Diabetologia (1994) 37: 985–993

Diabetologia

Immunoneutralization of endogenous glucagon with monoclonal glucagon antibody normalizes hyperglycaemia in moderately streptozotocin-diabetic rats

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Summary The role of glucagon in diabetic hyperglycaemia has been a matter of controversy because of difficulties in the production of selective glucagon deficiency. We developed a high-capacity (40 nmol/ml), high-affinity ($0.6 \cdot 10^{11}$ l/mol) monoclonal glucagon antibody (Glu-mAb) and gave i.v. injections (4 ml/kg) to rats in order to study the effect of selective glucagon deficiency on blood glucose. Controls received a mAb against trinitrophenyl. Glu-mAb completely abolished the hyperglycaemic effect of 2.86 nmol/kg glucagon in normal rats ($p < 0.05$, $n = 6$). In moderately hyperglycaemic rats injected with streptozotocin as neonates (N-STZ), Glu-mAb abolished a postprandial increase in blood glucose (from 11.2 ± 0.7 mmol/l to 17.3 ± 1.8 mmol/l in controls vs 10.5 ± 0.9 mmol/l to 9.3 ± 1.0 mmol/l; cross-over: $n = 6$, $p < 0.05$). No significant effect of Glu-mAb treatment was observed in more hyperglycaemic N-STZ rats (cross-over, $n = 4$) and in severely hyperglycaemic rats injected with STZ as adults ($n = 6$), but after insulin treatment of the latter, at doses partially restoring blood glucose levels (12.7 ± 4.3 mmol/l), Glu-mAb administration almost normalized blood glucose (maximal difference: 6.0 ± 3.8 mmol/l; cross-over: $n = 5$, $p < 0.05$). In conclusion, our results provide strong additional evidence for the hypothesis that glucagon is involved in the pathogenesis of diabetes. The hormone plays an important role in the development of STZ-diabetic hyperglycaemia, but glucagon neutralization only leads to normoglycaemia in the presence of insulin. [Diabetologia (1994) 37: 985–993]

Key words Immunoneutralization, monoclonal antibody, glucagon, insulin, streptozotocin, rat.

Diabetic hyperglycaemia is generally believed to be a bihormonal disorder where absolute or relative lack of insulin and excess of glucagon cause decreased peripheral glucose uptake and increased hepatic glucose production [1]. The "bihormonal abnormality" hypothesis was first proposed by Unger and Orci [2] in 1975 and supported later by the same group using somatostatin to suppress glucagon in dogs made insulin deficient by somatostatin, alloxan or pancreatectomy [3], and by Gerich et al. [4] using somatostatin to suppress glucagon in insulin-deficient diabetic patients. Subsequently, a variety of methods to block glucagon secretion [5, 6] or action [7–9] have been employed in order to study the role of glucagon in diabetic man and animals. The essential pathophysiologic role of glucagon in diabetes has been a matter of controversy [10–13] the most important arguments against the bihormonal hypothesis being development of diabetic ketoacidosis despite pancreatectomy in man [14] and reduced ketoacidosis in pituitary-ablated diabetic patients (the latter finding indi-

Received: 18 February 1994
and in revised form: 5 May 1994

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Abbreviations: A-STZ rats, Adult streptozotocin injected rats; AUC, area under the curve; BW, body weight; Con-mAb, monoclonal control antibody; Glu-mAb, monoclonal anti-glucagon antibody; K_a, constant of association; NIDDM, non-insulin-dependent diabetes mellitus; N-STZ rats, neonatal streptozotocin-injected rats; OGTT, oral glucose tolerance test; STZ, streptozotocin.

cating that pituitary factors might be involved) [15]. On the other hand, it seems probable that hyperglucagonaemia, a common finding in uncontrolled diabetes [16–19], contributes to the development of diabetic hyperglycaemia. In agreement with this notion increased hepatic glucose production is also commonly found [16, 18–21]. However, at least one study [19] found no correlation between increased hepatic glucose production and glucagon levels, a relationship not examined by other studies [16, 18]. But the glucose production rate has been demonstrated to correlate to the hyperglycaemic level [16, 18–21]. Furthermore, plasma glucagon concentrations have been shown in some [17, 22], but not all patients [22, 23], to correlate with the glycaemic level.

Direct evidence for the pathophysiologic role of glucagon in diabetes can only be provided by production of selective lack or suppression of glucagon. Suppression of glucagon secretion by somatostatin is not selective in that several non-glucagon-mediated mechanisms involved in glucose homeostasis are also affected [24–26]. Pancreatectomy as a model for diabetes without glucagon is still a matter of controversy, because the physiological role of extrapancreatic glucagon remains unclear [27]. Reduction of hyperglycaemia in experimental diabetes by the use of glucagon receptor antagonists has been demonstrated [8]. Attempts in our laboratory to reproduce this using the antagonist, des-His^1 [Glu^9] glucagon amide [8], in streptozotocin (STZ) diabetic rats was unsuccessful. On the contrary we found the analogue to act as a partial agonist with a maximal effect on blood glucose in rats amounting to 42 % of that of glucagon (Brand, C. L. et al., unpublished observations).

