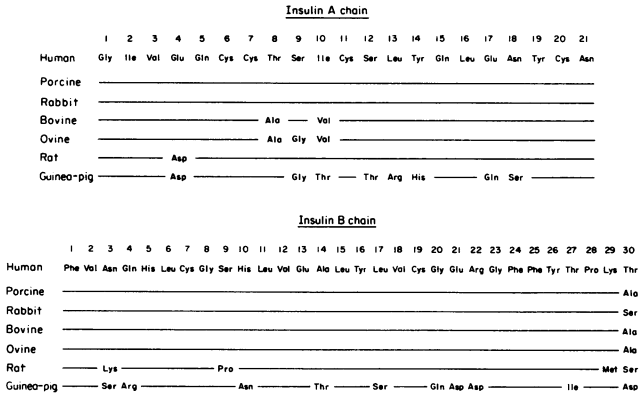


**Figure 1.** Amino acid sequences of insulins from different species.

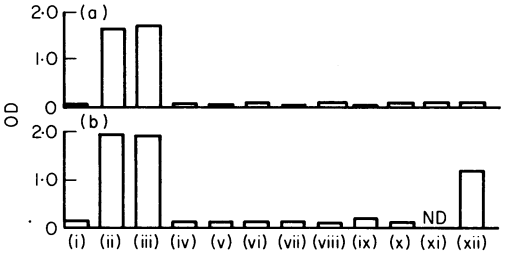


**Figure 3.** Binding of (a) D10 and (b) 3.15.G9.D8 monoclonal antibodies to a panel of insulin variants. Significant binding by both monoclonal antibodies is seen only with human insulin and human insulin B chain implicating the requirement of B30 threonine in the epitope. Insulin variants used: (i) human proinsulin; (ii) human; (iii) human B chain; (iv) porcine; (v) porcine B chain; (vi) desalanated porcine; (vii) bovine; (viii) guinea-pig; (ix) ovine; (x) porcine A chain; (xi) rat; (xii) rabbit.

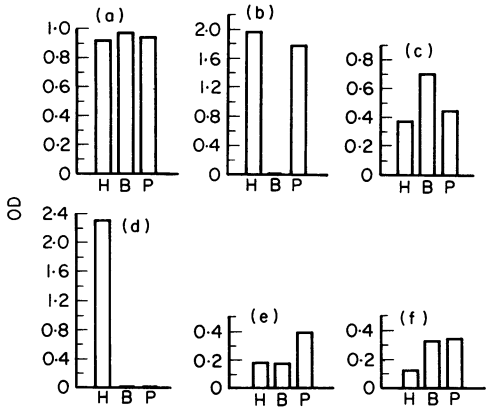


**Figure 2.** Spectrum of antibody specificities as measured by binding against human (H), bovine (B) and porcine (P) insulins. (a) Human, bovine and porcine; (b) human and porcine only; (c) bovine predominant; (d) human specific; (e) porcine predominant; (f) porcine and bovine predominant. Numbers of each type of monoclonal antibody in a group of 49 clones isolated from two lymph node cell fusions: (a) 36; (b) 2; (c) 2; (d) 2; (e) 2; (f) 4.

## MATERIALS AND METHODS

*Immunization protocols*

*Spleen cell fusions.* Female BALB/c mice (Animal House, Tenovus Laboratory, Southampton, Hants) were given subcutaneous injections of 50 μg human monocomponent soluble insulin (Novo, Copenhagen, Denmark) in complete Freund's adjuvant (Difco, Detroit, MI). One or more boosters of human monocomponent insulin were given in incomplete Freund's adjuvant subcutaneously at 2-week intervals. A final boost of 50 μg human insulin in phosphate-buffered saline (0·5 ml) was given intraperitoneally 4 days before removing the spleen for cell hybridization. The final boost was accompanied by an intraperitoneal injection (0·5 ml) of 10% glucose to prevent any hypoglycaemic attack.

*Lymph node fusions.* Ten female BALB/c mice were immunized with 20 μg human monocomponent insulin in complete Freund's adjuvant (0·1 ml) distributed equally between the rear footpads. On Day 14, the draining lymph node (popliteal and inguinal) were removed and pooled. There was no ulceration or gross swelling of the footpads that may have caused distress to the animals and the activity of the animals was not compromised in any way.

