Immunologic Improvement Resulting from the Transfer of Animal Insulin–treated Diabetic Subjects to Human Insulin (recombinant DNA)

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To study the immunologic effects of transfer of patients from animal insulins to human insulin (recombinant DNA), a double-blind comparative trial was begun in over 300 patients. Preliminary results are reported in 116 individuals. After maintenance on purified pork or mixed beef-pork insulins (PPI or MBP) for a minimum of 6 mo, 116 patients were either switched to human insulin or maintained on their previous insulin. Antibody levels were assessed at a baseline visit and then monthly. In PPI-maintained individuals as well as those switched to human insulin there was a significant decrease in qualitative antibody binding as indicated by species-specific binding of ^{125}I beef and human insulins (SBB and SBH), both $P < 0.005$. Quantitative binding, as indicated by bound insulin levels, decreased to a much greater extent in patients switched to human insulin, 52% versus 31%, $P < 0.005$. Parameters derived from formal antibody titration did not change. In patients maintained on MBP, bound insulin decreased (−36% at 6 mo, $P < 0.002$). When switched from MBP to human insulin, there was a marked reduction in all parameters of binding, both qualitative and quantitative: SBP, −68%; SBH, −61%; SBB, −57%; bound insulin, −67% (all $P < 0.001$) and decreases in high- and low-affinity binding capacities ($P < 0.02$). Thus, for patients treated previously with nonhomologous insulins, transfer to human insulin may result in significant immunologic improvement in anti-insulin antibody levels. DIABETES CARE 5 (SUPPL. 2): 107–113, 1982.

Since human insulin (recombinant DNA) is structurally and chemically homologous with endogenous insulin,[1] transfer to human insulin may have significant effects on antibody levels in individuals previously treated with commercial insulins of animal origin. In previous studies, during which patients were transferred from conventional insulins to purified insulins, insulin dose decreased by 7–30%, and this was accompanied by decreased antibody concentrations over time.[2,3] Reductions in allergic phenomena were also reported.[2,3] The purpose of this double-blind, randomized, comparative trial is to determine the effects of transferring insulin-treated diabetic patients from mixed beef-pork insulin (MBP) or purified pork insulin (PPI) to human insulin. Transferred patients are contrasted with patients who were maintained on MBP or PPI. This preliminary report in 116 out of over 300 patients will concentrate on alterations observed in antibody concentrations in both transferred and maintained groups of patients during the first 6 mo of the study.

METHODS

One hundred and sixteen patients from the private practices of six physicians (see ACKNOWLEDGMENTS) are participating in this clinical trial. Selection was limited to patients over 4 yr of age who were maintained on MBP or PPI for a minimum of 6 mo. Informed consent was obtained prior to entry, and protocols conformed to principles outlined in the Declaration of Helsinki. Patients were excluded who had a life expectancy of less than 3 yr, cancer, serum creatinine of >2.0 mg/dl, advanced cardiovascular disease, or who were pregnant.

During a 1-mo baseline period, optimal dietary compliance was attempted and a history and physical examination were completed. Antibody concentrations were assessed at baseline and monthly intervals thereafter. At the end of baseline, patients were assigned to either maintenance or transfer groups by means of a random number table. Patients and physicians were blinded with respect to the insulin assigned. Purified pork insulin used in these studies was manufactured from

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TABLE 1
Baseline characteristics*

	PPI-PPI	PPI–human insulin	MBP-MBP	MBP–human insulin	Comparisons†
Age	42.4 ± 2.5	39.8 ± 2.6	24.0 ± 2.8	34.2 ± 3.1	1 = 2, 3 < 4, P < 0.05
Duration (yr)	15.8 ± 1.3	15.7 ± 1.1	10.2 ± 2.0	8.2 ± 1.2	1 = 2, 3 = 4
% IBW	103 ± 2	107 ± 3	105 ± 4	114 ± 8	N.S.
Clinical type I/II	35/5	34/3	20/1	14/5	N.S.
M/F	23/17	15/22	15/6	13/6	N.S.
N	39	37	21	19	