Immunoneutralization of endogenous glucagon with glucagon antisera has been used as a model of selective glucagon deficiency in animal diabetes. Flatt and co-workers [7], in an uncontrolled study demonstrated that treatment of hyperglycaemic ob/ob mice with glucagon antibodies caused a normalization of blood glucose (after 6 h) paralleled by a 15–20 % decrease in plasma insulin. Contrary to this finding, treatment of hyperglycaemic STZ-diabetic rats with glucagon antiserum [9] did not cause changes in the resting blood glucose level, although the increment in blood glucose after an alanine load was markedly diminished by the antiserum. It has been discussed whether the variable results obtained in experiments involving immunoneutralization could be due to the nature of the antisera (low- and high-affinity clones and binding-sites occupied by glucagon originating from the donor rabbit) causing incomplete neutralization of endogenous glucagon [28].

In the present study we produced a state of selective glucagon deficiency employing excess amounts of a glucagon-free, high-affinity monoclonal glucagon antibody and studied the effect on blood glucose regulation in STZ-diabetic rats injected as neonates or adults. The studies were performed with and without insulin therapy designed to compensate partly for the insulin deficiency in severely diabetic rats injected with STZ as adults.

Materials and methods

Animals: Male Sprague Dawley rats weighing 180–220 g and pregnant rats (see below) were purchased from Møllegård breeding center (Lille Skensved, Denmark) at least 1 week before the experiments, and were allowed free access to standard rat pellets (licence produced Altromin no. 1324, C. Petersen A/S, Ringsted, Denmark) and water until the morning of the experiments. Rats were housed, four to six per cage with a 12-h dark: light cycle.

Anaesthesia and surgical procedures: All rats were anaesthetized with a mixture of Hypnorm (fentanyl, 0.05 mg/ml and fluanizone, 2.5 mg/ml: Janssen Pharma Ltd, Copenhagen, Denmark), and Dormicum (Midazolam, 1.25 mg/ml: Roche, Basel, Switzerland), 1.0 ml/kg BW s.c. and maintained at a light anaesthetic level by Hypnorm (fentanyl, 0.1 mg/ml and fluanizon, 5 mg/ml), 0.15–0.20 ml/rat i.m. every 30 min during the experiments. Decrease in normal body temperature induced by the anaesthesia was prevented using thermostatic heating blankets with probes (Homeothermic Heating Blanket System, Harvard Apparatus Ltd, Kent, UK). An indwelling catheter (Venflon 2, 22 G, Viggo-Spectramed, Helsingborg, Sweden) was placed in a tail vein for i.v. administration of compounds.

Streptozotocin (STZ) preparation: STZ (Sigma Chemical, Co., St. Louis, Mo., USA) was dissolved in ice cold citrate buffer (0.1 mol/l, pH 4.5). STZ solutions were kept on ice during the injection procedure and were used within 5 min of preparation.

Induction of moderate diabetes in neonates: Pregnant Sprague Dawley rats were received 1 week before term. Diabetes was induced in 48 2-day-old male neonates by intraperitoneal injection of STZ, 80 mg/kg BW (N-STZ rats). Citrate buffer was given to 16 control rats by the same procedure. The N-STZ rats were 16–18 months old when used for the antibody experiments (see below).

Induction of severe diabetes in adult rats. Severe diabetes was induced in CO_2-sedated, overnight-fasted adult rats by injection of STZ, 45 or 50 mg/kg BW in a tail vein (A-STZ rats). Fasting was continued for a further 2 h after STZ injection. The resulting untreated diabetes stabilized within 10 days and was characterized by means of blood glucose and body weight measurements twice weekly. Only rats with blood glucose values over 25 mmol/l were accepted as severely diabetic.

Production and characterization of monoclonal antibodies: The monoclonal antiglucagon antibody (Glu-mAb) (NovoClone GLU-001, Novo Nordisk, Bagsvaerd, Denmark) is a class IgG_1 antibody produced by a hybridoma selected from a fusion of BALB/c spleen cells and X63-AG8-6.5.3 myeloma cells. By a competitive radioimmunoassay method the association constant (K_a) was determined to $0.6 \cdot 10^{11}$ l/mol. Total binding capacity of Glu-mAb, as calculated from Scatchard analysis [28], was 40 nmol/ml. Binding studies with synthetic glucagon fragments indicated that the antibody-binding epitope was localized to segment 5–15 of the amino acid sequence of glucagon. The cross-reactivity against glicentin (a

glucagon-containing fragment of proglucagon [29]) was determined to be 100 %. There was no binding to glucagon-like peptide 1, gastric inhibitory polypeptide, glicentin-related pancreatic peptide, vasoactive intestinal polypeptide or pancreatic polypeptide. Glu-mAb was produced in cell-culture and purified and concentrated on a protein-A Sepharose column.

A hybridoma producing a monoclonal anti-2,4,6-trinitrophenyl antibody used as the control antibody (Con-mAb) was a gift from G. Köhler [30]. Con-mAb is a class IgG_1 antibody; production and concentration of the antibody were performed using the same methods as for Glu-mAb.

