Radioimmunoassay of Chemically Modified Insulins

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Summary. The reactions between four insulin antisera and eighteen insulin derivatives with modifications at the A₁, B₁ and B₂₉ positions have been studied using a standard double-antibody radioimmunoassay procedure. The derivatives studied had: a) single modifications at A₁, B₁ or B₂₉; b) modifications at two sites with or without a crosslink between them; c) modifications at all three sites with or without a crosslink. Analysis of the results showed a clear difference in the reactivity of the antisera. One antiserum (GP5) was highly sensitive to modifications of the B₁ residue and another (Ab1) was sensitive to A₁ and B₂₉ modifications. Thus, immunological potencies of insulin analogues derived on the basis of these reactions with the antisera give widely varying results. These antisera were used in discriminatory radioimmunoassays of chemically modified insulins in biological fluids for estimation of in vivo hypoglycaemic potencies by an infusion technique, where the knowledge of the specificity of the antisera was useful in assessing the immunological identity of immunoreactive material in plasma with the analogue infused.

Key words: Radioimmunoassay, chemically modified insulins, insulin antiserum specificity, in vivo biological activity.

The elucidation of the three-dimensional structure of insulin [1], and the use of specifically modified insulins have contributed greatly in recent years to the understanding of the relationship between the structure and biological activity of the hormone [2–4].

Freychet et al. [2] made an estimate of the relative potency of a number of insulin derivatives by their ability to displace radio-iodinated insulin from liver plasma membranes. Gliemann and Gammeltoft

[3] derived similar values by measuring their capacity to stimulate lipogenesis in isolated rat fat cells. Jones et al. [4] have shown that the in vivo activities of insulin derivatives are modified by their rate of degradation and thus estimates of biopotency are dependent on the radioimmunoassay of these materials in biological fluids. Estimations of the relative potency of chemically modified insulins have also been made by measuring the ability of these materials to compete with radio-iodinated insulin in binding to insulin antibodies in a radioimmunoassay [5–8].

This paper describes the results of studies made of the reaction between chemically modified insulins and antisera raised in guinea-pigs against either porcine insulin or porcine proinsulin.

Materials and Methods

Insulin and Insulin Derivatives

The reference insulin preparation used in all assays, was crystalline bovine insulin (Brunnengraber Lübeck, 24 IU mg⁻¹). This was starting material for the chemically modified insulins prepared by Brandenburg [9–11]. Bovine insulin was also the starting material for the preparations kindly donated by Lindsay [5, 12–14]. The insulin derivatives studied are listed in Table 1. Standard solutions of insulin and insulin derivatives were prepared for use in the radioimmunoassay by dissolving in 0.05 mol/l of sodium phosphate buffer, pH 7.4, containing 2 g/100 ml human serum albumin at a final concentration of 1.6 µg µl⁻¹ and were stored in small aliquots under nitrogen at –20 °C. ¹²⁵I-iodinated insulin was prepared by the method of Greenwood et al. [15] and purified by gel filtration on a Sephadex G-50 column.

Antisera

Twenty three antisera to porcine insulin and one to porcine proinsulin, raised in guinea-pigs, were tested initially to define the cross-reactivity of the insulin derivatives and insulin. Four of these antisera were then selected for the experiments described in this

Fig. 1a and b. Dilution curves of insulin (■) and $\text{A}_1\text{-B}_{29}$ dodecyl insulin (□). **a** curves obtained using antiserum GP5. **b** curves obtained using antiserum Ab1

paper. Three antisera, GP5, Ab1 and Ab12 had been raised against porcine insulin and the fourth antiserum, PPAB, had been raised against porcine proinsulin. The antisera were stored at -20°C either undiluted or at a dilution of 1:100 in 0.05 mol/l of sodium phosphate buffer, pH 7.4, containing 2 g/100 ml human serum albumin.