*Abbreviations used in this and subsequent tables include: PPI-maintained (PPI-PPI) and PPI-switched patients (PPI–human insulin), MBP-maintained (MBP-MBP) and -transferred patients (MBP–human insulin); % ideal body weight, IBW (Metropolitan Life Tables, 1959); clinical type of diabetes, IDDM = type I and NIDDM = type II; and male/female ratio, M/F. From left to right, columns are numbered 1, 2, 3 and 4.
†Columns 1 and 2, and 3 and 4 were compared by Student's t tests. Duration of disease in patients receiving PPI during baseline exceeded that in the two MBP groups ($P < 0.05$).

pancreases obtained from pork-only vendors and contained <5 PPM of proinsulin and had undetectable levels of beef insulin. Mixed beef-pork insulin contained <50 PPM of proinsulin and 70% beef insulin. Human insulin was supplied as regular or NPH formulations. Insulin dose was adjusted theoretically according to the individual needs of the patients. Home glucose monitoring was made available to each participant.

Anti-insulin antibody levels were measured in fasting sera obtained 12–24 h after the patient's last insulin injection. The ^{125}I insulins used in these studies were provided by Bruce Frank, Ph.D., of Eli Lilly Laboratories by his published methods.[4] Species-specific binding of ^{125}I insulin (human, pork, and beef: SBH, SBP, and SBB) was measured using a 1:10 dilution of charcoal-extracted sera, 1 μU/tube of insulin, a 2-h incubation at 37°C, and polyethylene glycol (PEG) precipitation of antibody-bound insulin.[5,6] Control serum binding was determined in each assay. Results were reported as % bound/total − control. Total and free insulin were measured using nonextracted aliquots of sera and PEG precipitation of anti-insulin antibodies.[7] Supernatant insulin concentrations were measured using Heding's method.[8] Recovery was determined in each assay. Insulin prebound to circulating anti-insulin antibodies (bound insulin) is the measured difference between total and free insulin when recovery is taken into account. Formal antibody titrations were carried out in selected subsets of patients using 1:10 dilutions of charcoal-extracted sera, species-specific nonradioactive and ^{125}I-insulins, and conditions as described above.[6] Analysis of parameters used nonlinear curve fitting techniques.[9,10]

Data were analyzed using statistical packages available through the Indiana University Computing Network. Repeated measures over time were compared between groups by two-way analysis of variance (RANOVA). Groups were treated as an independent factor and multiple values as a repeated measure.[10] Significant differences in behavior over time between experimental and control groups are reflected by the P value for the interaction terms in the tables. Absolute magnitude of change in percentage of species-specific antibody binding was found to be significantly related to initial magnitude $r = 0.42–0.50$ ($P < 0.01$). However, when data were treated as percentage of baseline, they were not related to initial magnitude of binding. When appropriate, comparisons were made using nonparametric methods of Friedman, the Wilcoxon test, and Mann-Whitney U tests.[10]

RESULTS

Baseline characteristics (*Table 1*). Both groups of patients treated with PPI (columns 1 and 2) were similar with respect to age, duration of disease, percentage of ideal body weight, clinical

TABLE 2
Percentage of species-specific binding of ^{125}I pork insulin*

	B	1	2	3	4	5	6	RANOVA†	Interaction‡
PPI-PPI	14.4	14.4	14.9	13.6	13.0	12.8	13.6	N.S.	N.S.
PPI–human insulin	22.1	22.9	26.4	22.4	20.6	21.2	21.3	<0.001	
MBP-MBP	19.7	21.4	22.8	19.1	19.2	21.0	19.3	<0.02	<0.001
MBP–human insulin	23.9	21.1	17.0	15.3	14.0	14.0	12.6	<0.001	

*Data were analyzed by two-way, repeated measures analysis of variance (RANOVA) with observations made at baseline (B) and monthly thereafter.
†P value for the significance of RANOVA *within* groups over time.
‡P values for the significance of changes *between* groups over time (interaction terms).

