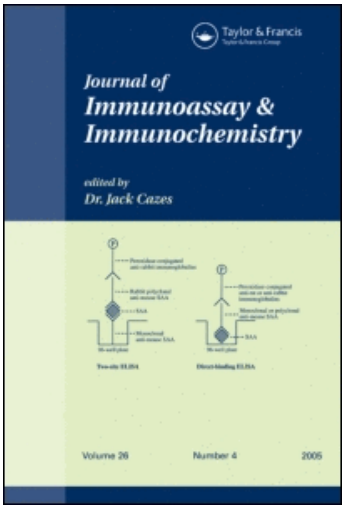

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SIMPLE METHOD FOR DEVELOPMENT OF SENSITIVE AND SPECIFIC ANTIINSULIN ANTISERA FOR LABORATORY USE

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ABSTRACT

Commercial sources provide good, though expensive antiinsulin antisera. We describe here a simple, fast and inexpensive method for the production of antiinsulin antisera. Purified pork insulin (Lente) was injected subcutaneously in oil/water/complete Freund adjuvant mixture. Three guinea pigs received 0.25 mg of insulin and three received 0.5 mg of insulin. Subsequent injections of the same dose were done 40 and 60 days later. Five animals developped antisera with titers superior to 1:10,000 40 days after the primary inoculation. Four out of five guinea pigs improved further their antibody titer after the 2nd and 3rd injection ($p<0.0005$). Good sensitivity was associated with titers superior to 1:50,000 and appeared only after the 2nd injection to improve further after the 3rd. Thus, four out of six animals developed antiinsulin antisera suitable for the radioimmunoassay (RIA). The antisera bound proinsulin on an equimolar basis with insulin while glucagon was not bound up to 100 ng/ml. The minimum detectable insulin concentration was about 12 pg (0.3 μU) at the optimum antiserum dilution. Six animals given a small dose of insulin (0.06 mg) developed antisera of a low titer and sensitivity, unsuitable for the RIA.

(KEY WORDS: Insulin, radioimmunoassay, antisera)

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INTRODUCTION

It is often stated that development of useful antisera against polypeptide hormones is more an art than a science (1) and numerous more or less complicated methods have been devised (2) to achieve this goal. Although good antisera against many polypeptide hormones (insulin included) are now commercially available, they tend to be expensive, especially if many radioimmunoassays are performed and even more so if antibodies are used for other purposes, such as affinity chromatography or immunoprecipitation where high concentrations of antisera are needed (3). Also, it is not convenient to switch from one antiserum to another within one experimental project since a variability of results can be introduced. Sometimes, one can secure a large supply from commercial sources, but the price is prohibitive for many investigators.

The production of «home-made» antibody will be useful and inexpensive only if one can avoid long trials of different doses of antigen, different animal species, a large animal colony and a time-consuming testing of titer and sensitivity of obtained antisera.

We developped highly sensitive and specific antiinsulin antisera in guinea pigs using three subcutaneous injections (40 and 60 days after the first injection) of 0.25 or 0.5 mg of Lente insulin in oil/lanolin emulsion. Intradermally injected insulin (0.06 mg) did not induce production of usable antiinsulin antisera in guinea pigs.

MATERIALS AND METHODS

Animals and Inoculations

Male albino guinea pigs (approximately 250 g) from Canadian Breeding Farm (St-Constant, Quebec) were used.

Method I (modified from ref. 4)

The insulin suspension for inoculation was prepared by mixing equal volumes of oily and aqueous phase.

Oily phase: heavy mineral oil (Baker) (7 ml) and lanolin (Lanolelle) (3 ml) are mixed in a water bath at 90°C until a homogenous suspension is obtained and then cooled at room temperature.

Aqueous phase: phenol 0.3% (w/v) acidified with HCl 1N to pH 2.3 (5.75 ml), pork insulin (Lente, Eli Lily) (1.25 ml - 125 U) and complete Freund adjuvant (Difco - 3 ml) are thoroughly mixed.

The oily phase is mixed with the aqueous phase by transfering between two connected syringes until the emulsion becomes thick and white.