*Cell hybridization and cloning of hybrid cell lines*

The myeloma cell line NSO was used for cell hybridization (Galfre & Milstein, 1981). 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% PEG 4000 over 1 min. After a 90-second incubation at 37°, the PEG was diluted out by the drop-wise addition of serum-free RPMI and cells gently spun down. Supernatant was aspirated, the cells resuspended in HAT selection medium (Flow Labs, Irvine, Ayrshire) and plated out onto four 96-well tissue culture plates with a macrophage feeder layer. Cells were fed with fresh HAT medium 7 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 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 (Wilkin, Nicholson & Casey, 1985). Briefly, the wells of an ELISA plate (Alpha Labs, Eastleigh, Hants) were coated with highly purified human, bovine and porcine insulin and other insulin variants (Eli Lilly, Indianapolis, IN) at 2·5 μg/ml in carbonate/bicarbonate buffer pH 9·6 at 4° overnight. Wells were washed three times with phosphate saline buffer + 0·05% Tween (PBST) and refilled with 200 μl of cell culture supernatant. Following a 2-hour incubation at room temperature and further washing, rabbit anti-mouse Ig (polyclonal) conjugated to horseradish peroxidase (Dako, High Wycombe, Bucks) was added at 1:1000 dilution in PBS + 1% BSA (Sigma, Poole, Dorset) + 1% normal rabbit serum and left at 4° overnight. The assay was

developed by the addition of o'phenylenediamine (Sigma). Colourimetric reaction was stopped with 34% $H_2SO_4$ and the colour intensity measured in a spectrophotometer (Kontron ST10) at 490 nm.

*Further expansion of the hybridoma colonies*

Hybridoma colonies were recloned twice by limiting dilution to ensure monoclonality and genetic stability before further expansion in 250-ml flasks using HAT medium.

*Isotyping assay*

A solid-phase ELISA capture assay was used to determine the subclass of monoclonal antibodies. Wells of an ELISA plate were coated with polyclonal rabbit anti-mouse IgG1, IgG2a, IgGb, IgG3, IgM (a gift of Dr M. Glennie, Tenovus Labs, Southampton, Hants) at 50 μg/ml in carbonate/bicarbonate buffer with an overnight incubation at 4°. After washing with PBST, wells were blocked with PBS+1% BSA for 1 hr at 37°. Wells were washed again and refilled with 200 l of culture supernatant containing monoclonal antibody at 1 : 500 dilution in PBS. Following a 9-min incubation at 37° and a further washing step, rabbit anti-mouse Ig (polyclonal) conjugated to horseradish peroxidase was added at 1:1000 dilution in PBS+1% BSA+1% normal rabbit serum and plate was incubated for 90 min at 37°. The plate was again washed and developed as previously described for the ELISA screening assay.

*Absorption studies*

The relative affinities of monoclonal antibodies to insulin variants were studied by using insulin coupled to Sepharose 4B beads (Pharmacia, Uppsala, Sweden). Culture cell supernatant containing monoclonal antibody was diluted 1 : 5 in PBS and incubated with increasing amounts of insulin variants coupled to sepharose beads in BSA-blocked Eppendorf tubes (Axen, Porath & Ernback, 1967). The protein coupled was qauntified by the method of Lowry *et al.* (1951). The total mass of Sepharose beads was kept constant. After an overnight incubation at 4° with end/end rotation, beads were spun down and the supernatant aspirated. The activity against human insulin remaining in the supernatant was measured by ELISA and expressed relative to that remaining after incubation with a given mass of uncoupled beads only.

## RESULTS

### Spleen cell fusions

Fusions of spleen cells using human insulin resulted in low specific fusion frequencies of hybridomas that secreted antibodies showing low binding reactions to human insulin when screened on a solid-phase ELISA.

### Lymph node fusions

In contrast, lymph node cell fusions resulted in higher specific fusion frequencies with higher binding signals in ELISA. Hybridomas producing insulin monoclonal antibodies were cloned by limiting dilution and further tested for insulin specificity by screening against human, bovine and porcine insulins. A spectrum of specificities were observed, namely (i)

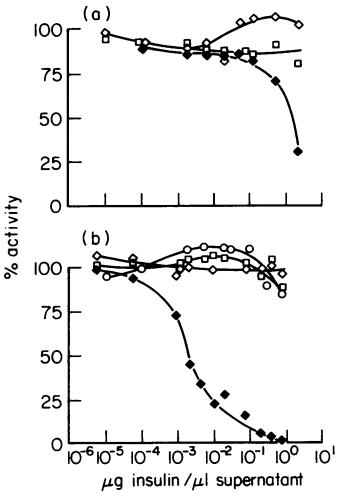


**Figure 4.** Immunospecific absorption of activity of monoclonals (a) 3.15.G9.D8 and (b) D10 with increasing amounts of insulin coupled to Sepharose beads. After incubation with insulin-coupled beads, the resulting antibody activity was measured against human insulin in ELISA. This activity was expressed as a percentage of the signal obtained after incubation of antibody with Sepharose beads blocked with BSA but not coupled to insulin. Absorption with: human insulin (♦); porcine (◊); bovine (□); desalanated insulin (○); ODs at 100% activity: 3.15.G9.D8, 0.597; D10, 1.20.

equal binding to human, bovine and porcine insulin in 36 out of 49 clones from two fusions; (ii) human and porcine predominant (3/49); (iii) bovine predominant (2/49); (iv) human specific (2/49); (v) porcine predominant (2/49); and (vi) porcine and bovine predominant (4/49). The majority of the lymph node-derived hybridomas secreted monoclonal antibodies of type (i). The different binding profiles are shown in Fig. 2. Monoclonals of type (iii), (v) and (vi) 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) (data for publication).

