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A novel insulin formulation can keep providing steady levels of insulin for much longer periods in-vivo

Mitsuko Takenaga, Yoko Yamaguchi, Aki Kitagawa, Yasuaki Ogawa, Yutaka Mizushima and Rie Igarashi

Abstract

We have recently succeeded in preparing insulin-loaded microcapsules that release the insulin in a strictly controlled manner with little initial rapid release in-vitro or in-vivo. We show here the superiority of the best formulation prepared with co-poly(D,L-lactic/glycolic) acids (PLGA) (mean MW 5800, L/G ratio 50:50) with a main diameter of 15 ~ 30 μm in-vivo. When 3.2% insulin-loaded PLGA microcapsules were subcutaneously given as a single dose to streptozotocin-induced hyperglycaemic rats (250 U kg^{-1}), plasma insulin levels gradually increased and constant levels (30.3–94.1 μU mL^{-1}) were sustained. Rats receiving the formulation once a week showed not only steady plasma insulin levels, but also gained weight at a similar speed to normal rats. Meanwhile, daily treatment with Humulin U (25 U kg^{-1}) caused a transient high insulin level (2723.9 μU mL^{-1} at 1 h) in plasma, but the body weight of the rats was little changed. A pharmacological study in female Cynomolgus monkeys also revealed that the microcapsular formulation provided a flat release of insulin for longer periods and showed no immunogenic activity. In the near future, therefore, this insulin formulation could become very beneficial as a provider of basal insulin levels for insulin-dependent diabetic patients.

Introduction

The beneficial effects have been firmly established of achieving blood glucose values as close to the normal range as possible in type I diabetic patients (Kawamori 1994; Haak 1999). Namely, this therapy contributes to the improvement of quality of life and the reduction of additional diseases. At present, patients have to keep taking one or more doses of intermediate- or long-acting insulin preparations, in addition to that taken every mealtime, to satisfy the basal insulin requirement. If there is an insulin formulation that could provide release in a strictly controlled fashion for longer periods, patients could be released partly from the necessity of multiple treatments.

Much attention has been focused on the development of a longer-acting and sustained-release formulation of insulin (Gershonov et al 1999; Owens et al 2000; Pieber et al 2000). Insulin glargine, one of them, is progressing towards clinical use (Pieber et al 2000). Studies in animals and healthy humans have demonstrated it to exhibit a protracted action profile compared with NPH insulin, which is characterized by a relatively flat plasma insulin concentration profile. However, the period of sustained action is confirmed as being within 24 h, and investigators have not succeeded in developing a formulation with a more sustained release for clinical application (Creque et al 1980; Goosen et al 1983). The main reason appears to be that the formulations have failed to prevent the initial rapid release (burst). This burst, which causes severe hypoglycaemia, is such a critical factor that it must be prevented. At the same time, a low peak/trough ratio is desirable to prevent hypoglycaemia. This is because the change in plasma insulin levels can sensitively affect the balance of glucose production and utilization (Rizza et al 1981). Our attention was therefore focused on developing a formulation to reduce the burst and achieve a prolonged, relatively flat, release of insulin.

As a result of our aggressive investigation, we have recently succeeded in developing a novel preparation, which continues to release insulin with little burst following a single

Institute of Medical Science, St Marianna University School of Medicine, Kawasaki, Japan

Mitsuko Takenaga, Yoko Yamaguchi, Aki Kitagawa, Yasuaki Ogawa, Yutaka Mizushima, Rie Igarashi

Correspondence: M. Takenaga, Institute of Medical Science, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8512, Japan. E-mail: m2take@marianna-u.ac.jp

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administration (Takenaga et al 2002; Yamaguchi et al 2002). The reduced burst from the formulation in-vivo was also observed in-vitro. There was a good correlation between the two. Further investigation resulted in the preparation of the best formulation. The aim of this study is to show that the best formulation could be promising in providing basal secretary insulin levels for insulin-dependent diabetes mellitus (IDDM) patients in the future. With this aim, we administered this insulin formulation not only to streptozotocin-induced hyperglycaemic rats but also to normal monkeys, and the plasma insulin levels were monitored. An intermittent treatment study was also performed to reveal a steady release of insulin from microcapsules for a longer period of time. In addition, a comparative study was carried out in hyperglycaemic rats, with a long-acting insulin used clinically.