Three guinea pigs were injected with 0.5 mg of insulin (12.5 U) in 4 ml of emulsion and three others received 0.25 mg of insulin (6.25 U) in 2 ml of emulsion at four subcutanoeus (s.c.) sites on the back. No shaving was necessary.

Method II (modified from ref. 5)

Five guinea pigs were injected with 0.06 mg of insulin (Lente) prepared in an emulsion of phenol 0.3%, pH 2.3 (500 µl), anti-BCG

(150 µg), complete Freund adjuvant (2.5 ml) and saline (2 ml) at 5 to 10 intradermal (i.d.) sites on the back. Backs had to be shaven for this procedure.

Sucrose 1% was given instead of water for three days after inoculation in order to prevent hypoglycemia.

Forty days after the initial inoculation about 0.5 ml of blood were drawn into heparinized tubes from a marginal vein of the ear. After centrifugation plasma was removed and kept at -20°C until the assay was performed. Second and third injections of the same composition as the initial inoculation were administered 40 and 60 days after the first injection and blood drawn 20 days later (at 60 and 80 days).

Evaluation of the titer and sensitivity of the antiserum

Purified pork insulin was used throughout the study (gift of Dr Ronald Chance, Eli Lilly). Labelled ^{125}I-insulin (250-350 mCi/mg) was prepared by the chloramine-T method of Freychet et al. (6), purified on a cellulose column and diluted for use to contain about 10,000 cpm in 100 µl. Barbital saline buffer 0.1 M, pH 7.4 containing 0.25% of human serum albumin was used in the assay. Incubation volume (1 ml) consisted of ^{125}I-insulin (about 25 pg) - 100 µl; antibody diluted in 3% of normal guinea pig serum from 1:100 to 1:100,000 - 100 µl; unlabelled insulin at concentrations of 0 to 25.6 ng/ml (0-640 µU/ml) - 100 µl and buffer, - 700 µl. In order to assess tracer damage the antibody was omitted in one series of tubes. (The damage was under 10% in all assays). After incubation (4 hours at 37°C plus 48 hours at 4°C) dextran-charcoal

mixture (Dextran 200-275, BDH, grade A: 0.5 g/100 ml of buffer, mixed with Novit A neutral charcoal (pharmaceutic grade): 5 g/100 ml of buffer) 1 or 2 ml was added. (No difference in results was seen whether 1 or 2 ml of dextran-charcoal were added). After being vortex mixed tubes were centrifuged at 1,500 g, 4°C for 25 minutes. The radioactivity of the pellet («free») and supernatant («bound») were determined in the gamma-counter (Beckman 8000).

The following terms are used when expressing and calculating results: binding in the absence of unlabeled insulin (B_0); binding at each point of the curve (B); radioactivity in the charcoal pellet in the absence of unlabelled hormone (F_0) and in the presence of unlabelled hormone (F).

R/R_0 is equal to $-\frac{B/F}{B_0/F_0}$. The minimal insulin dose detectable in the RIA is derived from the following formula:

$$100 - \frac{2 \text{ S.D. of } R_0}{\text{average of 12 } R_0} \times 100,$$ which indicates the percentage of I^{125}-insulin displaced by the minimal insulin dose.

Evaluation of the specificity of the antibody

In order to test the specificity of the antibody proinsulin (Eli-Lilly) 0.01 to 100 ng and glucagon (Eli-Lilly) 0.01 to 1000 ng were tested in the radioimmunoassay (RIA).

Statistical analysis

Statistical evaluation was done using Student's «t» test for paired data using a 5% confidence limit.

RESULTS

1. *Titer evaluation* (*Fig. 1, Table 1,2*)

The titer of an antiserum is defined as the dilution of antiserum in the assay at which 50% of tracer is bound. Titers equal or greater than 1:10,000 were achieved in all animals injected initially with 0.25 and 0.5 mg of insulin s.c. except in one guinea pig which died 24 hours after the initial injection. On the other hand, only one out of five guinea pigs injected with 0.06 mg of insulin i.d. developed a titer superior to 1:10,000 (Fig. 1). A further increase in titer is seen after the second inoculation in the two guinea pigs injected with 0.5 mg of insulin and two out of the three guinea pigs injected with 0.25 mg ($p<0.05$). In all these good responders titers either increase or stay stable at a high dilution of antisera (>1:100,000) after the third inoculation. The only guinea pig injected with 0.25 mg of insulin which exhibited a titer inferior to 1:10,000 after the second injection did not improve its titer appreciably after the third injection of insulin. All guinea pigs injected i.d. with 0.06 mg of insulin exhibited titers of less than 1:10,000 after the second and the third inoculation.