Assay Methods

The specificity of each antiserum was determined using the double-antibody radioimmunoassay procedure described by Morgan and Lazarow [16] as modified by Sönksen [17]. In this assay the binding of the hormone to the antibody is studied by measuring its ability to compete with ^{125}I -insulin using a fixed concentration of labelled insulin (approx 0.006 pmol per assay tube) and increasing concentrations of insulin or insulin derivative (0.0013–0.67 pmol per assay tube). Antiserum dilutions were chosen to give approximately 50% binding of labelled insulin in the zero tube (final dilutions: – GP5 – 1:700,000, Ab1 – 1:189,000, Ab12 – 1:217,000 and PPAB – 1:350,000). After a first incubation period of 48 h the guinea-pig immunoglobulin and antibody-bound insulin were precipitated using rabbit anti-guinea-pig immunoglobulin (1:450) with normal guinea-pig serum (1:14,400) as a carrier and incubating for a further 48 h at 4°C . The free and antibody-bound hormone were separated by centrifugation (1,700 g for 30 min at 4°C) and decanting the super-

Fig. 2a and b. Dilution curves of insulin (■) and B_1 -acetoacetyl insulin (□). **a** curves obtained using antiserum GP5. **b** curves obtained using antiserum Ab1

natant. The radioactivity in both the free and bound fractions was measured in a Wallac autogamma counter. The data are presented as plots of the percentage of the total counts in the bound fraction as a function of the hormone concentration.

In vivo biopotency of the chemically modified insulins was measured in anaesthetised greyhounds by a priming dose, constant infusion technique [4]. The relationship between the fall in plasma glucose concentration and the serum hormone concentration, measured by radioimmunoassay, was analysed by the procedure described by Finney [18] for parallel line bioassays. This procedure fits dose-response curves to pooled data for the reference insulin preparation and test preparation and assesses the validity of the data as a bioassay by testing them for linearity, nonparallelism and heterogeneity of variance. If these conditions were met, a potency was calculated for the analogue relative to insulin from the displacement of the curves, using a pooled slope, and 95% confidence limits were derived from the variance of the data.

Results

Dilution curves obtained with three chemically modified insulins using two different antisera are illustrated in Figures 1–3. In each figure the dilution curve of the insulin derivative is compared with that of bovine insulin obtained in the same assay.

Figure 1 shows the dilution curves for an A_1 - B_{29} crosslinked insulin derivative (A_1 - B_{29} dodecoyl insulin). In the assay system using antiserum GP5 (Fig. 1a) the insulin analogue shows an ability to compete with the labelled insulin almost identical to that of the standard preparation. In contrast, the dilution curve obtained for the same analogue in the system using antiserum Ab 1 (Fig. 1b) shows that this antiserum is sensitive to modifications at the A_1 and B_{29} sites. Thus the amount of labelled insulin that is antibody-bound in the presence of the analogue is greater than that bound in the presence of the same concentration of insulin showing that the affinity of the insulin derivative for the antibody was less than that of insulin.

The effect of a B_1 modification to the insulin molecule (B_1 acetoacetyl insulin) is shown in Figure 2. With the antiserum GP5 non-parallel dilution curves were obtained, indicating that this antiserum is sensitive to modifications at the B_1 position. Antiserum Ab 1, however, showed only a very small difference in the equilibrium constant for the binding of this derivative and insulin.

When all three sites of the insulin molecule are modified, as in A_1 , B_1 , B_{29} tricarbamyl insulin, both assay systems, using antiserum GP5 and antiserum Ab 1, show a marked impairment in the binding of the insulin derivative (Fig. 3). This analysis of the reaction between the four antisera used and each of the chemically modified insulins studied is summarised in Table 1.

The bioactivity of ten of the analogues studied was measured and is presented (Table 2) relative to beef insulin, which is given a value of 100%. These were calculated by relating the rate of fall in plasma glucose concentrations to the serum concentrations of the insulin analogues achieved during the experiment [4].

A measure of the immunological potency of the derivatives was obtained with each antiserum used by comparison of the amounts of insulin and derivative needed to cause half maximal displacement of the labelled insulin from the antibody (Table 2).

Discussion

These results show that antisera raised against insulin vary considerably in their ability to recognise modifications of the insulin molecule. Thus one antiserum, GP5, is able to distinguish between insulin and derivatives of insulin with modifications at the B_1 position, while another antiserum Ab 1, is most sensitive to modifications in the region of the A_1 and B_{29} residues. The two other antisera studied in detail showed no clear pattern of sensitivity to A_1 , B_1 or B_{29}