Materials and Methods

Reagents

Human recombinant insulin (MW 5807.6, 26 U mg^{-1}) was purchased from Wako Pure Chemical Ind. Ltd (Osaka, Japan). Co-poly(D,L-lactic/glycolic) acid (PLGA; mean MW 5800, L/G ratio 50:50) was synthesized and kindly provided by Wako Pure Chemical Ind. Ltd (Osaka). Humulin U was purchased from Eli Lilly Japan K. K. (Kobe, Japan).

Insulin-containing PLGA formulations

To minimize the size of insulin particles, human insulin solution (10 mg mL^{-1}) was dropped into liquid nitrogen and lyophilized.

Insulin-loaded PLGA microcapsules were prepared by the solvent evaporation method (Takenaga et al 2002) with a modification. In brief, 240 mg of insulin, 39.9 mg of zinc oxide and 7.84 g of PLGA dissolved in 8 mL of methylene chloride were mixed and agitated vigorously to form a solid/oil (S/O) suspension. At this time, glycerol (240 mg) and distilled water (108 mg) were added for the S/O suspension to become a solution (Takenaga et al 2002). This solution was poured into 1.6 L of 1.0% w/v polyvinyl alcohol (GOHSENOL:EG-25, average MW 45000; Nippon Gosei Kagaku Inc., Tokyo, Japan) solution under stirring. This emulsion was stirred at 1200 rev min^{-1} for 3 h to evaporate the organic solvent and obtain microcapsules. The microcapsules were washed 3 times with distilled water by centrifugation and then sieved with a 125-μm screen to remove larger particles. Mannitol was added to the resulting microcapsules to prevent aggregation, and they were then lyophilized. The insulin content of the prepared microcapsules was determined after extraction with methylene chloride and 0.01 M HCl according to the method of Lowry et al (1951), using the insulin as a standard. The encapsulation efficiency was more than 95%.

Electron microscopy

The morphology of formulations was examined under an SEM 4300 scanning electron microscope (Hitachi, Ibaraki) at an acceleration voltage of 1.0 kV.

Animals

Seven-week-old male Wistar rats (220–240 g) were purchased from SLC Experimental Animals (Shizuoka, Japan). Rats were housed at a constant temperature (23±1°C) and humidity (50–60%) with free access to a standard diet and water. The animal room had a 12-h light–dark cycle (lights on at 0630 h). The study protocol was approved by the animal experimentation committee of St Marianna University.

Female Cynomolgus monkeys were housed at the primate quality control centre of INA Research, Philippines, Inc. (Batangas, Philippines). They were housed individually in stainless-steel cages under monitored temperature and humidity. Each monkey was provided with biscuits and bananas daily in the afternoon.

Animal experiments

Streptozotocin-induced hyperglycaemic rats were used. Three days after rats intravenously received 60 mg kg^{-1} of streptozotocin dissolved in 10 mM citrate–sodium citrate buffer (pH 4.5), they were given a subcutaneous injection of insulin-loaded PLGA microcapsules. Just before administration, the insulin formulations were dispersed (20% w/v) with 5% mannitol solution pH 6.5 containing 0.5% carboxymethylcellulose and 0.1% Tween 80. Blood samples were taken from the inferior ophthalmic vein before and after treatment to measure plasma insulin and blood glucose concentrations. These samples were all taken in the morning (0900–1130 h), except the sample taken 6 h after dosing. Plasma insulin was determined using an RIA kit (Shionogi Seiyaku Co. Ltd, Osaka, Japan). Blood glucose levels were determined by the glucose oxidase method using a glucose analyser (Glucoster-M, Sankyo Co. Ltd, Tokyo, Japan) immediately after blood collection. Streptozotocin-treated rats tested in this study were all hyperglycaemic. Insulin levels in normal rats (non-treated) were in the range 20.4–51.4 μU mL^{-1}, and their blood glucose levels were 98–150 mg dL^{-1}. The blood glucose concentration was more than 290 mg dL^{-1}, when measured in the morning, and the plasma insulin level was 3.0–5.6 μU mL^{-1}. The insulin level in normal plasma (from non-treated rats) was 20.4–51.4 μU mL^{-1} and the normal blood glucose level was 98–150 mg dL^{-1}.

The monkeys of the test article administration groups were determined to be 4–9 years old with a body weight range of 3.62–4.26 kg at the time of initial administration. Their health records showed them to be in good health. Dosing was performed in a similar manner as in the rats. Blood samples were collected from the saphenous vein using a polypropylene syringe. Plasma samples in monkeys were used for the determination of insulin and C-peptide levels. C-peptide levels were determined using an RIA kit

(Shionogi, Osaka). Although both RIA kits were for humans, there was a positive correlation between insulin and C-peptide concentration in untreated normal monkeys (r = 0.716). Exogenous insulin levels after dosing were therefore calculated from both data. Plasma insulin levels before administration were 9.8–60.6 μU mL^{-1}.

