Production and characterization of specific antibodies for evaluation of glycated insulin in plasma and biological tissues

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Abstract

Previous studies have shown that glycation of insulin occurs in pancreatic β -cells under conditions of hyperglycaemia and that the site of glycation is the N-terminal Phe¹ of the insulin B-chain. To enable evaluation of glycated insulin in diabetes, specific antibodies were raised in rabbits and guinea-pigs by using two synthetic peptides (A: Phe-Val-Asn-Gln-His-Leu-Cys-Tyr, and B: Phe-Val-Asn-Gln-His-Leu-Tyr-Lys) modified by N-terminal glycation and corresponding closely to the N-terminal sequence of the glycated human insulin B-chain. For immunization, the glycated peptides were conjugated either to keyhole limpet haemocyanin or ovalbumin using glutaraldehyde, *m*-maleimidobenzoyl-N-hydroxy-succinimide ester or 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride. Antibody titration curves, obtained using I¹²⁵-tyrosylated tracer prepared from glycated peptide A, revealed high-titre antisera in five groups of animals immunized for 8–28 weeks. The highest titres were observed in rabbits and guinea-pigs immunized with peptide B coupled to ovalbumin using glutaraldehyde. Under radioimmunoassay conditions, these antisera exhib-

ited effective dose (median) (ED₅₀) values for glycated insulin of 0.3–15 ng/ml and 0.9–2.5 ng/ml respectively, with negligible cross-reactivity against insulin or other islet peptides. The degree of cross-reaction with glycated proinsulin was approximately 50%. Glycated insulin in plasma of control and hydrocortisone-treated diabetic rats measured using rabbit 3 antiserum (1:10 000 dilution; sensitivity <19 pg/ml) was 0.08 ± 0.01 and 1.5 ± 0.6 ng/ml ($P < 0.01$), corresponding to 4 and 16% of total circulating insulin concentration respectively. Immunocytochemistry studies of the pancreas of streptozotocin-treated diabetic rats using a 1:1000 dilution of guinea-pig 2 antiserum revealed clusters of fluorescent positively stained cells in islets. These studies document the successful production of polyclonal antisera specific for glycated insulin and their usefulness in radioimmunoassays and immunocytochemistry. The demonstration of glycated insulin in plasma and islets of animal models of diabetes supports the view that glycation of insulin is involved in the pathogenesis of this disease.

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Introduction

One of the major pathophysiological consequences of long-term elevation of plasma glucose in diabetes is an increase in the non-enzymatic glycation (glycosylation) of proteins (Brownlee 1995, Lyons & Jenkins 1997, Brownlee 2000). The glycation process is initiated by nucleophilic addition of a reducing-sugar aldehyde group with an available amino group of amino acids, peptides or proteins. This non-enzymatic reaction is a reversible condensation reaction that produces an aldimine or labile Schiff base which dissociates readily or else undergoes rearrangement to form the more stable Amadori product; this eventually leads to the formation of irreversible advanced glycation end-products (AGEs) (Brownlee 1995).

The glycation of structural proteins, such as lens crystallins, has long been known to contribute to the

development of many of the complications of diabetes (Perry *et al.* 1987). More recently, it has been established that glycation can disrupt normal cellular functions, including the activities of various enzymes such as Cu–Zn superoxide dismutase, glucose-6-phosphate dehydrogenase and glutathione reductase (Blakytyn & Harding 1992, Oda *et al.* 1994, Ganea & Harding 1994, Takahashi *et al.* 1995). Recent observations also suggest that glycation may contribute to the mechanisms through which hyperglycaemia exacerbates disturbances in pancreatic β -cell function and insulin action, which are the hallmark of glucose intolerance in type 2 diabetes (Unger & Grundy 1985, Flatt *et al.* 1994, 1997).

Increasing evidence now exists to support the idea that insulin is glycated in the pancreatic β -cell under conditions of hyperglycaemia and that such structural modification contributes to insulin resistance by impairing insulin

action (Lapolla *et al.* 1988, Flatt *et al.* 1997, Abdel-Wahab *et al.* 1997a). Thus, glycation of insulin and proinsulin has been shown to occur readily in the pancreata of normal and diabetic animal models (Abdel-Wahab *et al.* 1996, 1997b). Insulin is also rapidly glycated within, and secreted from, isolated pancreatic islets and clonal insulin-secreting cells maintained under hyperglycaemic conditions in tissue culture (Abdel-Wahab *et al.* 1996, 1997a). Studies have also shown that glycated insulin exhibits a reduced ability to regulate plasma glucose homeostasis *in vivo* and to stimulate adipose-tissue lipogenesis (Dolhofer & Wieland 1979) or glucose uptake and oxidation by isolated diaphragm and abdominal muscle *in vitro* (Boyd *et al.* 1996, Abdel-Wahab *et al.* 1997b). When hyperinsulinaemic euglycaemic clamps were used in healthy human volunteers, glycated insulin was also shown to exhibit significantly impaired insulin-mediated glucose uptake (Boyd *et al.* 1996, Hunter *et al.* 1996).