Fig. 3a and b. Dilution curves of insulin (■) and A_1 , B_1 , B_{29} tricarbamyl insulin (□). **a** curves obtained using antiserum GP5. **b** curves obtained using antiserum Ab 1

modifications. Their cross-reactivity with the insulin derivatives, however, did vary; Ab 12 was unable to distinguish between insulin and either B_1 carbamyl insulin or B_1 acetoacetyl insulin, while PPAB could not distinguish between insulin or A_1 acetoacetyl insulin and was more sensitive to the larger modifications of the insulin molecule (Table 2). It is possible that if a larger range of chemically modified insulins were available with changes at sites other than A_1 , B_1 or B_{29} , that the specificity of these two antisera might be more clearly defined. All the antisera studied showed a similar gradation of sensitivity to small and large modifications to the insulin molecule. Antiserum Ab 1, for example, showed small changes in the dilution curves of the derivatives with a single modification, a greater change with the crosslinked derivatives, and was highly sensitive to the derivatives modified in three positions or with modifications which change the three-dimensional structure of the insulin molecule such as A_1 - B_1 Z (LAL) insulin. Recent studies (unpublished data) have shown the high degree of specificity of the binding reaction

Table 1. Reactivity of chemically modified insulins with four different antisera, derived from qualitative analysis of the data. Two characteristics are presented: I – the displacement of the dilution curve of the derivative away from that of insulin (0 = no displacement; + + + = large displacement); II – the parallelism of the dilution curves of insulin and insulin derivative (0 = parallel; + = non-parallel)

Insulin derivative	Reference	Antiserum							
		GP5		Ab 1		Ab 12		PPAB	
		I	II	I	II	I	II	I	II
A ₁ carbamyl insulin	14	0	+	+	+	+	0	0	0
A ₁ acetoacetyl insulin	12	0	0	+	+	+	0	0	0
A ₁ succinyl insulin	^a	+	0	++	+	+	0	+	0
A ₁ thiazolidine insulin	5	+	0	+	+	+	+	+	0
B ₁ carbamyl insulin	14	++	+	+	0	0	0	+	0
B ₁ acetoacetyl insulin	12	++	+	+	0	0	0	+	0
B ₂₉ succinyl insulin	^a	+	0	+	+	++	0	+	0
A ₁ , B ₂₉ diacetyl insulin	9	0	0	+	+	0	0	0	0
A ₁ , B ₁ , B ₂₉ tricarbamyl insulin	14	+++	+	+++	+	+	0	++	0
A ₁ -B ₁ Z(LAL) insulin	^b	+++	+	+++	+	+++	+	++	+
A ₁ -B ₂₉ oxalyl insulin	10	+	0	++	+	+	0	+	0
A ₁ -B ₂₉ succinyl insulin	13	+	+	++	+	+	0	+	0
A ₁ -B ₂₉ suberoyl insulin	10	+	0	++	+	+	0	+	0
A ₁ -B ₂₉ dodecoyl insulin	10	+	0	++	+	+	0	+	0
A ₁ -B ₂₉ cystinyl insulin	10	+	0	++	+	+	0	+	+
A ₁ -B ₂₉ (BOC) ₂ cystinyl insulin	10	++	0	++	+	++	+	++	+
A ₁ -B ₂₉ Z(LAL) insulin	^b	+	0	++	+	+	0	+	+
A ₁ -B ₂₉ [des phe B ₁] suberoyl insulin	10	+++	+	+++	+	+	0	++	0

^a personal communication D. Lindsay

^b personal communication D. Brandenburg

Table 2. Biological potencies and immunopotencies of insulin derivatives relative to insulin. *Fat cell results are presented as the mean and total range of observations obtained from the published results of Ellis et al. [21]. In vivo results are presented as the mean potency with 95% fiducial limits. The immunopotency was calculated from the ratio of the amounts of derivative and insulin required to cause half-maximal displacement of label from the antiserum