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TABLE 3
Percentage of species-specific binding of ^{125}I beef insulin*

	B	1	2	3	4	5	6	RANOVA†	Interaction‡
PPI-PPI	16.4	15.5	15.5	15.6	14.6	14.0	14.5	N.S.	N.S.
PPI–human insulin	25.5	25.0	26.5	24.9	19.3	20.9	19.7	<0.001	
MBP-MBP	21.5	23.3	26.1	22.4	19.3	20.9	19.7	<0.001	<0.001
MBP–human insulin	26.7	24.2	21.2	18.3	16.5	16.4	14.9	<0.001	

*Data were analyzed by two-way, repeated measures analysis of variance (RANOVA) with observations made at baseline (B) and monthly thereafter.
†P value for the significance of RANOVA *within* groups over time.
‡P values for the significance of changes *between* groups over time (interaction terms).

type of diabetes, and proportion of males and females. The mean age of patients who were maintained on MBP was less than that in patients transferred from MBP to human insulin, $P < 0.05$. In previous unpublished observations, we have established that age is unrelated to antibody development at least in patients who were begun de nouveau on various types of insulin. Duration of diabetes was comparable in the patients who were treated with PPI during baseline, but this duration somewhat exceeded that seen in the individuals maintained on MBP during baseline.

Species-specific binding of insulin (Tables 2, 3, 4 and Figures 1, 2). Purified pork–maintained (PPI-PPI) and transferred (PPI–human insulin) patients had significantly different percentages of species-specific binding at baseline ($P < 0.05$). However, the behavior of percentage of SBP, SBB, and SBH did not differ between groups as the study progressed. Significant alterations in specific binding when analyzed by RANOVA did occur over time (Tables 2, 3, 4). This is best seen in Figure 1, where data are plotted as percentage of baseline (analyzed nonparametrically and by Friedman and Wilcoxon tests). In both PPI–human insulin and PPI–PPI patients SBH and SBB decreased significantly.

Mixed beef-pork–maintained individuals (MBP-MBP) also had significant fluctuations in specific binding, with most individuals tending to remain near elevated above baseline. Several patients increased from negligible to significant levels, thus distorting means upward in Figure 2. When individuals were transferred from MBP to human insulin, binding of all species of insulin decreased markedly.

Bound insulin (Table 5 and Figure 3). Unlike species-specific data, bound insulin values were not normally distributed and varied widely among individuals. Therefore this data had to be analyzed by nonparametric techniques. Bound insulin was significantly different at baseline in the two PPI groups but not in the MBP groups. When data were normalized to baseline, there was a 30% overall reduction in antibody-bound insulin over time (Figure 3), $P < 0.001$ in PPI-PPI. Reduction in bound insulin levels in the PPI–human insulin group, in contrast, amounted to 51% at 6 mo and was greater than in the former group, $P < 0.005$. Thus, the reduction in prebound insulin in this group was significantly greater than that seen in PPI-PPI.

As in the former groups, bound insulin fell with time even in MBP-MBP patients (Table 5 and Figure 3). At 6 mo, this reduction in prebound insulin was 36%. However, in the group of patients who were switched to human insulin there was an 80% reduction in levels at 4 mo and 67% at 6 mo. Group differences between MBP-MBP and MBP–human insulin were highly significant, $P < 0.001$.

Formal antibody titration (Tables 6 and 7). Antibody titration was carried out at baseline and 3 mo in all MBP–human insulin patients and only in those patients in the PPI–human

TABLE 4
Percentage of species-specific binding of ^{125}I human insulin*

	B	1	2	3	4	5	6	RANOVA†	Interaction‡
PPI-PPI	15.0	14.4	14.4	13.2	13.4	12.3	12.3	<0.001	N.S.
PPI–human insulin	23.1	23.3	24.7	22.6	21.8	21.0	20.7	<0.02	
MBP-MBP	20.5	21.4	23.6	19.5	18.6	20.3	18.5	<0.002	<0.001
MBP–human insulin	24.9	21.3	17.2	15.6	13.3	13.5	12.2	<0.001	

*Data were analyzed by two-way, repeated measures analysis of variance (RANOVA) with observations made at baseline (B) and monthly thereafter.
†P value for the significance of RANOVA *within* groups over time.
‡P values for the significance of changes between groups over time (interaction terms).