Despite these various observations, technology to date has not been sensitive enough or reliable enough to measure circulating glycated insulin in normal or pathophysiological states. Since recent peptide chemistry studies have identified the site of glycation of human insulin to be the amino-terminal Phe¹ of the B-chain (O'Harte *et al.* 1996), the present study was conducted in an attempt to raise polyclonal antibodies, of high affinity and specificity, to two glycated synthetic sequences from the insulin B-chain. This paper describes, for the first time, the successful production of polyclonal antisera specific for glycated insulin, the immunocytochemical demonstration of insulin in pancreatic islets and the development and use of a sensitive radioimmunoassay (RIA) for measuring glycated insulin in diabetic plasma.

Materials and Methods

Reagents

HPLC-grade acetonitrile was obtained from Rathburn (Walkersburn, UK). Sequencing-grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, UK). Keyhole limpet haemocyanin (KLH: *Megathura crenulata*) was obtained from Calbiochem (Nottingham, UK). All water used was purified using a Milli-Q water-purification system (Millipore, Milford, MA, USA). Glucose oxidase reagent was obtained from Beckman Instruments Ltd (Galway, Ireland). Dialysis tubing (M_r 2000 cut-off) was purchased from Spectra Medical Industries Inc. (Los Angeles, CA, USA). NaI¹²⁵ (100 mCi/ml) for iodination was obtained from Amersham International (Aylesbury, UK). Insulin-free serum was purchased from Biogenesis Ltd (Poole, UK). Peptide hormones were purchased from the American Peptide Company (Sunnyvale, CA, USA). All other chemicals, including glutaraldehyde (GLU), ovalbumin (OV), streptozotocin (STZ) and hydrocortisone, were of the highest quality available and were purchased from Sigma (Poole, UK).

Animals

Male New Zealand white rabbits ($n=12$, age 12 weeks) and Duncan Hartley guinea-pigs ($n=18$, 400–450 g) were purchased from Laboratory Services, Queen's University, Belfast, UK. Male Wistar rats aged between 12 and 18 weeks were obtained for experimental studies from the Behavioural and Biomedical Research Unit, University of Ulster, Coleraine. All animals were housed under controlled environmental conditions of temperature ($22 \pm 2^\circ\text{C}$), relative humidity (45–65%) and photoperiod (12 h light:12 h darkness). Pelleted diets for rabbits/guinea-pigs or for rats (SDS Ltd, Witham, Essex, UK) were freely available as appropriate. Animals were also allowed to drink water *ad libitum*. Hyperglycaemia was induced in rats by intraperitoneal injections of hydrocortisone (40 mg/kg body weight in 0.2 ml corn oil) twice daily for a period of 8 days. Control rats were injected twice daily with 0.2 ml corn oil. Diabetes was also induced in rats by a single intraperitoneal injection of STZ (200 mg/kg body weight) dissolved in 0.1 ml of 0.1 mol/l sodium citrate (pH 4.5). These animals were maintained for a period of 14 days. Blood was collected from the tip of the tail into heparinized microcentrifuge tubes and the plasma separated (by centrifugation at $2500 \times g$ for 10 min) and stored at -20°C until analysis. All animal studies were performed in accordance with the Animals (Scientific Procedures) Act 1986.

Preparation of synthetic insulin peptides

Previous studies have established that insulin is naturally glycated at the N-terminal Phe¹ of the insulin B-chain (O'Harte *et al.* 1996). For preparation of antibodies and an RIA specific for glycated insulin, two peptides that were closely related to the amino-terminal sequence of the insulin B-chain (Phe¹-Val²-Asn³-Gln⁴-His⁵-Leu⁶-Cys⁷-Gly⁸-) were synthesized. Peptide A comprised the naturally occurring 1–7 sequence with the addition of a Tyr at position 8 (Phe¹-Val²-Asn³-Gln⁴-His⁵-Leu⁶-Cys⁷-Tyr⁸), whereas peptide B was extended from position 6 with a Tyr and a Lys at positions 7 and 8 respectively (Phe¹-Val²-Asn³-Gln⁴-His⁵-Leu⁶-Tyr⁷-Lys⁸). Peptides were synthesized by Fmoc strategy and solid-phase peptide chemistry using a Applied Biosystems Synergy 432A peptide synthesizer (Foster City, CA, USA) starting from the Fmoc-Tyr-Wang and Fmoc-Lys-Wang resins respectively. After cleavage from the resin by TFA:water:thioanisole:ethanedithiol (90:2.5:5:2.5) (20 ml/g of resin), the peptide was removed by filtration and the filtrate volume was lowered under reduced pressure using a rotary evaporator. Diethyl ether was added slowly until a precipitate was observed. The precipitate was collected by low-speed centrifugation ($900 \times g$ for 20 min), resuspended in diethyl ether and centrifuged again, the procedure being carried out at least five times. The pellets were then dried

