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Insulin antibodies induced by bovine insulin therapy

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SUMMARY

Insulin antibodies are detectable in the sera of most patients within 3–4 months of starting treatment with conventional bovine insulins. Various factors determine the immunogenicity of insulin preparations including the genetic background of the recipient. This causes wide variation in response. Solid phase absorption studies as well as competitive binding in fluid phase indicate that the antibodies formed are almost always specific for determinants shared by the endogenous human molecule. This has important implications for the metabolic effect of insulin antibodies *in vivo* as well as for mechanisms of autoantibody production in man.

INTRODUCTION

When injections of bovine insulin first became available for the treatment of diabetes, the incidence of allergic manifestations was much less than many had feared (Reeves, 1980). The size and chemical nature of the insulin molecule led some to doubt its immunogenicity in unmodified form (Clutton, Harington & Yuill, 1938). Bovine insulin differs from the endogenous human molecule by only three out of a total of 51 amino acid residues (Fig. 1), and it was not until antisera were raised by immunizing animals possessing marked species differences (e.g. the guinea-pig) that a precipitating antiserum was obtained (Birkinshaw, Randall & Risdall, 1962). However, acute allergic reactions as well as immunologically-mediated resistance to the metabolic effect of insulin were soon recognized as rare but important complications of bovine insulin therapy (Witters *et al.*, 1977).

Soluble injections of bovine insulin have a short duration of action and various methods have been devised to prolong the metabolic effect e.g. increasing the zinc concentration or complexing with protamine (Sönksen, 1977). This has led to many different bovine insulin formulations and it is important to distinguish these when examining the effect of different physico-chemical factors on the immunogenicity of the native molecule. Conventional bovine insulin preparations are purified by sequential recrystallization and are not rendered free of contaminating proinsulin (see Reeves & Douglas, 1982). The preparations referred to in this paper are soluble, isophane (complexed with protamine), lente (high zinc level) and protamine zinc insulins. The latter (PZI) contains insulin complexed with protamine plus a high zinc concentration.

Various methods have been used to detect insulin antibody but relatively few have characterized the antibody immunochemically and some methods are prone to interference by the presence of free insulin in the test serum (Reeves, 1980). We have used a second antibody co-precipitation assay specific for IgG antibody with removal of free insulin prior to testing (Reeves & Kelly, 1980). Care is needed to ensure that the radio-iodinated insulin has been labelled optimally without physico-

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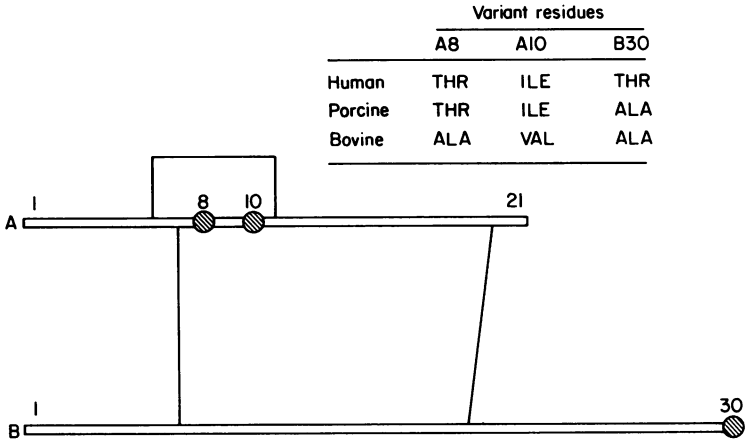

Fig. 1. Structure of the insulin molecule. Disulphide bridges linking A + B chains as well as the intra-chain bridge linking A6 and A11 are shown and amino acid variations between bovine, porcine and human molecules are also indicated (ALA = alanine, ILE = isoleucine, THR = threonine and VAL = valine).

chemical alteration. The use of ^{22}Na as a volume marker obviates the need to wash precipitates and gives a precise and sensitive method.

MATERIALS AND METHODS

Sera. Test sera were separated promptly and stored at −20°C or lower. They were obtained from patients who had been established on specified bovine insulin regimes i.e. isophane, soluble + isophane, lente or soluble + protamine zinc insulins. Data for sera obtained from patients treated with highly purified porcine insulins are also included for comparison.

