Immunological properties and biological effectiveness of insulin analogues substituted at position B30

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Summary. The aim of this investigation was to assess the immunological properties of several insulin analogues by examining both their antigenic and immunogenic behaviour. In addition, the hypoglycaemic activity was also determined and compared with values obtained with natural insulins. The modified insulin preparations were of porcine and bovine origin. All analogues had in common the fact that the alanine B30 had been exchanged by either leucine, threonine, tyrosine, phenylalanine or glycine. The blood glucose-lowering activity was determined in rabbits and dogs, while the stimulation of antibody development was studied in three different animal species; pigs, dogs and rabbits. The antigenic properties of analogues were evaluated in vitro by measuring their

binding capacity to pre-formed antibodies. In all cases the blood glucose lowering activity of the analogues was comparable to that of the respective natural insulin. There were remarkable differences in the binding capacity to pre-formed antibodies, with bovine Leu-B30 insulin competing only to 73% with the natural insulin tracer. With regard to antibody development, the analogues behaved similarly to the original hormones. These results show that there is little correlation between the antigenic make-up of the insulin molecule and its ability to provoke antibody stimulation

Key words: Insulin analogues, immunogenicity, antigenicity, efficacy

Compared with its porcine counterpart, beef insulin is distinguished by a rather high degree of immunogenicity [1–3]. The A-chain loop is believed to be responsible for this phenomenon, i.e. the main carrier determinants are presumably located in this molecular region [4]. However the immunological importance of other sites of the insulin molecule has also been reported [5–7].

Contrary to earlier statements [8], Kobayashi et al. [9] attributed a reduced antigenic activity to Leu-B30 insulin as well as to other B30 analogues, even though the original biological activity is maintained. The present investigation was designed to study the dependency of substitutions in position B30 on both the antigenicity and immunogenicity of the insulin molecule.

Materials and methods

Besides the original insulins, two analogues of beef insulin and six analogues of pork insulin were at our disposal. In the preparations, the B30 position was substituted with either threonine, phenylalanine, valine, leucine, glycine or tyrosine; in one case des-alanine B30 was employed. All insulin preparations were crystallized and thereafter chro-

matographed on a molecular sieve. In addition, another purification step in the form of ion exchange followed. To map out the biological properties of these novel substances, both the blood glucose lowering effect and the immunological behaviour were assessed and the data obtained compared with those of natural insulin preparations.

Domestic rabbits with an average weight of 2.2 kg and young adult beagle dogs were used for determining the blood glucose lowering activity. Following the subcutaneous administration of the respective insulin preparation, blood samples were collected at periodic intervals and, subsequently, glucose values were measured by means of the hexokinase method using a commercial kit (Boehringer, Mannheim). The immunological characteristics of the analogues were investigated by using two different approaches. To study the immunogenic behaviour, three different species (pigs, rabbits and dogs) served as experimental animals. Following the initial administration of 1 IU/kg body weight, weekly booster doses of a logarithmically reduced amount of the preparation was given subcutaneously. Starting from week 6 until the end of the 13-week trial, the uniform doses amounted to 0.2 IU/kg. The initial and following four booster doses were mixed with equal volumes of complete Freund's adjuvant immediately prior to the injections. Accordingly, control groups of experimental animals received the respective original insulin at the same time and in the same dosage.

The antigenicity of the insulin preparations was also evaluated in a model in vitro. Varying amounts of the analogues were added to an established system of labelled pork insulin and preformed anti-insulin antibodies. The degree of inhibition of tracer binding was used as parameter of antigenicity of the respective analogue.

Fig. 1. Blood glucose concentrations in rabbits after injection of original and modified pork insulins. Dissolved, chromatographed pork insulin (●—●), porcine Leu-B30 insulin (○—○), porcine Tyr-B30 insulin (■—■) and porcine Phe-B30 insulin (▽—▽) were administered subcutaneously in doses of 0.2 IU/kg body weight. Each experimental group consisted of five animals and the results are expressed as mean $\pm$ SEM

Statistical analyses

Immunogenicity and blood glucose lowering activities are reported as mean values $\pm$ SD or SEM. Statistical significance was calculated by two-tailed Student's *t*-test.