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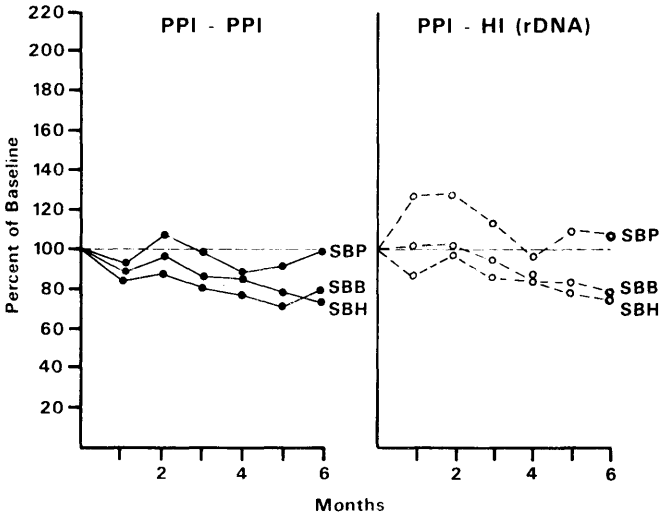

FIG. 1. Species-specific binding of ^{125}I insulin given as percentage of baseline in individuals who were treated with PPI during baseline. Data were analyzed by the Friedman test. In the PPI-PPI group SBP did not change significantly, while SBB (x^2 = 25, $P < 0.001$) and SBH (x^2 = 21, $P < 0.005$) decreased. In the PPI–human insulin [HI(rDNA)] group SBP increased (x^2 = 31, $P < 0.001$) and SBB (x^2 = 39, $P < 0.001$) and SBH (x^2 = 20, $P < 0.005$) decreased.

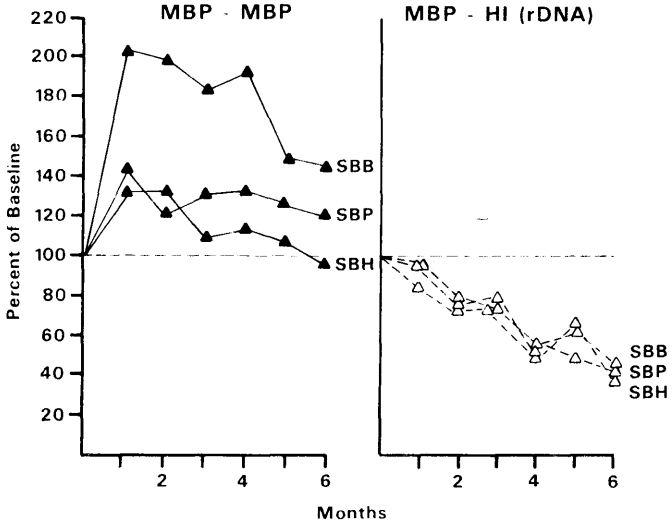

FIG. 2. Species-specific binding of ^{125}I insulin, given as percentage of baseline in individuals who were treated with MBP during baseline. In the MBP-MBP group SBB (x^2 = 29, $P < 0.001$), SBP (x^2 = 14, $P < 0.03$) and SBH (x^2 = 21, $P < 0.001$) all increased. In the MBP–human insulin group SBB (x^2 = 48, $P < 0.001$), SBP (x^2 = 44, $P < 0.001$) and SBH (x^2 = 51.5, $P < 0.001$) decreased. Antibody behavior was significantly different between groups, $P < 0.001$).

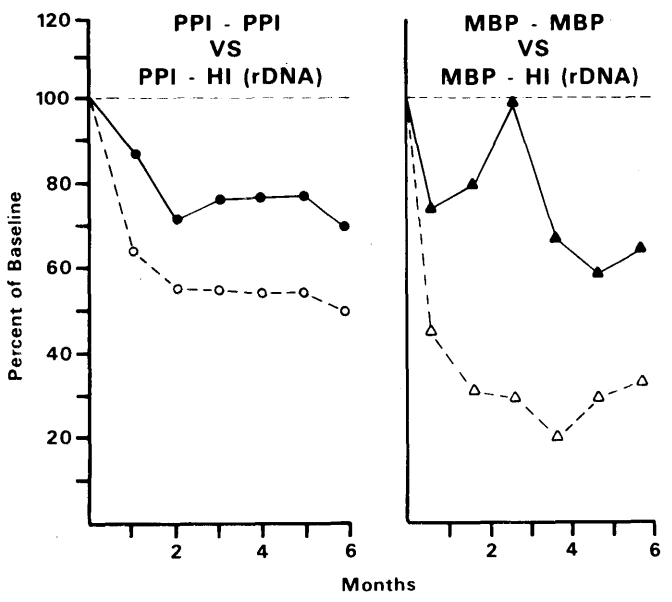

FIG. 3. Bound insulin as percentage of baseline compared in control and experimental groups. Bound insulin decreased significantly in PPI-PPI as well as in PPI–human insulin (x^2 = 35, $P < 0.001$ and x^2 = 35, $P < 0.001$, respectively), decreases in PPI–human insulin exceeded those in PPI-PPI, $P < 0.005$. Significant decreases over time in bound insulin occurred in MBP-MBP (x^2 = 29, $P < 0.001$) and in MBP (x^2 = 49, $P < 0.001$), decreases were greatest for MBP–human insulin and antibody behavior differed between groups (<0.005).