2. *Sensitivity evaluation* (*Tables 3 and 4*)

The sensitivity is defined here as a percentage of radioactivity displaced by 200 pg (5 μU) of unlabeled insulin.

After the first inoculation the sensitivity is poor, improves considerably after the second inoculation ($p<0.0125$) and increases

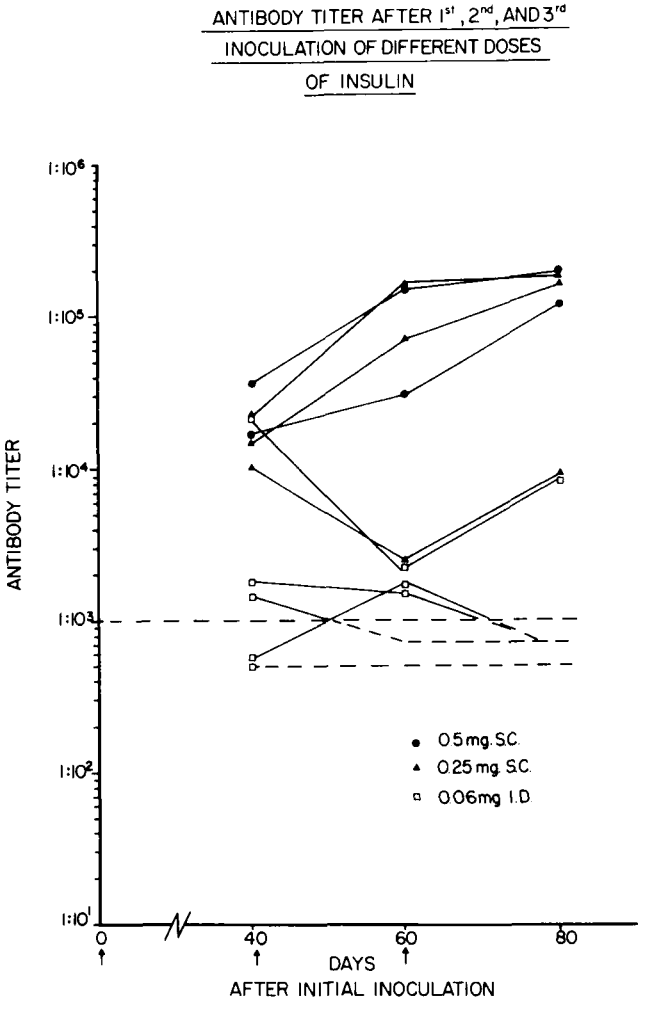

Figure 1: Antisera Titer after First, Second and Third Inoculation of Different Doses of Insulin.
Insulin 0.5 mg s.c. (——O——), 0.25 mg s.c. (——Δ——) and 0.06 mg i.d. (——□——) were injected on the day 0, 40 and 60 as indicated by arrows. Blood was drawn 40, 60 and 80 days after the initial inoculation. Highest concentration of antisera tested was 1:1,000 (broken line); more concentrated antisera were not tested at 60 and 80 days. Antiserum titer is the dilution of antibody in the assay at which 50% of tracer (^{125}I-insulin) is bound.

TABLE 1

Evolution of the Titer of Antiinsulin Antisera after the Primary and Subsequent Subcutaneous Injections

Antiserum number	Insulin dose	Titer ($X\ 10^{-3}$)* After the 1st injection	After the 2nd injection	After the 3rd injection
A77	0.5 mg s.c.	17.8	30.0	120.0
A86		37.2	150.0	180.0
A96	0.25 mg s.c.	23.5	150.0	150.0
A37		14.8	70.0	160.0
A99		10.0	2.5	9.0
Mean ± S.D.		20.7 ± 10.5	80.5 ± 67.8	124 ± 68

NS

p<0.05 | NS

p<0.005

*Antiserum dilution in the assay at which 50% of tracer ^{125}I-insulin are bound.