Immunogenic activity of microsphere formulations

Antibody formation was determined by incubating plasma with ^{125}I-labelled insulin, and precipitating the antigen–antibody complex with polyethylene glycol (PEG) (25%). After centrifugation, the pellets were counted. The formation against human insulin was evaluated by comparing the count with that of normal plasma.

Statistical analysis

Results are represented as the mean ($\pm$s.d.). Where the analysis of variance was significant ($P < 0.05$), post-hoc comparisons were performed using Bonferroni's test to compare significant differences among groups.

Results

Sustained insulin release from PLGA microcapsules in streptozotocin-induced hyperglycaemic rats and normal monkeys

Morphological examination using a scanning electron microphotograph revealed that the insulin-loaded PLGA microcapsules had smooth and spherical surfaces with a main diameter of 15–30 μm. When 250 U kg^{-1} of 3.2% insulin-loaded PLGA microcapsules was given to diabetic rats, plasma insulin levels were gradually increased (Figure 1). Plasma insulin levels of 12.9, 28.8 and 47.7 μU mL^{-1} were detected at 1, 2 and 6 h, respectively. The level was 58.2 μU mL^{-1} on day 1, and remained at 30.3–94.1 μU mL^{-1} from the second to the ninth day. The ratio of $AUC_{0\text{–day }1}/AUC_{0\text{–day }14}$ was calculated to be 10.5%. Little of the microcapsules remained at the injection site on the fourteenth day, when 8.8 μU mL^{-1} insulin was detected in plasma.

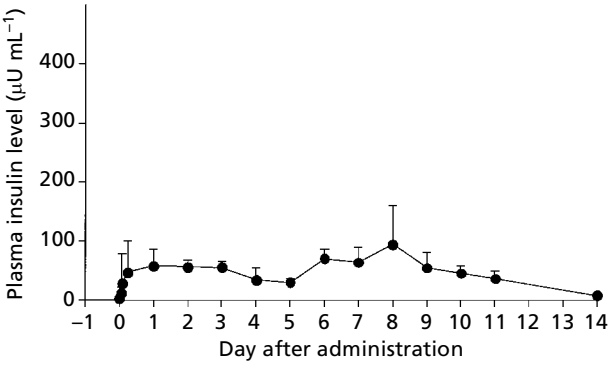

Figure 1 Plasma insulin levels following a single subcutaneous treatment of insulin-loaded PLGA microcapsules to diabetic rats. The 3.2% insulin-loaded PLGA microcapsules were subcutaneously administered as a single dose (250 U kg^{-1}) to streptozotocin-induced hyperglycaemic rats. Data are means$\pm$s.d., n = 5.

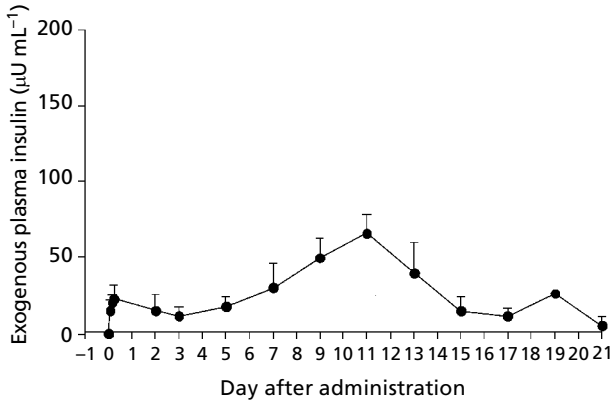

Figure 2 Exogenous insulin levels following a single subcutaneous dose (400 U/head) to female Cynomolgus monkeys. The 3.2% insulin-loaded PLGA microcapsules were subcutaneously administered as a single dose (400 U/head) to female Cynomolgus monkeys. Exogenous plasma insulin levels were calculated from insulin and C-peptide levels. Data are means$\pm$s.d., n = 4.