Analyses of plasma glucose, insulin, glucagon and glucagon binding capacity: Blood was collected into heparinized glass capillary tubes by puncture of the capillary vessels at the tail tip in order to determine the glucose concentration by the immobilized glucose oxidase method. Whole blood (10 µl) was diluted in 500 µl reagent buffer and analysed on a EBIO 6666 autoanalyser (Eppendorf, Germany). In order to determine plasma insulin (rat insulin radioimmunoassay kit, Novo Nordisk) and glucagon (pancreatic glucagon radioimmunoassay kit, Novo Nordisk) blood was collected from the orbita of lightly CO_2-sedated rats into chilled heparinized syringes which contained aprotinin (Aprotinin Novo, 500 Kallikrein inhibitor units/ml blood, Novo Nordisk). Rat insulin and porcine glucagon (identical to rat glucagon) were used as standards (Novo Nordisk). Blood for determination of glucagon binding capacity in plasma was collected in 20 µl heparinized glass capillary tubes and dissolved in 480 µl chilled saline (0.9 %) containing aprotinin. Samples were centrifuged, and glucagon binding capacity of the supernatant was determined as described elsewhere [28, 31]. The plasma glucagon binding capacity was controlled 2–3 times during the experiments and, most important, at the end of the immunoneutralization. The binding capacity was never below 30–60 pmol/ml plasma in Glu-mAb treated rats and did not decrease for the duration of the experiments.

Experimental protocols

Five different studies were performed to investigate the effects of selective glucagon deficiency on blood glucose regulation under various conditions. In cross-over designs, one half of the group of rats received Glu-mAb and the other half Con-mAb on day 1 and vice versa on day 2 of the experiments. The recovery time between two experiments in the same rat was at least 7 days. There was no measurable glucagon binding capacity in plasma obtained on day 2 in rats treated with Glu-mAb on day 1. In independent designs, rats were randomly allocated into two equally sized groups (one Con-mAb and one Glu-mAb treated) and killed after the experiment. In all experiments the mAbs were administered via the tail vein catheter at time zero as a single, 4 ml/kg BW, bolus. The Glu-mAb dose corresponded to a glucagon-binding capacity of 160 nmol/kg BW. Acute insulin treatment (Actrapid, Novo Nordisk) was accomplished by intraperitoneal injection 30 min before administration of the mAbs. Chronic insulin treatment was performed by s. c. injection of a single, daily bolus at 14.00 hours (heat-treated Ultralente, Novo Nordisk) as described by Rasch [32]. The last dose of Ultralente was given 18 h before treatment with the antibodies. Porcine glucagon (Novo Nordisk) was administered via the tail vein catheter. All experiments were performed in fed animals between 08.00 h and 13.00 hours.

1. Immunoneutralization of exogenous glucagon in normal rats. The in vivo efficacy of the dose of Glu-mAb employed was tested by immunoneutralization of exogenous glucagon. Twelve normal rats (six Glu-mAb and six Con-mAb treated) received glucagon, 2.86 nmol/kg BW 5 min after administration of the mAbs. Blood samples were collected for glucose determination at time zero and at 5-min intervals for 30 min.

2. Immunoneutralization of endogenous glucagon in N-STZ rats. In order to investigate the potential blood glucose lowering effect of immunoneutralization of endogenous glucagon in moderately diabetic rats, the N-STZ model was used. Prior to the treatment with the mAbs, plasma insulin, glucagon and blood glucose were measured in the fasted and fed state. An OGTT, 2 g/kg BW was performed in the fasted state. Glucose tolerance was quantified by measuring the incremental area under the glucose tolerance curve. Blood glucose values were determined 0, 30, 60 and 120 min after the oral glucose load (AUC_{OGTT}) and were compared to the group of normal controls. Ten N-STZ rats, with an approximate four-fold elevation in AUC_{OGTT}, were selected for a cross-over experiment with Glu-mAb and Con-mAb. Blood samples for glucose analysis were collected at 10-min intervals for 30 min and at 15-min intervals for a further 90 min. For statistical evaluation and graphic presentation, the N-STZ rats were allocated into two groups according to the initial blood glucose value (the time zero value at the day of the experiments): (Group A $\geqslant$ 15 mmol/l and Group B < 15 mmol/l).

3. Immunoneutralization of endogenous glucagon in non-insulin-treated A-STZ rats. In order to investigate the effect of combined insulin (judged by severe hyperglycaemia [33]) and glucagon deficiency on blood glucose regulation, glucagon immunoneutralization was studied in non-insulin-treated A-STZ rats. Diabetes was induced in 16 adult rats by STZ injection, 50 mg/kg. After 10 days, six rats with blood glucose values of approximately 26 mmol/l in the fed state were given the two mAbs in a cross-over design. Blood samples for glucose analysis were collected as in study 2.