Insulin derivative	Biological potency (%)		Immunopotency (%)			
	Fat cell assay*	In vivo assay	Ab 1	Ab 12	GP5	PPAB
A ₁ carbamyl insulin	34 (32–35)	29.0 (17.9–45.7)	30.7	71.4	10.4	95.3
A ₁ acetoacetyl insulin	27 (24–31)	27.2 (15.6–47.3)	40.5	65.7	92.0	100.0
A ₁ succinyl insulin	17 (13–19)	–	21.3	49.1	73.6	63.1
A ₁ thiazolidine insulin	18 (17–20)	34.0 (20.1–57.3)	38.7	48.7	56.8	65.0
B ₁ carbamyl insulin	116 (114–118)	102.0 (58.9–177.5)	62.3	100.0	31.6	77.5
B ₁ acetoacetyl insulin	86 (85–87)	96.1 (51.4–162.4)	52.3	100.0	15.9	65.5
B ₂₉ succinyl insulin	80 (73–84)	69.0 (42.3–113.3)	110.0	34.0	65.1	71.9
A ₁ , B ₂₉ diacetyl insulin	29 (23–38)	30.3 (20.8–44.2)	57.8	100.0	100.0	95.5
A ₁ , B ₁ , B ₂₉ tricarbamyl insulin	10 (10–10)	–	11.7	50.6	13.4	36.8
A ₁ -B ₁ Z(LAL) insulin	0.4 (0.3–8.5)	–	3.9	7.5	2.8	10.3
A ₁ -B ₂₉ oxalyl insulin	5.1 (4.4–6.6)	5.2 (1.9–8.2)	10.8	53.3	54.6	58.6
A ₁ -B ₂₉ succinyl insulin	8.1 (7.6–8.7)	–	16.2	67.0	72.3	73.2
A ₁ -B ₂₉ suberoyl insulin	5.4 (4.4–7.2)	6.9 (3.4–14.0)	12.7	62.6	67.3	68.0
A ₁ -B ₂₉ dodecoyl insulin	6.8 (5.5–9.4)	16.1 (10.3–25.1)	12.7	55.3	63.6	56.2
A ₁ -B ₂₉ cystinyl insulin	3.1 (3.0–3.2)	–	20.0	44.2	66.1	45.0
A ₁ -B ₂₉ (BOC) ₂ cystinyl insulin	2.2 (1.7–2.6)	–	9.8	42.5	45.8	36.0
A ₁ -B ₂₉ Z(LAL) insulin	3.7 (3.3–4.1)	–	7.6	61.1	52.5	35.5
A ₁ -B ₂₉ [des phe B ₁] suberoyl insulin	6.3 (6.3–6.3)	–	3.0	63.7	5.6	53.0

where antiserum GP5 was able to distinguish between native insulin and [D-Phe]B₁ insulin.

The shape of the dilution curves of the chemically modified insulins can be useful in the radioimmunoassay of these materials in biological fluids. The measurement of an insulin derivative is best achieved in an assay system using an antibody with a high

affinity for that derivative, thus yielding the most sensitive assay system. These antisera, however, are those that do not distinguish between the derivative and insulin itself and hence the derivatives can only be measured accurately in *in vitro* experiments in the absence of insulin [19] or in *in vivo* experiments where endogenous insulin secretion may be assumed

to be negligible [4]. The possible conversion of a chemically modified insulin back to insulin during the course of an experiment may be examined using antisera that are sensitive to the modification involved [4] by testing the immunological identity of the samples with the appropriate standards [20]. Using this technique we were able to show that there was no significant conversion of the analogue into insulin during the experimental procedure. The *in vivo* bioactivities measured correspond closely to those obtained by assessing the ability of the various analogues to stimulate lipogenesis in isolated fat cells [21] (Table 2).

The comparison of the antibody binding of insulin derivatives with their *in vivo* or *in vitro* biopotencies will be subject to error unless the antibody and the insulin receptor are known to bind to the insulin molecule in a similar fashion. Table 2 illustrates this where the 'immunopotency' of eighteen insulin analogues measured with each of the four different antisera is compared with their biopotency measured *in vivo*. It is clear that there is no general agreement between the *in vivo* potency and 'immunopotency'. Antiserum Ab1, however, gives values of 'immunopotency' which are more similar to the *in vivo* and *in vitro* biopotencies while antiserum GP5, which binds at a site of the insulin molecule removed from the site of receptor binding [22], gives values of 'immunopotency' which are far removed from the biological activity of the insulin derivatives.

Acknowledgements. We wish to thank Dr. B. Hurn, Wellcome Research Laboratories, Beckenham, Kent, U.K. for the gift of insulin antisera and Dr. D. Lindsay for chemically modified insulins. This research was supported by grants from the British Diabetic Association, St. Thomas' Hospital Research (Endowments) Committee and the Wellcome Trust.

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Received: February 5, 1979,
and in revised form: July 13, 1979

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