Results

The blood glucose-lowering efficacy of the analogues is reflected in one representative graph (Fig. 1). In rabbits both the original and the modified insulins caused a decrease of the blood glucose levels of approximately 2.0–2.5 mmol/l within 30 min after subcutaneous administration. Not only the other porcine B30 analogues

but the preparations of bovine origin also acted in a similar manner. As expected, the extent of the blood glucose reduction was not as pronounced in dogs, but again no significant difference between the natural hormones and the respective analogue could be interpreted.

Studies of the immunogenic behaviour revealed similar results, i.e. the individual analogues stimulated antibody development to the same extent as did the respective original hormone. Beef insulin and its two analogues proved to be highly immunogenic, while pork insulin and the seven B30 derivatives thereof were not able to provoke antibody production to any considerable extent – at least not within the 12 weeks of the trial. The bovine Thr-B30 insulin was just as immunogenic in rabbits and dogs as it was in pigs.

The investigations concerning the antigenic properties of B30-substituted insulin analogues yielded somewhat different results. In our *in vitro* system, the degree of competitive binding of the preparations ranged between 73 and 100% (Fig. 2).

The original pork insulin was set at 100%, since the assay system is based on porcine insulin. The values obtained proved that B30 derivatives of the porcine hormones were less antigenic than the original porcine hormone itself. In our assay system, this reduction ranged between 10% and 20%. At first sight, the binding quotas of beef insulin and its analogues to our antibody system surprised because of the low values measured. Beef insulin exhibited only 80% binding capacity, whereas the binding of the B30-Leu analogue showed an even further reduction to 73%. Interestingly, the binding capacity of the B30-Thr compound was more comparable to pork insulin and its analogues than it was to the beef insulin preparations.

Fig. 2. Antigenicity of insulin B30 derivatives expressed by their competitive binding to preformed anti-pork-insulin antibodies (guinea pig origin). Symbols indicate the insulin or analogue concerned: porcine insulin *; bovine insulin ○; Leu-B30 insulin^(b) ●; Thr-B30 insulin^(b) □; Des-Ala-B30 insulin^(p) ■; human insulin (semi-synthetic)^(p) △; Tyr-B30 insulin^(p) ▲; Phe-B30 insulin^(p) ○; Leu-B30 insulin^(p) ●; Gly-B30 insulin^(p) +. Assay system: anti-p-insulin-AB/p-insulin tracer PEG. ^(p) = porcine origin, ^(b) = bovine origin

Discussion

The observation that the antigenicity of certain insulin analogues is diminished agrees with the results of earlier studies reported by different groups. Kerp et al. [5] stated that, in comparison with bovine insulin, the binding capacities of high affinity antibodies against des-Phe-B1 insulin or des-(Phe-Val)B1-2 insulin are reduced by 77% and 76.3% respectively, with a further decrease to 68% for des-(phe-Val-Asn)B1-3 [Pyr-B4] insulin. Similar results with other analogues were published by Geiger et al. [6] and later by Kobayashi et al. [9]. All these studies come to the conclusion that certain changes in the amino-acid sequence may influence antigenic behaviour. With regard to the immunogenic properties of insulin preparations, there is general agreement that species-specificity plays a major part in antibody development. Our findings are in accordance with these earlier reports.

However, simultaneous studies of immunogenicity and antigenicity of the individual analogues prompted us to compare the two properties with each other. It appeared of interest to learn whether or not a correlation between the degree of binding to pre-formed antibodies and stimulation of antibody development does exist. A reduction of binding affinity to pre-formed antibodies does not justify the conclusion that a substance must also be less active with regard to antibody stimulation and vice versa.

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