Table 1 Immunization groups used in the production of antibodies against glycated insulin. Groups of three rabbits or guinea-pigs were immunized with either glycated insulin peptide A or glycated insulin peptide B linked to a carrier protein by a coupling agent. Animals received a primary immunization followed by booster injections at 4-weekly intervals. At least six booster injections were given, with blood being taken 10 days after immunization. Antibody titres are expressed according to log-inverse transformation as means $\pm$ S.E.M. of all bleeds (8–28 weeks) at 50% binding, with the number of animals being given in parentheses

Group	Species (<i>n</i> = 3) ¹	Antigen peptide	Coupling agent ²	Carrier protein ³	Antibody titre (log inverse)
1	GP	A	MBS	OV	–
2	R	A	CDI	OV	2.98 $\pm$ 0.08 (15)
3	R	A	MBS	KLH	–
4	GP	A	MBS	KLH	–
5	GP	A	CDI	KLH	–
6	GP	B	GLU	OV	3.76 $\pm$ 0.06 (18)
7	R	B	GLU	OV	4.44 $\pm$ 0.10 (18)
8	GP	B	CDI	OV	2.64 $\pm$ 0.18 (6)
9	GP	B	GLU	KLH	–
10	R	B	GLU	KLH	1.10 $\pm$ 0.03 (4)

¹GP, guinea-pig; R, rabbit.

²MBS, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester; CDI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; GLU, glutaraldehyde.

³OV, ovalbumin; KLH, keyhole limpet haemocyanin.

in vacuo, cleaned up by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters MILLENNIUM 2010 chromatography (software version 2.1.5) and molecular identity was confirmed as described below using a high-resolution LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). The SWISSPROT and SWISSPROTNEW databases did not reveal any known peptides or proteins containing sequences similar to peptides A and B, other than insulin and proinsulin.

Preparation and purification of glycated insulin peptides

Synthetic insulin peptides A and B were stably glycated by exposure to D-glucose (220 mmol/l) for 24 h at 37 °C in the presence of the reducing agent sodium cyanoborohydride, as previously described (O'Harte *et al.* 1996). Glycated and non-glycated peptides were separated by RP-HPLC (O'Harte *et al.* 1996) using a Vydac (C-18) 218TP54 (4.6 $\times$ 250 mm) analytical column (The Separations Group, Hesperia, CA, USA) at a flow rate of 1.0 ml/min. The mobile phase consisted of solvent A (0.12% (v/v) TFA in water) and solvent B (0.1% (v/v) TFA in 70% acetonitrile/29.9% water). A linear gradient of 0–20% (v/v) acetonitrile over 10 min was used; this was followed by a gradient of 20–60% (v/v) acetonitrile over 30 min. Elution profiles were monitored at 206 nm and peptide peaks collected manually. Pooled glycated peptide fractions were concentrated under vacuum using an AES 1000 Speed Vac Concentrator (Life Sciences International, Runcorn, UK).

Confirmation of structure by electrospray ionization-mass spectrometry

Glycated and non-glycated peptides were identified by electrospray ionization/mass spectrometry (ESI-MS). The peptides were dissolved in 0.12% (v/v) TFA in water and applied at a flow rate of 10 μ l/min by syringe injection to the electrospray ionization source of an LCQ ion-trap mass spectrometer (Finnigan MAT). Spectra were obtained from a quadrupole ion-trap mass analyser. The mass range of the detector was *m/z* 150–2000. For direct injection, the interface to the mass spectrometer was operated with the heated capillary at a temperature of 220 °C. The molecular masses of glycated and native peptides were determined using prominent multiple charged ions and the equation $M_r = iM_i - M_h$, where M_r is molecular mass, M_i is the *m/z* ratio, *i* is the number of charges, and M_h is the mass of a proton.

Preparation of immunogen

Ten groups each containing three rabbits or guinea-pigs were immunized with either glycated peptide A or glycated peptide B linked to two different carrier proteins (KLH or OV) using a range of conjugation agents (Table 1). Glycated peptide A was linked to the carrier protein via a Cys⁷ side chain (using the *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, MBS) or the terminal carboxylic group (using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, CDI). Glycated peptide B

was linked to the carrier protein via the ϵ -amino group of Lys (using GLU) or the terminal carboxylic group (using CDI). Correct orientation and coupling of the carrier protein to the C-terminus of the glycated peptides were critical, and overcoupling was limited by the amount of linker used.