insulin group whose initial percentage of binding was >15%. In the PPI–human insulin group, reduction of prebound insulin was not reflected by altered binding capacities or affinity constants. However, in MBP–human insulin patients (Table 7), high- and low-affinity binding capacities decreased significantly, $P < 0.02$, and the high-affinity constants doubled, $P < 0.02$.

DISCUSSION

Patients included in this study had diabetes for an average duration of about 10 yr. Therefore, it is likely that their immunologic backgrounds included exposure to conventionally purified, more immunogenic insulins.[11] It is also probable that considerable "washout" of higher antibody concentrations occurred during the 6-mo prestudy maintenance period on MBP or PPI. In keeping with this premise, there was a significant reduction in total circulating anti-insulin immunoglobulins in the control groups as indicated by bound insulin measurements. Changes in qualitative binding of species-specific insulin were significant but of a minor nature in these two groups. SBH and SBB in the PPI-PPI decreased significantly over time (see Table 4 and Figure 1), and in the MBP-MBP group, SBH, SBB, and SBP transiently increased and then returned toward baseline (see Tables 2 and 3, and Figures 1 and 2). At 6 mo these changes amounted to −24% for SBH ($P < 0.01$) in PPI-PPI and −7% (P

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TABLE 5
Bound insulin μU/ml*

	B	1	2	3	4	5	6	Friedman†	Mann-Whitney U tests‡
PPI-PPI	262	231	231	228	236	240	205	N.S.	<0.01
PPI–human insulin	500	395	365	379	415	389	395	<0.06	
MBP-MBP	358	270	280	389	235	204	210	<0.001	<0.001
MBP–human insulin	673	556	307	319	281	200	323	<0.002	

*Abbreviations used include: PPI-maintained (PPI-PPI) and PPI-switched patients (PPI–human insulin), MBP-maintained (MBP-MBP) and -transferred patients (MBP–human insulin). Observations were made at baseline (B) and monthly thereafter.
†Data were not normally distributed and were therefore analyzed by nonparametric methods. P values for the significance of changes over time within groups, as analyzed by the Friedman test, are noted.
‡P values for the significance of between group changes over time are given (analyzed by Mann-Whitney U tests).

value N.S.) in MBP-MBP. In the MBP-MBP group SBP increased by 16% and SBB by 35% (both P values N.S.). Thus, when continued on PPI cross-reacting antibodies to human insulins eventually decreased whereas antibodies to the immunogen(s) used did not change significantly.

Of potential concern were baseline differences in percentage of specific binding and bound insulins between groups. However, for data that were normally distributed, the interaction term of RANOVA detected significant difference in patterns of behavior between control and experimental groups over time. Further, when data were analyzed as percentage of baseline over time, they were not correlated with initial magnitude of either bound insulin (Figure 3) or specific binding (Figures 1 and 2).

As can be clearly seen in Tables 2, 3, and 4, specific binding decreased dramatically in the MBP–human insulin groups. At 6 mo percentage of SBH, SBP, and SBB decreased by 61, 58, and 57%, respectively. All decreases were highly significant, $P < 0.001$. This was in marked contrast to the fluctuations in SBP and SBB and the minor decrease in SBH at 6 mo, as noted above in the MBP-MBP group. Further, these changes in specific binding were fully reflected by similar reductions in circulating prebound insulin (Table 5 and Figure 3) and a quantitative reduction in antibody binding capacity as indicated by formal antibody titration (Table 7).