4. Immunoneutralization of endogenous glucagon in A-STZ rats receiving chronic and acute insulin treatment. In order to quantify the importance of insulin for a blood glucose lowering effect of glucagon deficiency, glucagon immunoneutralization was studied in insulin-treated, severely-hyperglycaemic A-STZ rats. Diabetes was induced in 15 adult rats by STZ injection, 50 mg/kg and the rats were left untreated for 1 week. Five rats with blood glucose values over 25 mmol/l were selected for chronic low-dose insulin therapy (2 IU · rat^{-1} · day^{-1}) for 1 week. The rats were then anaesthetized and subsequently injected with Actrapid 30 min prior to administration of the two mAbs in a cross-over design. Blood for glucose analysis were collected at –30 min, zero and at 15-min intervals for 120 min. The chronic insulin therapy was continued between the 2 days of experiments. Actrapid doses were adjusted according to the blood glucose value before the experiment measured using a Refloux 2 M apparatus and Haemo-Glucotest 1-44R sticks (Boehringer Mannheim, Mannheim, Germany). The purpose of the Actrapid administration was to lower the blood glucose concentration to a level of 10–12 mmol/l during the experiment. For statistical analysis, Actrapid doses injected were calculated as number of mU insulin per mmol/l blood glucose at time –30 min as well as number of mU insulin per rat and number of units insulin per kg BW.

5. Immunoneutralization of endogenous glucagon in A-STZ rats receiving chronic insulin. In order to eliminate the variability of the effects of acute insulin treatment as given in study 4, a more stringent chronic insulin therapy was introduced designed to cause a moderate hyperglycaemic level in A-STZ rats. Diabetes was induced in 30 adult rats by injection of

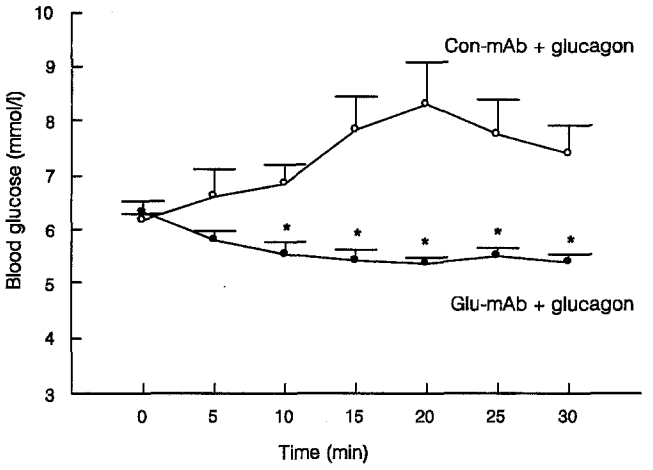

Fig. 1. Neutralization of exogenous glucagon by Glu-mAb (●, $n = 6$) in normal rats. Con-mAb was used as control antibody (○, $n = 6$). Antibodies (4 ml/kg) were given at time zero and glucagon (2.86 nmol/kg) 5 min later. Values are means ± SEM. * $p < 0.05$ vs the control group

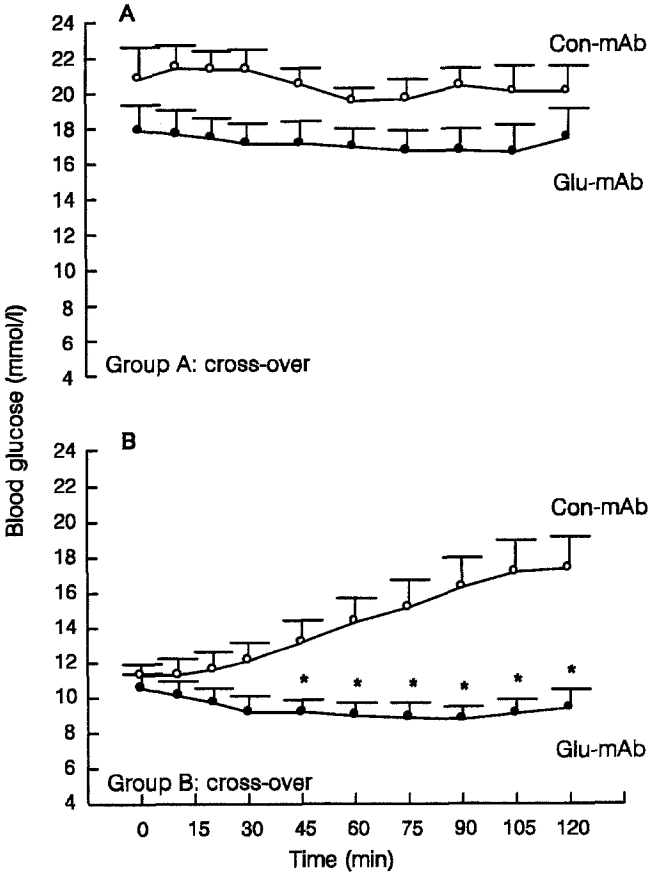

Fig. 2 (A, B). Effects of Glu-mAb treatment (●) on blood glucose in severely (Group A, $n = 4$) and moderately (Group B, $n = 6$) diabetic rats injected with STZ as neonates. Con-mAb was used in control studies (○). Antibodies (4 ml/kg) were given at time zero in a cross-over design. Values are means ± SEM. * $p < 0.05$ vs the Con-mAb group

STZ, 45 mg/kg. After 10 days rats with blood glucose levels over 25 mmol/l ($n = 27$) received a chronic medium-dose insulin therapy (3 IU · rat^{-1} · day^{-1}) for 2 weeks. The rats were then anaesthetized and a blood sample was taken from each rat in order to determine the glucose concentration. Based on these glucose measurements, 18 rats with values at approximately 10 mmol/l were randomly allocated into two equally-sized groups given either Glu-mAb or Con-mAb at time zero. Blood was sampled for glucose measurements as in study 2.