TABLE 2

Evolution of the Titer of Antiinsulin Antisera after the Primary and Subsequent Intradermal Injections

Antiserum number	Insulin dose	Titer (x 10^{-3})* After the 1st injection	After the 2nd injection	After the 3rd injection
A30	0.06 mg i.d.	22.4	2.5	8.0
A92		1.4	<1.0	<1.0
A82		<1.0	<1.0	<1.0
A46		<1.0	1.5	<1.0
A85		<1.0	<1.0	<1.0
Mean ± S.D.		5.4 ± 9.5	1.4 ± 0.7	2.4 ± 3.1

NS | NS

NS

*Antiserum dilution in the assay at which 50% of the tracer are bound.

TABLE 3

Evolution of the Sensitivity of Antiinsulin Antisera in Good Responders after the Primary and Subsequent Subcutaneous Injections.

Antiserum number	Insulin dose	Sensitivity (%)* After the 1st injection	After the 2nd injection	After the 3rd injection
A77	0.5 mg s.c.	7	17	38
A86		19	54	70
A96	0.25 mg s.c.	27	51	60
A37		33	54	60
Mean ±S.D.		22 ± 11	44 ± 18	57 ± 13
		p<0.0125	p<0.025	
		p<0.0005		

*Percentage of ^{125}I-insulin displaced from antiserum by addition of 200 pg (5μU) of unlabelled insulin.

TABLE 4

Comparison of Different Dilutions of Antiserum (A37)

Antibody dilution in the RIA	B_0*	Minimal detectable* insulin concentration (pg)
1:250,000	60.2	21
1:500,000	39.0	11
1:750,000	26.7	12
1:1,000,000	18.7	97

* Definitions in Methods.

further after the third ($p<0.025$ vs sensitivity after the second inoculation) (Table II). Titers superior to 1:50,000 were associated with a good sensitivity.

Since the sensitivity of the assay can be dependent on the antiserum dilution we tested different dilutions to assess the minimal quantity of insulin which can be detected (Table III). As maximum binding decreased from 60% at 1:250,000 dilution to 27% at 1:750,000 dilution sensitivity improved up to a certain point. However, when the B_0 dropped under 20%, counts were low, counting error large and the sensitivity worsened. Best sensitivity was obtained with antisera dilution which gives a maximum binding between 25 and 40%. Under those conditions about 12 pg (0.3 μU) of insulin could be detected in the RIA.

3. *Specificity testing* (*Fig. 2*)

Glucagon, which is immunologically unrelated to insulin, did not displace ^{125}I-insulin from the antisera in concentrations up to 100 ng/ml. Proinsulin, on the other hand, contains the whole immunological structure of insulin. Our antisera bind proinsulin in an identical fashion as insulin when expressed on a molar basis.

DISCUSSION

Insulin has many advantages as an immunogen when compared to other polypeptide hormones. Mammalian insulin is often highly immunogenic when injected into other mammalian species (7), e.g. porcine insulin administered to guinea pigs. Also, large quantities of a purified hormone are available for immunization. A