Reagents. Highly purified bovine and human insulins were obtained from the Novo Research Institute, Copenhagen. Monospecific IgG fractionated rabbit anti-human IgG was obtained from Dako Immunoglobulins Ltd., Copenhagen.

Insulin antibody determination. This was performed according to our previously described method (Reeves & Kelly, 1980) with the following modifications. Positive and negative quality control sera were used in each assay batch to validate the performance of the ^{125}I-labelled insulins which were prepared using a low level chloramine t method. Tests were performed in duplicate and results expressed as percentage bound.

Solid phase adsorption studies. Highly purified bovine insulin, human insulin and ovalbumin (Sigma) were linked to CH-sepharose (Pharmacia). Six milligrams of protein was incubated with 100 mg (dry weight) CH-sepharose at pH 8. Dextran–charcoal adsorbed sera (Reeves & Kelly, 1980) were then incubated overnight with the conjugated sepharose prior to determination of the insulin binding of these adsorbed sera.

Fluid phase competitive binding studies. Different concentrations of unlabelled bovine or human insulin were added to 0·25 ng aliquots of ^{125}I-insulin prior to determination of insulin binding in the usual way. The reduction in percentage binding of the labelled insulin was then plotted against increasing concentrations of the cold competitor.

RESULTS

Levels of antibody reactive with labelled bovine insulin determined for five different serum groups are shown in Fig. 2 and contrasted with data for 12 normal sera. Each of the four bovine insulin regimes induce a wide range of insulin antibody levels although none of the sera from patients on

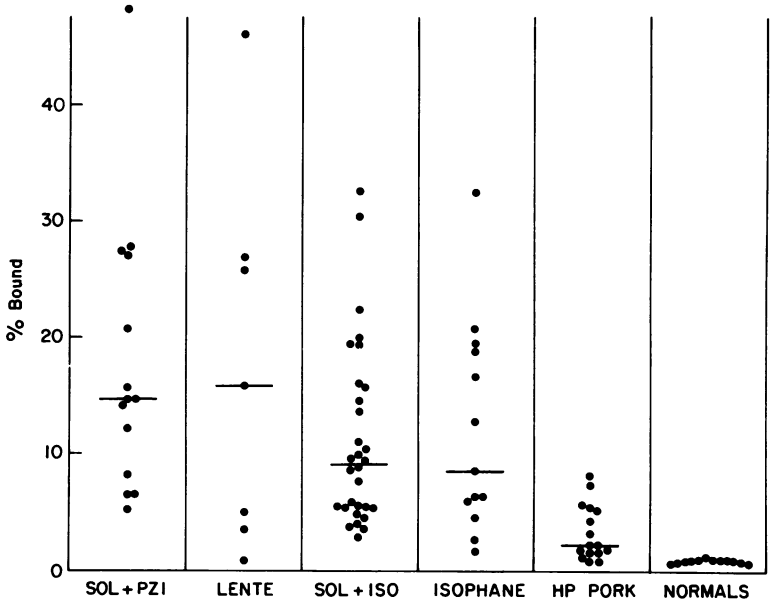

Fig. 2. Levels of antibody reactive with labelled bovine insulin in sera from patients established on four different bovine insulin regimes: soluble+protamine zinc insulins (SOL+PZI); lente; soluble+isophane (SOL+ISO) and isophane insulins compared with 16 patients receiving highly purified (HP) pork insulins (11 isophane+five lente) and normal sera.

soluble+protamine zinc insulins show $< 5\%$ binding. A minority of patients receiving other bovine insulins show levels below this; in some instances overlapping with those seen with normal sera. Binding levels for 16 sera from patients receiving highly purified porcine insulins (in isophane or lente formulation) are included for comparison. The considerable variation in response to a standard bovine insulin regime is illustrated in Fig. 3 which contains data for 127 patients treated with soluble and isophane insulins. Some sera show levels indistinguishable from normal whereas others extend as high as 54% binding.