Statistical analyses

Results are presented as means ± SEM. Student's *t*-test for unpaired and paired data was performed in order to test differences between two groups in unpaired and cross-over designs, respectively. Means from more than two samples were compared using analysis of variance (ANOVA) including the Bartlett test for homogeneity of group variance. Correlation studies were performed using univariate linear regression analysis. Delta-AUC_{BG} (incremental or decremental area under the blood glucose curve) was calculated for each blood glucose profile in order to test differences in the overall effects of the compounds. *P*-values less than 0.05 were considered significant. All statistics were calculated using Systat 5.1 for Macintosh computer program.

Results

In vivo glucagon-neutralizing efficacy of Glu-mAb in normal rats. As indicated in Figure 1, glucagon administration to normal rats caused a significant maximal increase in the blood glucose level of 2.1 ± 0.8 mmol/l above the initial value (6.2 ± 0.1) in the Con-mAb group after 20 min (ANOVA: $p < 0.01$) whereas the glucose level decreased by 1.0 ± 0.2 mmol/l below the initial value (6.3 ± 0.2) in the Glu-mAb treated rats (ANOVA: $p < 0.01$). Calculated as delta-AUC_{BG}, the overall effect of Glu-mAb and glucagon was −22 ± 7 vs 36 ± 15 min · mmol/l in the control experiment ($p < 0.005$).

Glucagon deficiency in moderately diabetic N-STZ rats. Characteristics of the two subgroups of N-STZ rats (A and B; see Methods section) compared to normal controls are presented in Table 1. The overall AUC_{OGTT} was increased 3.5 to 4.2-fold ($p < 0.0005$ vs controls). In the fasted state the blood glucose level did not differ from that of the normal controls whereas all diabetic rats were significantly hyperglycaemic in the fed state although the glycaemic levels of the two groups varied. In the fed state, plasma glucagon was only significantly elevated in group A ($p < 0.001$ vs normal controls), but for the whole group plasma glucagon levels correlated positively to the glycaemic levels ($r = 0.548$, $p < 0.004$).

Figure 2 a and 2 b show the data obtained from the N-STZ group A and B, respectively. In the less hyperglycaemic group B (initial values in mmol/l: 10.4 ± 0.9 before Glu-mAb and 11.2 ± 0.7 before Con-mAb

Table 1. Basal characteristics of the two groups (A and B) of diabetic rats injected with STZ as neonates (N-STZ rats) in the fed and fasted (18 h) state compared to a normal control group injected with citrate buffer

Group *n*	Fed rats			Fasted rats			
	BG (mmol/l)	IRI (pmol/l)	IRG (pmol/l)	BG (mmol/l)	IRI (pmol/l)	IRG (pmol/l)	AUC_{OGTT} (min · mmol/l)
Control 16	5.7 ± 0.1	361 ± 39	24 ± 2	4.2 ± 0.1	35 ± 5	20 ± 2	255 ± 16
N-STZ group A 4	16.0 ± 2.0[a]	415 ± 135	41 ± 6[a]	4.3 ± 0.4	70 ± 19	13 ± 3	1081 ± 87[a]
N-STZ group B 6	10.0 ± 0.9[a]	439 ± 80	29 ± 1	4.4 ± 0.2	79 ± 37	14 ± 2	890 ± 40[a]

BG, Blood glucose, IRI and IRG, immunoreactive plasma insulin and glucagon. AUC_{OGTT} (area under the glucose tolerance curve) was based on blood glucose values determined at time 0, 30, 60 and 120 min after an oral glucose load (2 g/kg) in the overnight fasted rats (see Methods). Values are presented as mean ± SEM. [a] $p < 0.001$ vs controls (see also Fig. 2 a, b)

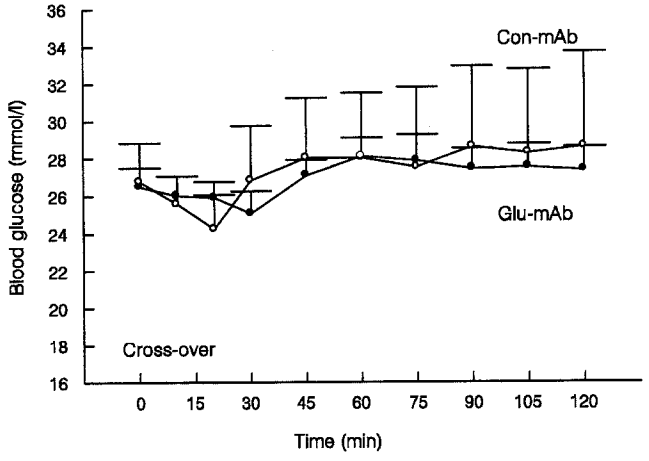

Fig. 3. Effects of Glu-mAb treatment (●) on blood glucose in severely diabetic rats injected with STZ as adults. Con-mAb was used in control studies (○). Antibodies (4 ml/kg) were given at time zero in a cross-over design ($n = 6$). Values are means ± SEM