The insulin-loaded PLGA microcapsules were also subcutaneously administered as a single dose (400 U/head) to monkeys (n = 4). Since they secrete insulin in response to blood glucose levels, their exogenous insulin levels needed to be calculated from their insulin and C-peptide levels. Although both RIA kits were for humans, blood sampling was also performed in untreated monkeys (n = 2) at the same time as in treated ones, and a positive correlation between insulin and C-peptide concentration was confirmed (r = 0.716). Exogenous insulin levels of 14.8, 20.1 and 22.7 μU mL^{-1} were detected at 2, 4 and 6 h, respectively (Figure 2). The level was 15.2 μU mL^{-1} on day 2, and remained at 15.4–65.8 μU mL^{-1} from the third to the seventeenth day. The ratio of $AUC_{0\text{–day }2}/AUC_{0\text{–day }21}$ was calculated to be 12.3%. In the monkeys receiving the PLGA formulation, the second release from the microcapsules created a peak of 65.8 μU mL^{-1} on day 11.

Intermittent treatment of streptozotocin-induced hyperglycaemic rats and normal monkeys with the insulin formulation

Insulin-loaded PLGA (125 U kg^{-1}) was given to diabetic rats once a week, and plasma insulin levels were monitored. As shown in Figure 3A, plasma insulin levels were relatively constant (45.8–124.4 μU mL^{-1}). After each administration, constant weight gain was observed (Figure 3B); the speed of weight gain was as fast as that in normal rats. Once the treatment was stopped, body weight gradually fell with pretty low plasma insulin level. After a time, no weight gain was observed as a result of the administration of streptozotocin alone.

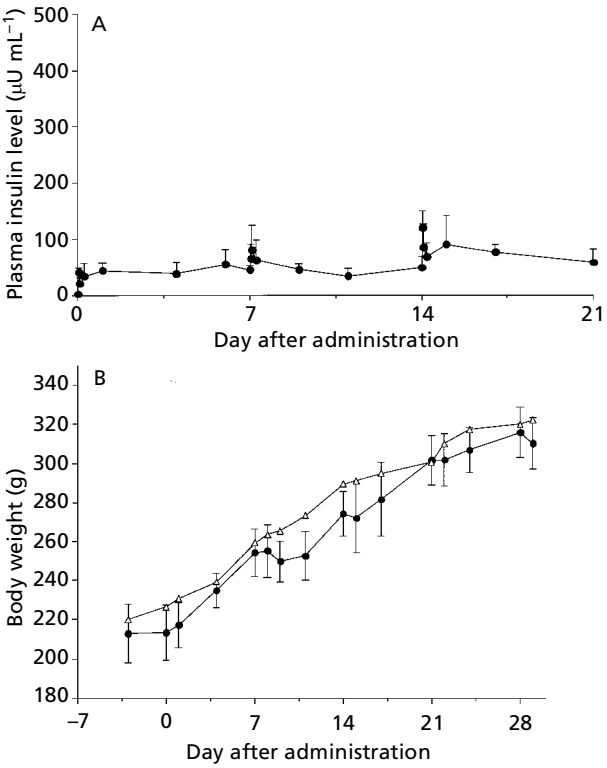

Figure 3 Plasma insulin levels (A) and body weight (B) of diabetic rats following intermittent subcutaneous treatment with insulin-loaded PLGA microcapsules. The 3.2% insulin-loaded PLGA microcapsules were subcutaneously administered (125 U kg^{-1}) once a week to streptozotocin-induced hyperglycaemic rats. Data are means±s.d., n = 5. Mean body weight of normal animals (B, open triangles) are also shown, n = 5.

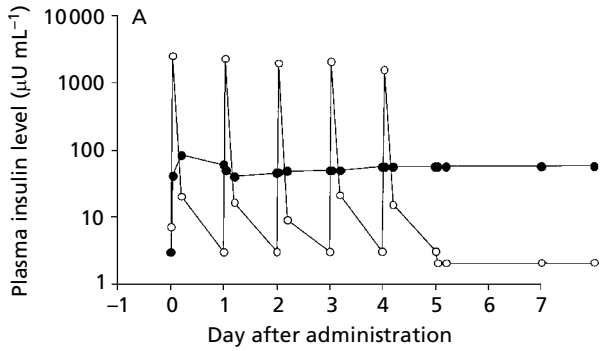

Figure 4 Comparison of plasma insulin levels in diabetic rats following successive once-daily treatments with Humulin U (open circles) and those of a single treatment of insulin-loaded PLGA microcapsules (closed circles). Long-acting insulin preparation, Humulin U (25 U kg^{-1}), was subcutaneously administered to streptozotocin-induced hyperglycaemic rats once a day; treatments were carried out 5 times (arrows), n = 5. The single treatment with the insulin-loaded PLGA microcapsules was given in a dose of 125 U kg^{-1}, n = 5.