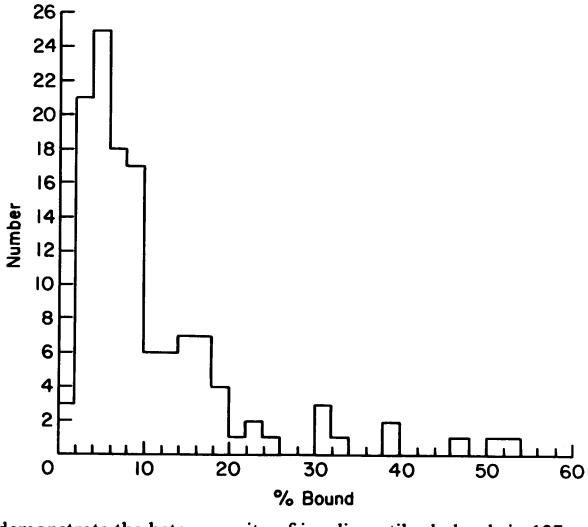

Fig. 3. Histogram to demonstrate the heterogeneity of insulin antibody levels in 127 patients established on a combination of bovine soluble and isophane insulins.

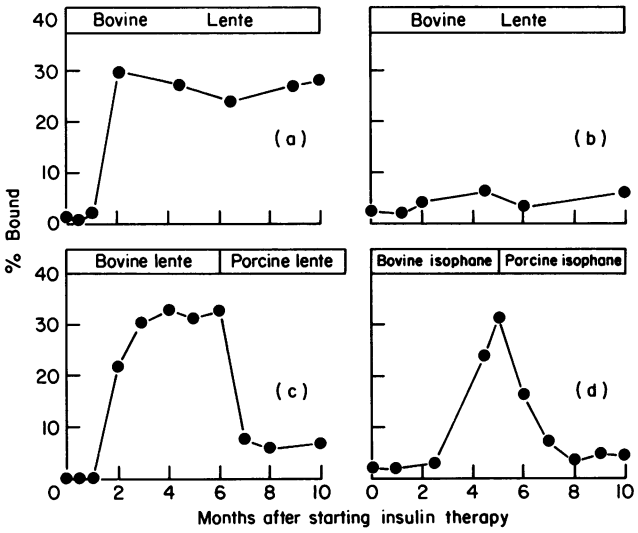

Fig. 4. Development of antibody reactive with bovine insulin in four patients treated initially with ordinary bovine insulins. Patients (a) and (b) were both treated with bovine lente once daily. Patients (c) and (d) were started on bovine lente and bovine isophane respectively and then switched to highly purified porcine equivalents.

Kinetics of antibody production

Antibodies are usually detectable within 2–4 months of commencing insulin injections (Fig. 4a, c & d) and reach a plateau level within 3–6 months of continuous therapy (Fig. 4a & c). However, some patients produce little or no response whatsoever (Fig. 4b). If the insulin is changed to a highly purified porcine equivalent then the antibody levels of responder patients fall back toward the normal range fairly promptly (Fig. 4c and d).

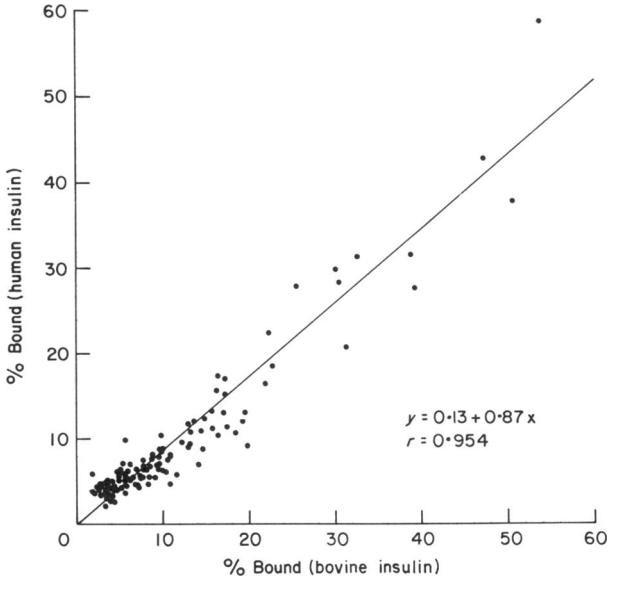

Fig. 5. Plot of binding of 127 sera from patients on bovine soluble and isophane insulins for bovine and human insulins.