treatment, NS), administration of Glu-mAb caused no significant decrease in blood glucose (nadir: 8.7 ± 0.8 mmol/l at 90 min, ANOVA: NS). However, a strong increase in blood glucose was observed in the control experiment (maximum: 17.3 ± 1.8 mmol/l at 120 min, ANOVA: $p < 0.004$). The overall effect as calculated by delta-AUC_{BG} was −151 ± 15 for Glu-mAb vs 358 ± 86 min · mmol/l for Con-mAb ($p < 0.003$). The maximal difference in blood glucose in the two experiments was 7.9 ± 1.2 mmol/l ($p < 0.005$). No significant effect of Glu-mAb was observed in the more hyperglycaemic group A (initial values in mmol/l: 17.9 ± 1.4 before Glu-mAb and 20.8 ± 1.8 before Con-mAb treatment, NS). The delta-AUC_{BG} was −106 ± 53 for Glu-mAb vs −42 ± 95 min · mmol/l for Con-mAb (NS).

Glucagon deficiency in non-insulin-treated severely hyperglycaemic A-STZ rats. As indicated in Figure 3, no effect of Glu-mAb on the glucose level was observed in non-insulin-treated severely hyperglycaemic A-STZ (ANOVA: NS) (initial values in mmol/l: 26.5 ± 1.0 before Glu-mAb and 26.7 ± 2.1 before Con-mAb treatment, NS). Calculated as delta-AUC_{BG}, the effect of Glu-mAb was 60 ± 80 vs 79 ± 196 min · mmol/l for Con-mAb (NS).

Glucagon deficiency combined with chronic plus acute insulin treatment in A-STZ rats. The chronic insulin treatment resulted in blood glucose values ranging from 20 to 24 mmol/l, The initial blood glucose values and doses of Actrapid injected 30 min prior to the mAb treatment are presented in Table 2. There were neither significant differences between the initial blood glucose values nor between the acutely administered insulin doses in the two experiments. In the control study (Actrapid + Con-mAb) the blood glucose level decreased from 22.1 ± 2.3 mmol/l at −30 min to 12.7 ± 4.3 mmol/l at 120 min while it decreased from 23.5 ± 3.5 mmol/l to 8.0 ± 1.4 mmol/l during the same period of time as a consequence of Actrapid and subsequent Glu-mAb treatment. As indicated in Figure 4, blood glucose was calculated as changes from the initial value (delta values) in order to compensate for the slight, non-significant difference in the initial glucose values in the two experiments. At the end of the experiment the difference in blood glucose in the two experiments was 6.0 ± 3.4 mmol/l ($p < 0.05$). Calculated as delta-AUC_{BG}, the overall effect of the combined insulin and Glu-mAb treatment was −1424 ± 172 vs −915 ± 281 min · mmol/l for insulin and Con-mAb ($p < 0.05$). The changes in blood glucose from time zero (time for mAb treatment) to 120 min was only significant after the Glu-mAb treatment (ANOVA: $p < 0.045$).

Combined glucagon deficiency and chronic insulin treatment in A-STZ rats. The chronic insulin treatment led to initial blood glucose values at 10.6 ± 0.7 mmol/l in the Glu-mAb group and 10.0 ± 0.8 mmol/l in the Con-mAb group (NS). As indicated in Figure 5, a significant decrease in blood glucose of 1.0–2.0 mmol/l below the initial value was observed 20–60 min after the Glu-mAb administra-

Table 2. Initial blood glucose (BG) values and acute Actrapid doses given 30 min prior to the antibody treatment in diabetic rats injected with STZ as adults (A-STZ rats)

Group Cross-over $n = 5$	Initial BG (mmol/l)	Insulin[a] (mU/rat)	Insulin[b] (U/kg)	Insulin[c] (mU · (mmol/l)$^{-1}$)
Con-mAb + insulin	22.1 ± 2.3	295 ± 61	1.26 ± 0.28	12.7 ± 2.1
Glu-mAb + insulin	23.5 ± 3.5	297 ± 33	1.38 ± 0.16	13.0 ± 1.2

Con-mAb, Control antibody; Glu-mAb, glucagon antibody. Acute insulin doses given are expressed as; [a] number of mU insulin given per rat; [b] number of U insulin given per kg body weight; [c] number of mU insulin given per mmol/l blood glucose determined at time –30 min (see also Fig. 4)