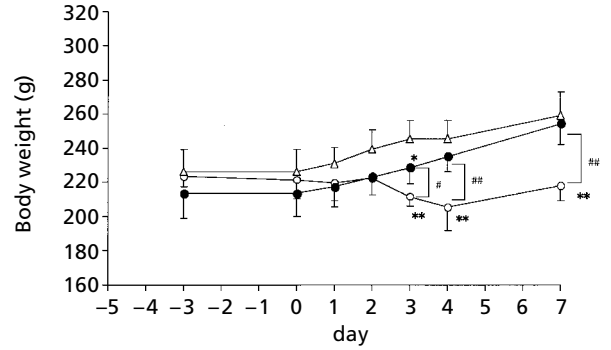

Figure 5 Comparison of body-weight change following a single treatment with insulin-loaded PLGA microcapsules with that following successive treatments of Humulin U in diabetic rats. Body weights were monitored before and after administration of insulin preparation (closed circles, 125 U kg^{-1} of insulin-loaded PLGA microcapsules; open circles, 25 U kg^{-1} daily for 5 days of Humulin U, with administration indicated by arrows) to streptozotocin-induced hyperglycaemic rats. Body weight of normal rats (triangles) was also monitored. Data are means±s.d., n = 5. $*P < 0.05$, $**P < 0.01$ vs normal rats; $\#P < 0.05$, $\#\#P < 0.01$ insulin-loaded PLGA microcapsules-treated group vs Humulin U-treated group (Bonferroni's test).

The immunogenic potency of insulin released from the microspheres was checked after multiple treatments. The precipitating count of normal plasma (untreated rats) with PEG was around 20% of the applied total radioactivity (^{125}I-human insulin) (n = 5). Three out of eight treated rats had counts of more than 30% (37%, 40%, 94%). An intermittent treatment study was also performed in monkeys for the determination of immunogenic potency of PLGA formulation. There were no anti-insulin antibodies in monkey plasma at the end of three once-weekly treatments (200 U/head) (22.3%, 23.5%, 23.9%, 27.0%, 24.2% vs the control 22.4%, 25.0%).

Comparison of plasma insulin levels following a single subcutaneous administration of insulin-loaded PLGA with those following daily administration of insulin solution

When a long-acting insulin preparation, Humulin U, was subcutaneously administered to hyperglycaemic rats (25 U kg^{-1}), the rapid insulin increase in plasma, associated with hypoglycaemia, was observed (less than 50 mg dL^{-1}). The insulin levels sharply increased up to 2723.9 μU mL^{-1} at 1 h, 2788.9 μU mL^{-1} at 2 h, and then decreased quickly. Six hours later, it was less than 3 μU mL^{-1}.

Figure 4 shows plasma insulin levels following once-daily treatment of rats with Humulin U (25 U kg^{-1}) for 5 days. The data following a single dose of 125 U kg^{-1} of the insulin-loaded PLGA microcapsules are also shown. It was obvious that a single injection of this preparation gave diabetic rats a lower but constant level of insulin.

During treatment of rats with Humulin U, their body weight changed little (Figure 5), being in the range of

200–229 g on the first dosing day and 210–222 g on day 7. The insulin-loaded PLGA augmented their body weight (197–235 g on the dosing day and 236–271 g on day 7). There was a significant difference on the third, fourth and the seventh day between the two. Significant difference in body weight was not observed on the first dosing day and day 1 between the Humulin U-treated rats and normal rats, but on the other days, there were significant differences. The body weight of rats treated with the microsphere insulin formulation only differed significantly from the body weight of normal rats on day 3.

Discussion

Our novel formulation provided a relatively flat release of insulin from microcapsules for longer periods in diabetic rats and normal monkeys. It is clear from study of diabetic animals that a basal insulin supply may help to improve their condition. Hyperglycaemic rats receiving the insulin-loaded PLGA formulation gained body weight at the same rate as normal rats. Intermittent treatment study also revealed the benefit. Meanwhile, daily treatment of diabetic rats with Humulin U rapidly produced a transient high level of insulin, with little body weight gain (or a loss).