Coupling of glycated insulin to carrier proteins using glutaraldehyde

Carrier protein (0.1 μ mol, 45 mg KLH or 4.5 mg OV) was added to glycated insulin peptide B (4 mg/ml in PBS) at the correct molar ratio (approximately 1 mole peptide per 50 amino acids of carrier) and stirred for 2 h at 25 °C (Harlow & Lane 1988). An equal volume of freshly prepared 0.2% GLU in PBS was added dropwise to the peptide/carrier protein solution and incubated with constant agitation for 1 h at 25 °C. Excessive cross-linking of GLU was indicated by the insolubility of KLH. The peptide solution was incubated for 1 h following the addition of 1 M glycine/PBS (pH 7.2) (final concentration 0.2 mol/l) to quench unreacted aldehyde. The peptide/carrier protein conjugate was separated from free peptide by dialysis with three changes of PBS overnight at 4 °C.

Coupling of glycated insulin to carrier proteins using MBS

As MBS can link peptides to carrier proteins through cysteines and free amino groups, glycated insulin peptide A was initially treated with citraconic anhydride to block free amino groups in the peptide. An equal volume of citraconic anhydride (10 mg/ml) was added to glycated insulin peptide A (10 mg/ml, pH 8.5). The pH was monitored and kept between 8 and 9. The solution was incubated for 1 h at 25 °C; this was followed by the addition of 0.1 volume of 1 mol/l sodium phosphate (pH 7.2). MBS in dimethylformamide (final concentration 3 mg/ml) was added to the peptide solution and incubated with agitation for 30 min at 25 °C. To remove any remaining maleimide, mercaptoethanol (final concentration 35 mmol/l) was added and incubated for 1 h at 25 °C. Sufficient carrier protein (0.25 μ mol, 112.5 mg KLH or 11.3 mg OV) was added to the activated peptide to obtain a final ratio of approximately 1 mole of peptide for every 50 amino acids of carrier protein (Harlow & Lane 1988). The peptide/protein conjugate was incubated for 3 h and dialysed against PBS overnight to remove uncoupled peptide.

Coupling of glycated peptide to carrier proteins using carbodiimide

To stop the cross-linking of one peptide to another, an equal volume of citraconic anhydride (10 mg/ml) was added to glycated insulin peptides A and B (10 mg/ml, pH 8.5) and the solution incubated for 1 h at 25 °C. The

peptide was diluted to 1 mg/ml, and, following the addition of CDI (final concentration 10 mg/ml), the solution was incubated for 5 min at 25 °C. An equal volume of carrier protein (0.25 μ mol, 112.5 mg KLH or 11.3 mg OV) was added to yield a molar ratio of approximately 1 mole peptide to 50 amino acids of protein (Harlow & Lowe 1988). The conjugate was incubated for 4 h at 25 °C and the reaction was stopped with 100 mmol/l sodium acetate (pH 4.2) and then incubated for a further hour. Uncoupled peptide was removed by dialysis against sodium acetate (pH 4.2) for 3 h and then against PBS overnight at 4 °C.

Immunization procedure

Rabbits and guinea-pigs were injected at multiple (5–7) subcutaneous sites with conjugate (Table 1) in an emulsion formed with equal volumes of Freund's adjuvant (1–2 ml/animal) prepared immediately before injection. A pre-immune sample was taken. Rabbits ($n=3$) and guinea-pigs ($n=3$) were given 0.5 and 0.3 mg immunogen at primary immunizations and then 0.17 and 0.1 mg respectively for subsequent booster injections at 4-week intervals. All animals received at least six boosters and blood samples were taken 10 days after immunization. Blood was stored at 4 °C overnight and the serum separated after centrifugation ($2500 \times g$ for 10 min), aliquoted and then stored at -20 °C.

Preparation of iodinated tracer

A high-specific-activity mono-iodinated I^{125} -tyrosylated glycated peptide tracer was prepared from glycated insulin peptide A using the solid-phase iodogen method (Salacinski *et al.* 1981). Iodinated peptide was purified by RP-HPLC, using a Vydac (C-8) (4.6×250 mm) analytical column at a flow rate of 1.0 ml/min. A linear gradient of 0–42% acetonitrile over 40 min was used. Fractions corresponding to the mono-iodinated glycated peptide tracer were pooled, aliquoted and then stored at 4 °C.