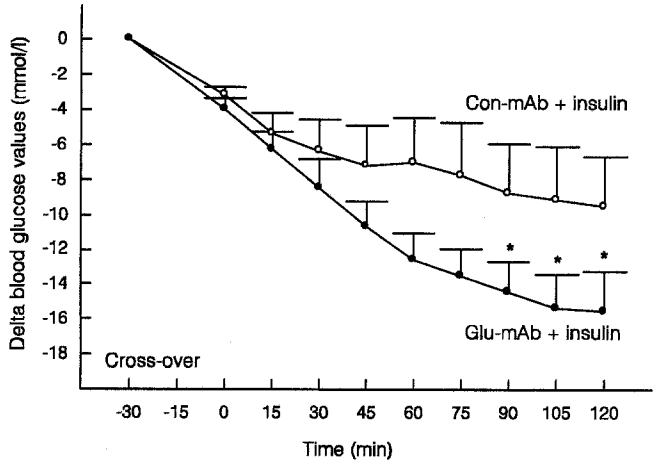

Fig. 4. Effects of Glu-mAb treatment (●) on blood glucose in chronic insulin treated (2 IU · rat^{-1} · day^{-1}) severely diabetic rats injected with STZ as adults. The rats were acutely injected with Actrapid 30 min prior to the antibody treatment (–30 min). Con-mAb was used in control studies (○). Antibodies (4 ml/kg) were given at time zero in a cross-over design ($n = 5$). See Table 2 for absolute, initial blood glucose values (–30 min) and Actrapid doses. Values are means ± SEM. * $p < 0.05$ vs the Con-mAb group

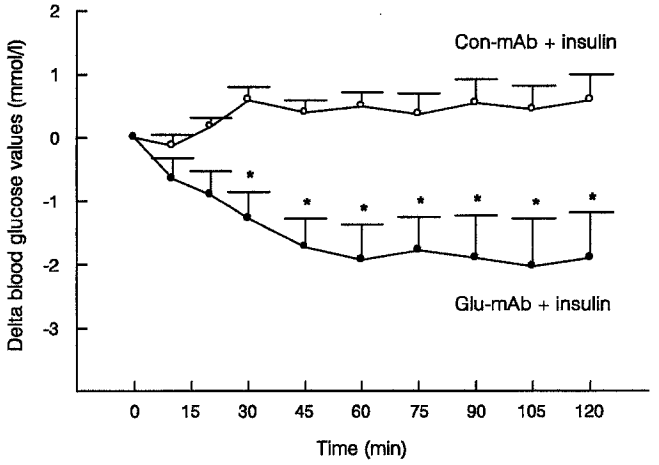

Fig. 5. Effects of Glu-mAb treatment (●, $n = 9$) on blood glucose in chronic insulin treated (3 IU · rat^{-1} · day^{-1}) severely diabetic rats injected with STZ as adults. Con-mAb was used in control studies (○, $n = 9$). Antibodies (4 ml/kg) were given at time zero. Values are means ± SEM. * $p < 0.05$ vs the Con-mAb group

tion (ANOVA: $p < 0.025$) whereas no changes in blood glucose occurred in the Con-mAb group (ANOVA: NS). Delta-AUC_{BG} in the Glu-mAb group was –187 ± 59 vs 31 ± 31 min · mmol/l in the Con-mAb group ($p < 0.005$).

Discussion

In the present investigation we produced an extensive immunoneutralization of endogenous glucagon in diabetic rats using excess amounts of a high-affinity, glucagon-free mAb in order to obtain reliable data concerning the effect of selective glucagon deficiency on blood glucose regulation. The association constant of Glu-mAb calculated by Scatchard plot analysis was more than 200 times higher than those reported for the native [34] and the cloned [35] rat glucagon receptor (K_a: 2.5 · 10^8 and 0.27 · 10^8 l/mol, respectively) favouring binding to Glu-mAb rather than to the receptor. The in vitro binding capacity of Glu-mAb was 40 nmol per ml antibody solution corresponding to a glucagon binding capacity of approximately 40 nmol glucagon per rat (4 ml/kg) which equals more than 10 times the total pancreatic glucagon content (3 nmol glucagon per pancreas from rats weighing 300 g) [36]. During the experiments, plasma binding capacity was never below 30–60 pmol/ml plasma corresponding to 700–1400 times the highest plasma glucagon concentration measured in the diabetic rats. The glucagon binding capacity in plasma obtained in this study is 35–70 times higher than that obtained in previous studies performed with polyclonal glucagon antisera which, furthermore, mainly contain clones with lower affinity than Glu-mAb [9, 37]. Taken into account that a moderately stimulated rat pancreas releases 0.032–0.052 nmol glucagon per h [38] the binding capacity of the amounts of Glu-mAb employed should therefore greatly exceed the amounts of endogenous glucagon expected to be secreted within the duration of the present experiments (2 h).

These considerations are supported by our observation that the hyperglycaemic effect of a large dose of glucagon given to the normal rats (0.76 nmol per 266 g BW) was completely abolished by Glu-mAb. The amount of exogenous glucagon administered in

this set of experiments equals more than one quarter of the total glucagon content in a rat pancreas [36] and more than seven times the glucagon released from pancreas over a 2-h period [38]. Furthermore, the dose of glucagon neutralized in the present experiment was 10 to 100 times higher than that immunoneutralized in previous studies using polyclonal glucagon antisera [28, 39]. Concentrations of free glucagon after Glu-mAb treatment were not measured in this study, but results from similar experiments with polyclonal glucagon antisera containing mixtures of clones with equal or lower affinities than Glu-mAb indicate that the plasma concentration of free and thereby active glucagon should be very low [28]. In agreement with our assumption that most of the endogenous glucagon was efficiently neutralized by Glu-mAb we found that immunoneutralization of exogenous glucagon caused blood glucose levels to fall below the initial value, a result which could not be demonstrated in similar experiments with polyclonal antisera [28, 39, 40].