Since pork and human insulins differ by only one amino acid, it was surprising that PPI and human insulin could be differentiated as revealed by decreases in antibody levels different from those observed in the control subjects. Similar reductions in SBB were seen in PPI-PPI and PPI–human insulin patients at 6 mo (−22% for both), overall for both, $P < 0.001$, suggesting that these individuals had been previously exposed to beef insulins. SBP did not change significantly in PPI-PPI, but SBH decreased ($P < 0.005$) after switching to human insulin or while being maintained on PPI ($P < 0.005$). This suggested that changes were occurring in cross-reacting antibodies and that human and pork insulins might be antigenically equivalent. However, bound insulin,

TABLE 6
Antibody titration: PPI to human insulin group* (N = 17)

	Baseline	3 mo	P by Wilcoxon test†
N_1mU/ml	0.105 (0.001–2.590)	0.094 (0.002–3.080)	N.S.
N_2mU/ml	6.91 (0.287–322.2)	3.987 (0.385–424.9)	N.S.
K_1L/M	1.010×10^9 (0.160–43.300)	0.942×10^9 (0.178–4.150)	N.S.
K_2L/M	6.010×10^6 (0.910–94.300)	4.050×10^6 (1.19–63.1)	N.S.

*Parameters were determined by nonlinear curve fitting of bound/total counts using pork ^{125}I tracer and nonradioactive pork insulin in a 12–16 point formal titration assay. N_1 and N_2 refer to high- and low-affinity binding capacities in mU/ml. K_1 and K_2 are the association constants of high- and low-affinity sites. The median value for the various parameters is given, with the ranges observed given below in parentheses.
†Data were not normally distributed and were analyzed by the Wilcoxon test.

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TABLE 7
Antibody titration: MBP to human insulin group* (N = 19)

	Baseline	3 mo	P by Wilcoxon test†
N_1mU/ml	0.061 (0.002–4.56)	0.016 (0–1.93)	<0.012
N_2mU/ml	1.000 (0.008–26.100)	0.27 (0.012–23.8)	<0.018
K_1L/M	0.599×10^9 (0.158–5.570)	1.210×10^9 (0.135–57.000)	<0.020
K_2L/M	7.96×10^6 (2.36–77.00)	11.100×10^6 (1.52–73.4)	N.S.

*See footnote to Table 6.
†See footnote to Table 6.

an index of quantitative, in vivo antibody concentrations, decreased by 30% in control subjects whereas levels decreased by 51% in patients who were transferred ($P < 0.005$), indicating that the two insulins were differentiated. A number of PPI–human insulin individuals did have reductions in antibody concentrations by formal titration. However, for the group as a whole, reductions in total anti-insulin immunoglobulins were not reflected by changes in binding parameters. Major changes in antibody concentrations may have been necessary before such alterations could have been detected by titration assays; or such changes may have occurred primarily in low-affinity antibodies, which would have been more difficult to detect.

In summary, in patients with diverse immunologic backgrounds, total immunoglobulin concentrations decreased over 6 mo while being maintained on MBP or PPI. Similar reductions over time have been reported previously.[13] Qualitative binding of SBB and SBH also decreased to a minor but significant extent in PPI-treated patients but not in MBP-treated patients. When PPI-treated patients were switched to human insulin, decreases in qualitative binding for beef and human insulins (when analyzed by percentage of baseline) in the PPI–human insulin were similar to those seen in the control group, but bound insulin decreased to a greater extent than in the control group. Dramatic reductions in all quantitative and qualitative binding as indicated by markers of insulin antibodies were seen in patients transferred from MBP insulins to human insulin. Human insulin may result in immunologic improvement in individuals who have previously been treated with nonhomologous insulins.

ACKNOWLEDGMENTS: We express our gratitude to the following physicians and their patients who participated in this study: T. G. Duncan, M.D., Philadelphia, PA; D. D. Etzweiler, M.D., Diabetes Education Center, Minneapolis, MN; R. A. Guthrie, M.D., Wichita, KS; A. Krosnick, M.D., Trenton, NJ; R. L. Nielson, M.D., Mason Clinic, Seattle, WA; and F. W. Whitehouse, M.D., Detroit, MI.

The assistance of the following personnel of Eli Lilly and Company is greatly appreciated: B. H. Frank, Ph.D., for assistance with preparation of ^{125}I insulins; T. Spradlin for study design; J. Inguilli-Fattic, D. Allemenos, and G. Cokinos for assistance with preliminary data handling and sorting. Preparation of this manuscript was made possible by the secretarial assistance of M. J. Smith.

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