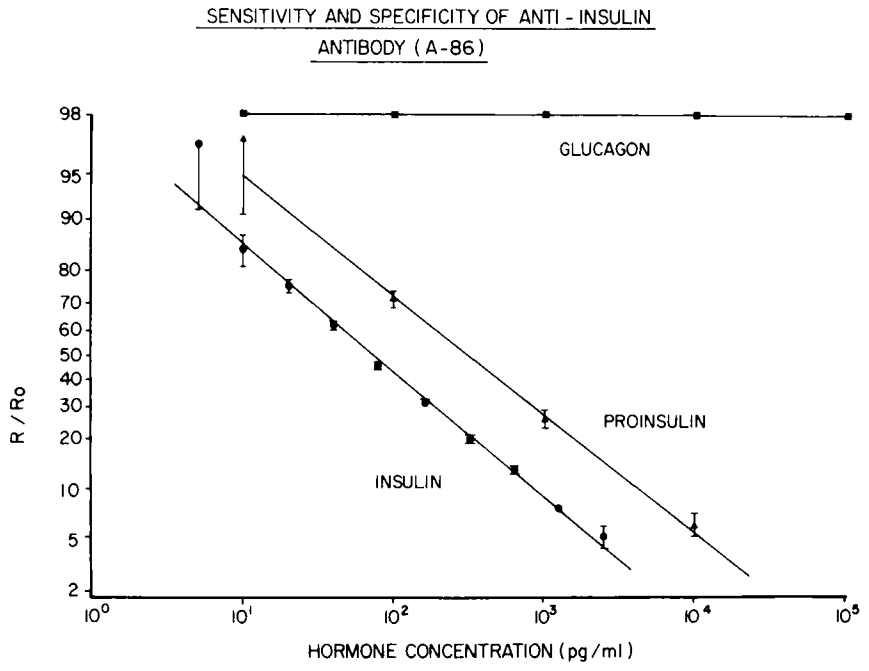

Figure 2: Sensitivity and Specificity of Antiinsulin Antibody (A-86). The radioimmunoassay was performed (cf. Methods) using insulin (——0——), proinsulin (——Δ——) and glucagon (——□——). Anti-insulin antibody was diluted 1:600,000 in the assay and B_0 was 33%.

$$R/R_0 = \frac{B/F}{B_0F_0}$$ (for definitions see Methods).

Mean of duplicates ±S.D. are plotted and lines are computer fitted.

disadvantage is its biological activity leading to hypoglycemia, which in practice limits the quantity of insulin which can be used for immunization.

There is little recent literature on the development of anti-insulin antisera probably because it is so easy to obtain good antisera from commercial sources. These may, however, be cost advantages when large quantities are used. Titer, sensitivity and specificity can also be readily evaluated and the same antiserum

can be used for a prolonged period of time, minimizing variability of results.

In this study, four out of five guinea pigs injected subcutaneously with three doses of 0.25 or 0.5 mg of insulin developped sensitive and specific antiinsulin antisera. On the other hand, none of those injected intradermally with 0.06 mg developped antisera useful for the RIA. Makulu (4) also concluded that small doses of porcine insulin are inefficient in inducing the production of antiinsulin antisera and suggested a minimal insulin dose of 0.5 mg. In the present work, two of three guinea pigs injected with 0.25 mg of porcine insulin developed good antisera. Although very small doses (μg) of immunogen can be used for some polypeptide hormones (5) this is clearly not suitable for insulin. We suggest use of 0.25 to 0.5 mg of insulin per guinea pig with replacement of water by sucrose 5% for two days after inoculation to prevent a hypoglycemic reaction. Guinea pigs with an antiserum titer greater than 1:10,000 forty days after the first inoculation should be reinjected with the same dose since the titer increases thereafter. If the initial titer is less than 1:10,000 animals should be probably discarded since the titer does not increase appreciably and the assay sensitivity obtained is poor when the titer is low. If a drop in titer is observed after the second inoculation the animal should be probably discarded.

Titers determined on the blood drawn from a marginal ear vein are lower than those observed after the animal is bled completely, due probably to dilution of blood with heparin. The antiserum used in the RIA will be ultimately even more diluted since one aims at

less than 50% of binding in order to achieve a maximum sensitivity. For example, the antibody 86 (A86) the titer of which was determined to be 1:180,000 during testing (Table I) was eventually used in the RIA at a dilution of 1:600,000 with B_0 of 33% and minimum detectable insulin concentration of 12 pg.

The ability of commercially available antiserum to bind proinsulin varies from one commercial lot to another (8). Our antisera detected proinsulin on an equimolar basis with insulin, which can be useful when proinsulin is of interest, such as in insulinoma patients.

The method described is inexpensive; the quantity of useful antisera we obtained in about two months of work would cost, if purchased from commercial sources, in excess of $1,000,000. Enough antiserum was obtained from one guinea pig for 3 X 10^6 radioimmunoassays, assuming that 6 ml of serum can be obtained from one guinea pig and 1:500,000 dilution is used in the RIA.

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