Evaluation of antisera by radioimmunoassay

Antisera to glycated insulin peptides were screened by dextran-coated charcoal RIA based on the method described by Flatt & Bailey (1981). Antibody dilution curves were constructed in 40 mmol/l sodium phosphate buffer (5 mg/ml BSA; pH 7.4) by incubating (for 48 h at 4 °C) I^{125} -glycated peptide tracer (approx. 10 000 c.p.m./100 μ l) with each of the antisera at final dilutions of 1:10, 1:100, 1:1000, 1:2000, 1:5000, 1:10 000 and 1:50 000. To evaluate the affinity and specificity of the antisera, standard curves were prepared using 100 μ l I^{125} -glycated peptide tracer, 100 μ l antisera, 100 μ l of 40 mmol/l sodium phosphate buffer and 100 μ l insulin or 100 μ l glycated

insulin standards (range 0.019–40 ng/ml). The 48 h incubation period was preceded by 24 h of incubation prior to addition of tracer. The cross-reactivity of antisera with other hormones (10 mg/ml) was determined with insulin, proinsulin, glycosylated proinsulin, glucagon, somatostatin, C-peptide, and advanced glycation endproduct-BSA (AGE-BSA). Glycosylated insulin and proinsulin were prepared and quantified as described previously (O'Harte *et al.* 1996). AGE-BSA was prepared by incubating BSA (1 mg/ml) with D-glucose (220 mmol/l) in the presence of sodium cyanoborohydride for 6 weeks at 37 °C. For blood assays, the glycosylated insulin standard curve was constructed in the presence of immunostripped human insulin-free serum obtained from Biogenesis Ltd.

Immunocytochemistry

Pancreatic tissue from rats with STZ-induced diabetes (plasma glucose >15 mmol/l) and controls (no treatment, plasma glucose 2.5 mmol/l) was fixed in 4% paraformaldehyde/PBS, embedded in paraffin and sections cut at 7 µm. After permeabilization in 0.3% Triton X-100 and blocking in normal goat serum (Vector Laboratories, Burlingame, CA, USA), tissue sections were incubated in primary antibody for 1 h, then incubated for 45 min with a fluorescein isothiocyanate (FITC)-labelled secondary antibody (1:40 dilution, goat anti-rabbit; Vector Laboratories). All steps were carried out at 25 °C. Stained sections were visualized using an FITC-filter on an Optiphot 2 microscope (Nikon, Chiyoda-ku, Tokyo, Japan) and the images digitally recorded using the IMAGEDOK program (Kinetic Imaging Ltd, Liverpool, UK).

Glucose and insulin assays

Glucose concentrations were measured by an automated glucose oxidase method (Stevens 1971) using a Beckman Glucose Analyzer 2 (Beckman Instruments Ltd). Insulin concentrations were determined by dextran-coated charcoal RIA using cross-reacting guinea-pig antiporcine insulin antiserum, I¹²⁵-bovine insulin tracer and a rat-insulin standard as described previously (Flatt & Bailey 1981). This antibody does not discriminate between glycosylated and native insulin (Abdel-Wahab *et al.* 1997a).

Statistical analysis

All experimental data are expressed as means ± S.E.M. Significant differences between groups of data were assessed using the unpaired Student's *t*-test and statistical significance was assumed for *P* < 0.05.

Results

Purification of glycosylated peptide epitopes

Two synthetic insulin peptides (A, *M_r* 1022.5; B, *M_r* 1047.5) were stably glycosylated under hyperglycaemic

reducing conditions and purified by RP-HPLC. The primary structures of the glycosylated peptides were confirmed by ESI-MS (Fig. 1). Prominent multiply charged species (*M*+*H*)¹⁺ and (*M*+*H*)²⁺ were detected for glycosylated insulin peptide A at *m/z* values of 1187.3 and 594.3 respectively, corresponding to an intact molecular mass of 1186.3 Da (theoretical *M_r* 1186.2). The multiple charged species (*M*+*H*)¹⁺ and (*M*+*H*)²⁺ were obtained for glycosylated insulin peptide B at *m/z* values of 1212.5 and 606.9 and corresponded to a molecular mass of 1211.5 Da (theoretical *M_r* 1211.2). The difference between the *M_r* values of the glycosylated and non-glycosylated peptides (164.1 and 164.0) corresponded exactly to the addition of a single glucitol adduct (theoretical *M_r* 164).

Antibody production

Guinea-pigs (GP) and rabbits (R) were immunized with a number of combinations of glycosylated insulin peptide (A or B), linker (MBS, CDI or GLU) and carrier protein (OV or KLH) (Table 1). Serum samples obtained 10 days after each monthly booster were screened by RIA using mono-iodinated glycosylated insulin peptide A tracer purified by HPLC (Fig. 2). There was no evidence of any binding of tracer to pre-immune sera. Five out of the 10 immunized groups of animals developed antibodies against glycosylated insulin (Table 1). The unsuccessful combinations included group 1 (GP/A/MBS/OV), group 3 (R/A/MBS/KLH), group 4 (GP/A/MBS/KLH), group 5 (GP/A/CDI/KLH) and group 9 (GP/B/GLU/KLH).