To achieve our aim, human recombinant insulin was encapsulated into an injectable, biodegradable polymer composed of co-poly (D,L-lactic/glycolic) acid (PLGA). PLGA is an attractive biodegradable carrier for drugs. LHRH analogue-loaded PLGA microcapsules have already been used in the clinical setting, and have made a contribution to improving the condition of patients with prostate cancer, ovarian hyperthecosis, early precocious puberty, endometriosis, and so on. Therefore, there is no problem about its safety. Drug release from PLGA microcapsules is initiated by hydration with the surrounding aqueous media, and then the drug dissolves and diffuses through aqueous-filled pores within the particles, and subsequently the polymer degrades by hydrolysis associated with drug release (Ogawa 1997). PLGA has accordingly been used to achieve a sustained release not only of insulin, but also of other drugs (Okada et al 1994; Takada et al 1995, 1997a, b; Yanai et al 1995; Ogawa 1997; Barichello et al 1999a, b; Lam et al 2000).

We have already shown that the insulin formulation produces release with little burst. Before development of the preparation, the ratio of $AUC_{0-day\ 1}/AUC_{0-day\ 14}$ was 57.6% (Takenaga et al 2002). It was noted that this value was 10.5% in the case of the same treatment to diabetic rats, showing little insulin burst. The use of glycerol and distilled water in the preparation process made the resultant microcapsules suppress the rapid insulin release after contacting aqueous media. It has already been confirmed that the additives changed the insulin localization among microcapsules (Yamaguchi et al 2002). In particular, the inclusion of glycerol must have contributed to the reduction of burst by causing PLGA microcapsules to swell, with lowering of the glass transition temperature. This is now under further investigation.

The release pattern of insulin from PLGA microcapsules needs to be strictly flat, since a high value can cause hypoglycaemia, as reported by Rizza et al (1981). We therefore chose PLGAs with a mean molecular weight of 5800. This polymer contains lower-molecular-weight PLGAs, which in-vivo can degrade earlier by hydrolysis with the drug. As a result, the formulation managed to release the insulin in a more flat fashion. The mean peak/trough ratio with this formulation was 3.0 in diabetic rats and 4.2 in monkeys and would be concluded to approach acceptability.

The pattern of plasma insulin levels after dosing in normal monkeys was similar to that in diabetic rats although there was a slight difference in the later insulin release profile. The peak was observed on the eleventh day in monkeys. Furthermore, their levels were detectable for longer. The later drug release phase is considered to be dependent on the degradation of PLGA (Ramchandani & Robinson 1998). Although degradation of PLGAs in monkeys has not been studied, a difference between the two would be expected.

Rats developed antibodies against human insulin after intermittent treatment. One explanation is that the rat and human insulin sequences differ by four amino acids (Cordell et al 1979). Another possibility could be that the immunogenicity of insulin released from the microcapsules provoked anti-insulin antibody formation. In fact, PLGA formulations of vaccines have been shown to display enhanced immunogenicity (Vordermeier et al 1995; Partidos et al 1997; Jabbal-Gill et al 2001). We confirmed that intermittent treatment with Humulin U also induced antibody formation against human insulin in one of 5 diabetic rats. Taken together, the immunogenic activity of the insulin formulation may be more potent but the main reason would be ascribed to the different amino-acid sequences. Antibodies against insulin were not detected in monkeys after 3 treatments. However, monkey and human insulin sequences differ by five amino acids (Yu et al 1990). Low immunogenicity would be partly because the monkeys were normal. The number of monkeys in this study was relatively small. If a large number were tested, the possibility of antibody formation would arise. Anyway, the incidence would be reduced in the case of man.

We showed that the insulin-loaded PLGA microcapsules presented here could provide a relatively flat release of insulin in diabetic rats and normal monkeys. Intermittent treatment studies also demonstrated the release of insulin from microcapsules at a steady level for longer periods. It is clear that a basal insulin supply may help to improve the conditions for IDDM. In the near future, we are sure that this insulin formulation will prove very beneficial as a provider of basal insulin levels for IDDM patients.

Conclusion

We prepared an injectable formulation that released insulin in a relatively constant fashion for a prolonged time in diabetic rats and normal monkeys. Rats receiving the formulation once a week had not only steady plasma insulin

levels, but also gained weight at a similar speed as seen in normal rats. Meanwhile, daily treatments with Humulin U (25 U kg^{-1}) caused a transient high level in plasma insulin, but had little effect on body weight. It is possible that this new therapy may contribute not only to the improvement of quality of life but also may reduce the incidence of additional diseases. In the near future, this formulation would therefore become very beneficial as a provider of basal insulin levels for IDDM patients.

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