Specificity

Fig. 5 correlates the binding of highly purified human and bovine insulins for the same 127 sera depicted in Fig. 3 and demonstrates the marked similarity in the binding levels of individual sera for these two ligands. In order to determine whether these sera contain two populations of antibody reactive with human and bovine insulins respectively or a single population reactive with determinants common to both species variants, adsorption studies were performed using human insulin, bovine insulin and ovalbumin (as control protein) linked to CH-sepharose. Table 1 sets out the binding levels of three sera for labelled bovine and human insulins respectively after adsorption with each of these three solid phase linked proteins. Sera A and B are typical in showing comparable

Table 1. IgG binding levels for bovine and human insulins measured in sera A, B and C after adsorption with ovalbumin, bovine insulin or human insulin linked to CH-sepharose. The values for an unadsorbed normal human serum are given for comparison

Serum	Sepharose-linked protein used for adsorption	Insulin binding	
		Bovine (%)	Human (%)
A	Ovalbumin	32·0	20·9
	Bovine insulin	0·4	1·2
	Human insulin	1·5	1·2
B	Ovalbumin	28·9	20·6
	Bovine insulin	0·02	1·3
	Human insulin	1·5	0·9
C	Ovalbumin	55·8	8·6
	Bovine insulin	0·5	2·7
	Human insulin	3·8	1·4
NHS	—	1·0	3·1

levels of binding to bovine and human insulins before and after adsorption with ovalbumin. When either of these sera are adsorbed with bovine insulin, all the reactivity for labelled bovine or human insulin is removed and a similar result is produced by adsorption with human insulin on solid phase. Although comparable binding to human and bovine insulins applies to the very large majority of sera from patients immunized with bovine insulins, occasional individuals who have developed insulin resistance do show preferential binding to bovine insulin (e.g. serum C, Table 1). Even with this serum, however, adsorption with human insulin removes almost all the reactivity against labelled bovine material and vice versa.

In order to analyse the degree of cross-reactivity more closely, liquid phase competitive binding studies were performed in which a range of doses of cold bovine or human insulins were added to aliquots of sera A, B or C in the presence of a standard amount of radio-labelled bovine insulin. Fig. 6 indicates that there is no significant difference in the ability of unlabelled 'cold' bovine or human insulin to inhibit the binding of labelled bovine insulin to sera A or B whereas with the atypical serum C human insulin is 200 times less effective than its bovine counterpart.

DISCUSSION

A majority of patients treated with conventional bovine insulin develop insulin antibody and a minority of patients receiving highly purified porcine insulin produce antibody of similar specificity (Fig. 2). A combination of soluble and PZI insulins is probably the most immunogenic regime, with little difference between lente, isophane and soluble + isophane insulins in terms of the range of

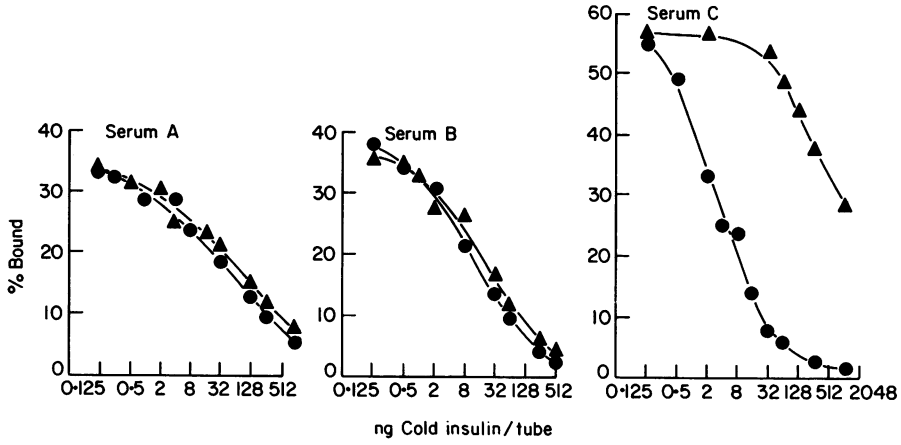

Fig. 6. Competitive binding curves obtained using sera A, B and C (specified in the text and Table 1). Aliquots of serum were incubated with 0·25 ng of ^{125}I-bovine insulin in the presence of different concentrations of unlabelled bovine or human insulin as indicated on the abscissa.

antibody levels produced. However, the highest levels of insulin antibody are usually found in patients with insulin resistance i.e. requiring more than 120 units of insulin per day (Reeves, 1980) and patients with injection site lipo-atrophy show higher levels of insulin antibody than those without this complication (Reeves, Allen & Tattersall, 1980).