The construction of antibody titration curves showed that all three animals in group 2 (R/A/CDI/OV), group 6 (GP/B/GLU/OV) and group 7 (R/B/GLU/OV) generated high-titre antibodies (Figs 3 and 4). One guinea-pig from group 8 (GP/B/CDI/OV) also responded reasonably well (Fig. 4). Group 10 (R/B/GLU/KLH) contained one rabbit that yielded low-titre antibodies (data not shown). Antibodies in the four successful groups of guinea-pigs and rabbits were evident at 8 weeks, and 50% maximum binding titres tended to increase over the 28-week period (Figs 3 and 4). As shown in Table 1, mean titres ± S.E.M. (expressed as log-inverse transforms) for groups 2, 6, 7 and 8 were 2.98 ± 0.08 (*n* = 15), 3.76 ± 0.06 (*n* = 18), 4.44 ± 0.10 (*n* = 18) and 2.64 ± 0.18 (*n* = 6). The single rabbit in group 10 had antibody titres of 1.10 ± 0.03 (*n* = 4). Comparison of titres by Student's *t*-test revealed the order group 7 > group 6 > group 2 > group 8 > group 10. The most successful immunogen in rabbits (group 7) and guinea-pigs (group 6) was peptide B coupled to OV using GLU. Peptides A (group 2) or B (group 8) coupled to OV with CDI appeared to be the next most effective. KLH was weakly immunogenic coupled to peptide B using GLU (group 10), whereas the two groups with MBS as the coupling agent failed to generate antibodies.

Figure 1 ESI-MS of (A) glycated insulin peptide A (M_r 1186.3) and (B) glycated insulin peptide B (M_r 1211.5). Peptides were dissolved in 0.1% TFA in water and applied by direct syringe injection to an ESI-MS at a flow rate of 0.01 ml/min in 35% acetonitrile/water. Spectra were collected using full-ion scan mode over the m/z ratio of 150–2000 and the M_r was determined using the equation $M_r = iM_1 - iM_n$ (see Materials and Methods section).

Sensitivity and specificity of antibodies

The reactivity of antisera from high-titre groups 6 and 7 with insulin and glycated insulin was determined using

Figure 2 Purification of iodinated glycated insulin peptide A by RP-HPLC. The ^{125}I -labelled peptide was prepared by the solid-phase iodogen method and purified by RP-HPLC using a Vydac (C-8) (4.6 × 250 mm) analytical column, with a linear gradient of 0–42% acetonitrile at a flow rate of 1.0 ml/min. The peak fraction containing the mono-iodinated glycated peptide tracer was used for RIA.

binding displacement curves over a range of peptide concentrations (0.019–10 ng/ml). The index B/B_0 (where B_0 and B represent counts bound to antibody in the absence and presence of glycated or native insulin respectively) allowed evaluation of displacement. The ED_{50} values for glycated insulin for guinea-pigs 1–3 in group 6 (final bleed; number vi) were 2.5, 1.25 and 0.9 ng/ml, respectively (Fig. 5). No cross-reactivity was found with human insulin over the range of concentrations. ED_{50} values for rabbits 1–3 in group 7 (final bleed; number vi) were 15, 9.6 and 0.3 ng/ml respectively, with no displacement by human insulin (Fig. 6). The assay using bleed vi from rabbit 3 had a sensitivity of <19 pg/ml, an intra-assay precision of 8.7% (coefficient of variance, CV). CVs for standard curve parameters (≤ 2.5 ng/ml) were in the range 12–22%. The antisera from these guinea-pigs or rabbits did not cross-react (<0.001%) with other islet hormones (10 ng/ml), including proinsulin, C-peptide, glucagon, somatostatin or AGE-BSA, but there was 52–56% cross-reactivity with glycated proinsulin (data not shown). Antiserum from rabbit 3 (group 7: R3/B/GLU/OV/vi) was selected to assess glycated insulin in biological samples. This antiserum was used at a dilution of 1:10 000 in RIA (log-inverse titre 4.0) and the standard curve was free from serum effects as assessed using insulin-free serum (data not shown).

Figure 3 Antisera evaluation of rabbits in group 2 (R/A/CDI/OV) and guinea-pigs in group 6 (GP/B/GLU/OV). The logarithm of the inverse final working dilution of the antiserum that will bind 50% of the label was determined by antibody titration curves. Mean values are shown for animals 1 (●), 2 (◆) and 3 (■), the overall mean of the group being shown by a horizontal bar.

Figure 4 Antisera evaluation of rabbits in group 7 (R/B/GLU/OV) and guinea-pigs in group 8 (GP/B/CDI/OV). The logarithm of the inverse final working dilution of the antiserum that will bind 50% of the label was determined by antibody titration curves. Mean values are shown for animals 1 (●), 2 (◆) and 3 (■), the overall mean of the group being shown by a horizontal bar.