The diabetes of the N-STZ rats has several of the characteristics of NIDDM in man [41]. The animals are not ketosis prone, they retain substantial beta-cell function, and hyperglycaemia is most pronounced in connection with food intake. The effect of STZ in neonates varies from rat to rat, and it was therefore necessary to characterize and group the animals. The individual animals will also display some day-to-day variation, as reflected in the minor differences between the blood glucose levels at the initial screening and on the days of the experiments. We therefore mainly used cross-over designs in our studies. The N-STZrats could be divided into groups with high and low levels of resting blood glucose. The difference in blood glucose between the more severe diabetic group A and the milder form in group B may be explained by a lower insulin/glucagon ratio in the former group. The diabetic state of both groups can be described as mild, as the fasting glucose levels did not differ from that of normal controls, but in the fed state there was, nevertheless, a more pronounced hyperglycaemia in group A compared to B, and we therefore considered group A to have a more severe form of non-insulin-dependent STZ diabetes.

Immunoneutralization of glucagon did not have any effect on blood glucose in the N-STZ rats from group A, whereas development of a further postprandial increase in blood glucose was prevented by Glu-mAb treatment in the milder diabetic group. Lickley et al. [42] demonstrated that suppression of glucagon by somatostatin only lowers blood glucose in alloxan diabetic dogs with preserved residual insulin capacity but not in totally insulin insufficient pancreatectomized dogs (dogs secrete "true" glucagon (IRG^{3500}) from the gastric fundus [43]). However, in our study none of the diabetic N-STZ rats were totally insulin deficient, but rather tended to be hyperinsulinaemic. Thus, our data seem to indicate that both insulin secretion and insulin resistance influence the effect of glucagon on blood glucose. Our observations lead to the working hypothesis that a decrease in blood glucose, caused by immunoneutralization of endogenous glucagon, could only be expected in conditions where metabolism was not markedly affected by severe insulin deficiency in which case peripheral glucose disposal is at a minimum and lipolysis and substrate-driven hepatic gluconeogenesis at a maximum. In order to test this hypothesis further we turned to the severely insulin-deficient A-STZ model. In these animals with postprandial blood glucose values around 27 mmol/l Glu-mAb treatment also, as expected, had no effect. However, after lowering of blood glucose to moderately hyperglycaemic levels by exogenous insulin, glucagon neutralization induced near normal glucose levels in two independent designs, in agreement with the hypothesis. Furthermore, our results may also explain the previously demonstrated lack of a glucose lowering effect of immunoneutralization with polyclonal glucagon antiserum in severely STZ-diabetic rats [9]. Our findings are in agreement with biochemical studies by McGarry and Foster [44] who demonstrated that insulin deficiency increases glucagon secretion which causes an unopposed increase in cAMP-mediated activation of protein kinase A in liver cells. Only insulin can reverse this; i.e. glucagon neutralization will not reverse the increased protein kinase A once it has been established.

The results obtained in the less diabetic N-STZ rats from group B and in the insulin-treated severely-diabetic A-STZ rats demonstrate that the effect of immunoneutralization persisted throughout the 2-h study period with no signs of blood glucose returning to the initial level, indicating that no adaptation to glucagon deficiency occurred. Furthermore, in the N-STZ rats the hyperglycaemic levels seen in the control experiments as opposed to the Glu-mAb experiments indicate that the circulating unneutralized glucagon apparently exerted a persistent hyperglycaemic effect.

Another important finding was that none of the animals became hypoglycaemic. The reasons for this are not clear, but several counterregulatory mechanisms may be responsible (ex. decreased insulin secretion and/or increased activity of the sympathoadrenergic system). These mechanisms are evaluated in our laboratory at present using the Glu-mAb.

Our findings represent acute and complete glucagon deficiency in experimental diabetes, and the results, therefore, cannot be easily extended to the relative and chronic hyperglucagonemia observed in diabetic subjects. However, they might indicate that glucagon antagonism in diabetic subjects could be beneficial, but potent and clinically useful glucagon antagonists are not available at present.

In conclusion, monoclonal glucagon antibody therapy normalizes blood glucose levels in moderately diabetic N-STZ rats. Severely diabetic STZ rats are insensitive to glucagon neutralization unless the diabetes is moderately controlled by insulin during or prior to the experiment. Immunoneutralization of endogenous glucagon does not cause hypoglycaemia in STZ-diabetic rats. The data support the hypothesis that glucagon antagonism could provide a therapeutic approach to the treatment of hyperglycaemia in moderate NIDDM.

Acknowledgements. The authors wish to thank Ms. K. F. Press, Ms. B. Hansen, Ms. L. Rabenhøj, B. Hansen and Ms. K. Arevad for their excellent technical assistance and Richard D. Carr and Mads K. Thomsen for their advice in the preparation of this manuscript.

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