Figs 2, 3 & 4 demonstrate that sera from some patients show binding levels indistinguishable from normal sera. The distribution data for 127 patients receiving soluble and isophane insulins (Fig. 3) are suggestive of a bimodal distribution. It is debatable whether such a group of patients should be divided into those who are 'unresponsive' vs those who are 'responsive' or into those with 'low' and 'high' responsiveness. Accumulating evidence indicates that this constitutional variation in response is linked to HLA phenotype (Rotter, 1981). Patients possessing the B8/DR3 haplotype are much more likely to show a reduced response to bovine insulin although whether there is a 'hyper-responsive' haplotype is less clear. The insulin antibody method used, its sensitivity and the threshold at which the cut-off point is set, are of importance in determining such associations. The ability to develop insulin antibody associates with major histocompatibility genes in other species e.g. mice and guinea-pigs (Keck, 1975; Barcinski & Rosenthal, 1977).

The fact that 2–3 months elapses before antibody is readily detectable in responding patients may reflect the somewhat unconventional mode of immunization i.e. subcutaneous injections once or twice daily in the absence of adjuvant. Experimentally, it is possible to inhibit responsiveness completely by the administration of frequent small doses of otherwise immunogenic insulins (Kerp *et al.*, 1970; Menzel *et al.*, 1971) and breaks in bovine insulin therapy are often followed by the development of high levels of insulin antibody and the development of insulin resistance. The fall in antibody level on switching to a less immunogenic insulin usually takes place over a period of several months (Fig. 4c & d). We have recently demonstrated that such falls in antibody are related to a change in insulin dose requirement and that an increase in antibody follows rechallenge with the more immunogenic regime (Walford, Allison & Reeves, 1982).

At least two changes occur when conventional recrystallized bovine insulin is switched to porcine insulin rendered highly purified by chromatographic techniques: a species change and a considerable reduction in contamination with other islet peptides. The precursor protein, proinsulin, is present in conventional bovine insulin preparations to a level of $\geq$ 10,000 p.p.m. (i.e. $\geq$ 1%) and its connecting or C-peptide contains 14 residue differences from the human sequence. Thus, differences within both insulin and C-peptide sequences may be important in determining the immunogenicity of conventional bovine preparations. Inbred strains of mice unresponsive to purified insulin, even in the presence of adjuvant, can produce antibodies reactive with insulin following immunization with the corresponding proinsulin molecule (Kapp & Strayer, 1978). The

production of antibodies to C-peptide are considered in the accompanying paper (Reeves & Douglas, 1982).

The observation that a minority of patients produce insulin antibody following injection with highly purified porcine insulins, which are effectively free of proinsulin contamination and in which there is only a single amino acid residue difference from the human molecule, suggests that physico-chemical factors may be important. The immunogenicity of insulin can be affected by pH, zinc concentration, aggregation and deamidation (Berson & Yalow, 1966; Arquilla *et al.*, 1978; Rosenthal *et al.*, 1980; Hansen, Nielsen & Welinder, 1981) and this may explain the development of insulin antibodies following the injection of homologous insulin in sheep, cows and humans (Deckert *et al.*, 1972; Neubauer & Schone, 1978).

It has often been proposed that insulin antibodies bind preferentially to regions of the bovine insulin molecule where its sequence differs from human insulin (Kumar, 1979; Irvine, 1980). The experiments outlined here, as well as studies using a despentapeptide preparation (Reeves & Dodson, 1981), do not confirm this indicating that antibodies induced by bovine insulin react with determinants shared by the human molecule. A few sera from patients with insulin resistance show preferential binding to the bovine molecule although still retaining partial cross-reactivity for human insulin (Fig. 6). The appearance of antibody reactive with the endogenous molecule following immunization with heterologous insulin has also been demonstrated in the guinea-pig and rabbit (Barcinski & Rosenthal, 1977; Keck *et al.*, 1980). Reduction in the remission period of insulin-dependent diabetes as well as interference with insulin–receptor interaction (Andersen, 1976; de Pirro *et al.*, 1980) suggest that these autoreactive insulin antibodies may cause important metabolic effects *in vivo*. Such antibodies are able to sequester and/or inhibit considerable quantities of injected insulin in the insulin-dependent diabetic (Bolinger *et al.*, 1964; Rendell *et al.*, 1981; Vaughan *et al.*, 1981).

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