Detection of glycated insulin in plasma and islets in diabetes

Hydrocortisone-treated rats exhibited hyperglycaemia and hyperinsulinaemia, indicative of a severe state of insulin resistance (Fig. 7). Glycated insulin was readily detectable by RIA in plasma from control and hydrocortisone-treated rats. The circulating concentration in control animals was 0.08 ± 0.01 ng/ml ($n=4$), representing about 4% of total plasma insulin measured using a cross-reacting insulin antibody (Fig. 7). The concentration of glycated insulin in plasma from hydrocortisone-treated rats was elevated 19-fold ($P<0.01$), corresponding to 1.5 ± 0.6 ng/ml ($n=4$). As shown in Fig. 7, glycated insulin represented approximately 16% of total plasma insulin in the hydro-

cortisone-treated diabetic rats ($P<0.01$ compared with controls).

Immunocytochemical studies

Immunocytochemical studies were performed using pancreatic tissue sections derived from rats with STZ-induced diabetes and non-diabetic control rats, using FITC-labelled secondary antibody. These studies were performed using a 1:1000 dilution of antiserum from guinea-pig 2, group 6 (GP2/B/GLU/OV/vi). Figure 8A illustrates clusters of fluorescent positively stained cells in a diabetic islet, with surrounding non-stained exocrine cells.

Figure 5 Cross-reactivity of antisera from guinea-pigs 1–3 (A–C) from group 6 (GP/B/GLU/OV) with glycated insulin (◆) and insulin (□). Results are expressed as an index given by the equation $100 \times B_0 - B/B_0$, where B_0 and B represent counts bound to antibody in the absence and presence of glycated or native insulin respectively.

Figure 6 Cross-reactivity of antisera from rabbits 1–3 (A–C) from group 7 (R/B/GLU/OV) with glycated insulin (◆) and insulin (□). Results are expressed as an index given by the equation $100 \times B_0 - B/B_0$, where B_0 and B represent counts bound to antibody in the absence and presence of glycated or native insulin respectively.

The islets of control rats also contained numerous positive cells but these were less intensely stained by the glycated insulin antibody (Fig. 8B).

Discussion

It is now 15 years since it was first proposed that hyperglycaemia could be an inducer as well as consequence of pancreatic β -cell dysfunction and insulin resistance (Unger & Grundy 1985). The concept of glucose toxicity is now a well-recognized phenomenon (Flatt *et al.* 1997, Lyons & Jenkins 1997), and considerable effort has been devoted, in particular, to understanding the mechanisms by which elevated glucose concentrations cause β -cell dysfunction and impaired insulin secretion in type 2 diabetes (Lapolla *et al.* 1988, Boyd *et al.* 1996, Abdel-Wahab *et al.* 1997b, Flatt *et al.* 1997). As part of this effort, we have explored the possible involvement of glycation of functional β -cell

proteins (Flatt *et al.* 1994, 1997), demonstrating that both proinsulin and insulin can be glycated within the β -cells during the stages of biosynthesis and storage (Abdel-Wahab *et al.* 1996, 1997a).

Evidence for the glycation of β -cell proteins includes the demonstration of glycated insulin in pancreatic extracts from various animal models of diabetes (Abdel-Wahab *et al.* 1996) and the time- and concentration-dependent glycation of insulin in both isolated mouse islets and clonal insulin-secreting cell lines under hyperglycaemic culture (Abdel-Wahab *et al.* 1996, 1997a). Thus the milieu within the pancreatic β -cells appears to represent a particularly favourable environment for the glycation of insulin (Abdel-Wahab *et al.* 1996, 1997a, O'Harte *et al.* 1997). This might reflect the fact that glucose is efficiently transported into the β -cell by GLUT transporters and rapidly metabolized by glucokinase to the particularly potent glycating agent glucose-6-phosphate (Leahy 1990, Flatt 1992, Avigad *et al.* 1996, Abdel-Wahab *et al.* 1997a,

Figure 7 Plasma glucose, insulin, glycated insulin concentrations and percent glycated insulin of control and hydrocortisone-treated rats. Rats received intraperitoneal injections of hydrocortisone (40 mg/kg body weight in 0.2 ml corn oil) or corn oil alone (controls) twice daily for a period of 8 days. Glycated insulin was measured by RIA using antiserum from rabbit 3, group 7 (R3/B/GLU/OV/vi). Results are expressed as means $\pm$ S.E.M. for four animals. ** $P < 0.01$; *** $P < 0.001$, compared with controls.

O'Harte *et al.* 1997). Since glyated insulin has been shown to exhibit reduced bioactivity and glucose-lowering ability (Dolhofer & Wieland 1979, Lapolla *et al.* 1988, Hunter *et al.* 1996, Abdel-Wahab *et al.* 1997b), an involvement in the glucose toxicity, insulin resistance and glucose intolerance of type 2 diabetes has been postulated (Flatt *et al.* 1994, 1997).