Out of two independent lymph node fusions, two human insulin-specific monoclonals were isolated. These were subtyped as IgG2b and IgM and their binding against a panel of insulin variants is shown in Fig. 3. Neither of these monoclonal antibodies bound bovine or porcine insulins, which differ from human only at B30. Absence of a B30 residue, as in desalanated insulin, also abolished binding. Both antibodies bound to isolated human B chain but not A chain. Furthermore, there was no binding to human proinsulin in which the B30 residue threonine is joined to A1 by a connecting peptide. The presence of the C peptide appeared to mask the epitope of these two antibodies, the data together suggesting that the epitope was on the B chain of human insulin and critically dependent on the B30 residue threonine. One of these antibodies (3.15G9D8) also showed reduced binding to rabbit insulin, which was serine at B30 instead of threonine. Monoclonal antibody D10, however, did not bind to rabbit insulin (Fig. 3). Its binding was therefore inhibited by the presence of an extra methyl group at the B30 residue serine (Fig. 3).

In order to characterize this epitope further, a solid-phase absorption assay was devised to measure the relative affinity for different insulin variants. Any antibody activity remaining after incubation with insulin variants was expressed relative to the

maximum obtainable signal, i.e. activity remaining after incubation with a fixed amount of protein-blocked but insulin-uncoated beads. Absorption curves for the monoclonals D10 and 3-15.G9.D8 are shown in Fig. 4. The A50 value of each absorption curve (i.e. the insulin concentration required to absorb 50% of antibody activity) was used to provide a measure of relative affinity of these antibodies for different insulins.

The ratio of the A50 for each insulin variant to the A50 for human insulin correlated with differences in amino acid sequences of the insulin variants (Fig. 1). This confirmed that both monoclonal antibodies were directed against the residue B30 threonine, since both bound five to 100-fold less strongly to bovine and porcine insulins which have alanine at B30, than to human.

## DISCUSSION

Human insulin-specific antibodies are known to occur in autoimmune individuals (Wilkin & Nicholson, 1984; Diaz & Wilkin, 1987). Such antibodies bind to insulin when the B30 residue is threonine, but not when it is alanine. This observation led us to investigate, using monoclonal antibodies, the contribution of the B30 carboxyl terminal residue to the antigenicity of insulin. In order to maximize the diversity of monoclonal antibodies in the analysis, both spleen and lymph node cell fusions were examined. After immunization of BALB/c mice with human insulin, a wide spectrum of antibody specificities was seen. Two independent fusions produced two B30 threonine (human insulin)-specific monoclonal antibodies. A number of heteroclitic antibodies was produced as well as others which bound predominantly to human and porcine insulins.

The isolation of IgG and IgM antibodies dependent on an epitope involving the terminal carboxyl residue of a globular protein such as insulin is interesting because it correlates with the observations of increased immunogenicity of the C and N terminals seen with synthetic peptide and other globular proteins (Williams & Moore, 1985). The antigenicity of the B30 terminal may be related to the increased mobility of this region. Its highly flexible and increased segmental mobility has been shown to play an important part in antigenicity (Tainer *et al.*, 1985). The antigenicity of a protein region is also correlated with such factors as surface accessibility and variability of residues in comparison to evolutionary related proteins (Berzofsky, 1985). The carboxy terminal (B30) residue of insulin is also surface accessible and subject to frequent evolutionary mutation. The isolation of monoclonals critically dependent upon the presence of B30 threonine contributes to our understanding of the molecular basis of antibody–antigen binding because it provides evidence that a single amino acid can determine the antigenicity of a protein and suggests that the footprint theory of antibody–antigen binding might be extended to include the notion of 'grip' dependent on a single feature of the footprint. Thus, although an antibody may bind an epitope spanning two to four amino acid residues (Marks, Yip & Wilson, 1985), a change in a single amino acid (e.g. threonine, alanine, serine), or its absence as in desalanated insulin, may cause the antibody to lose 'grip', manifested as a loss of affinity. Furthermore, molecular binding between antibody and antigen may be critically determined by a subresidue feature, e.g. presence or absence of a single methyl group, as shown by the binding of monoclonal antibody D10 to human insulin (threonine at B30), but no binding to rabbit insulin (serine at B30).

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