Observations of glyated insulin have hitherto relied on the rather crude approach of separating glyated and non-glyated proteins by boronate affinity chromatography, followed by the measurement of insulin in both fractions using cross-reacting RIA (Abdel-Wahab *et al.* 1996, 1997a). With the elucidation of the site of glycation of insulin (i.e. the N-terminal Phe¹ of the insulin B-chain; O'Harte *et al.* 1996), it became possible to envisage development, for the first time, of specific antibodies to the glyated region of the insulin molecule. The present study employed two glyated synthetic peptides for immunogen preparation, representing either the insulin B-chain (1–7) with Tyr at position 8 (peptide A) or the

insulin B-chain (1–6) with Tyr⁷ and Lys⁸ (peptide B). The rationale for these modifications concerned the use of Tyr for subsequent iodination (Salacinski *et al.* 1981) and the Lys side chain of peptide B for coupling to carrier protein using glutaraldehyde (Harlow & Lane 1988).

In all, 10 animal groups were employed; rabbits or guinea-pigs were immunized with glyated peptide A or B linked to OV or KLH using either GLU, CDI or MBS. Antisera were evaluated after 8–28 weeks of immunization with mono-iodinated I¹²⁵-tracer prepared from glyated peptide A, using the solid-phase iodogen method followed by HPLC purification (O'Harte *et al.* 1996). This analysis revealed that 5 out of the 10 immunized groups developed antibodies against glyated insulin. These included four groups with OV coupled to (i) peptide A using CDI in rabbits (group 2), (ii) peptide B using GLU in guinea-pigs (group 6) or rabbits (group 7), or (iii) peptide B using CDI in guinea-pigs (group 8). However, only one of three animals responded in the latter group, as did only one rabbit in group 10 when immunized with peptide A

Figure 8 Immunocytochemical demonstration of glycated insulin in pancreatic tissue from (A) a rat with streptozotocin-induced diabetes (plasma glucose >15 mmol/l) and (B) a control rat. Pancreatic tissue sections were immunostained using antisera from guinea-pig 2, group 6 (GP2/B/GLU/OV/vi), and FITC-labelled secondary antibody. Clusters of fluorescent positively stained islet cells are particularly evident in diabetic tissue (A) surrounded by non-stained exocrine tissue. Scale bar = 100 μ m.

coupled to KLH using GLU. As a general observation, conjugates prepared with peptide A or peptide B using KLH, MBS or CDI were either weakly immunogenic or non-immunogenic in guinea-pigs and rabbits.

The highest titre antisera (corresponding to 1:7000–1:70 000) were observed in guinea-pigs and rabbits receiving peptide B coupled to OV using GLU (groups 6 and 7, respectively). Evaluation by dis-equilibrium RIA revealed complete displacement of the tracer by glycated insulin standards. ED₅₀ values for three guinea-pig antisera (group 6) were 2.5, 1.25 and 0.9 ng/ml, corresponding values of 15, 9.6 and 0.3 ng/ml being recorded for the three rabbit antisera (group 7). Notably, these antisera exhibited no cross-reaction (<0.001%) with insulin, proinsulin, C-peptide, glucagon, somatostatin or AGE-BSA. This suggests that coupling of the glycated insulin fragment to OV through Lys⁸ yielded specific high affinity antibodies to glycated Phe¹ and the adjacent N-terminal region of the insulin B-chain.

To check the usefulness of these antisera, the final bleeds of rabbit 3 (ED₅₀ 0.3 ng/ml) and guinea-pig 2 (ED₅₀ 1.25 ng/ml) were employed for radioimmunoassay and immunocytochemical studies respectively, using two commonly employed animal models of diabetes. Pancreatic tissue from mice with STZ-induced diabetes and hydrocortisone-treated rats have been shown previously (by affinity chromatography and cross-reacting RIA) to contain appreciable amounts of glycated insulin (Abdel-Wahab *et al.* 1996). Consistent with these observations, the immunocytochemistry of pancreas resected from rats with STZ-induced diabetes revealed clusters of fluorescent

positively stained cells in islets surrounded by non-stained exocrine tissue. Control rats also exhibited numerous positive cells – an observation which is in accord not only with the earlier study (Abdel-Wahab *et al.* 1996), but also with specific RIA-measured circulating values of 0.08 ng/ml glycated insulin, estimated at 4% of total insulin in blood. Glycated insulin in the plasma of hydrocortisone-treated diabetic rats was elevated approximately 19-fold at 1.5 ng/ml, representing approximately 16% of total circulating insulin in this severely insulin-resistant hyperinsulinaemic animal model.

In conclusion, this paper describes, for the first time, the development and characterization of polyclonal antisera with high specificity and affinity for glycated insulin. RIA and immunocytochemical studies revealed appreciable amounts of glycated insulin in both the pancreas and the circulation of animal models of diabetes. These observations provide the impetus and the means to evaluate the possible involvement of glycated insulin in the glucose toxicity, pancreatic β -cell dysfunction and insulin resistance of type 2 diabetes.

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