Original Articles

Immunologic Aspects of Human Proinsulin Therapy

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We investigated the immunogenicity of human proinsulin (HPI) when used as the sole or principal insulin agonist in insulin-naive patients with insulin-dependent (type I) and non-insulin-dependent (type II) diabetes mellitus. Sixty-one patients (13 type I, 48 type II) were treated with rDNA human insulin (NPH HI with or without regular HI) and 53 were treated with HPI (8 type I, 45 type II). At 6 mo, virtually identical levels of HbA_{1c} (5.2 vs. 5.3%, P = NS) were achieved. However, regular HI was added less often to the treatment regimen in HPI-treated patients (16 vs. 32 patients, $P < .001$). Overall, there was no significant increase in proinsulin-specific antibodies in either treatment group. However, 8 of 51 (1 transiently) patients in the HPI group developed low levels of binding of HPI (highest percentage bound was 5%). Two patients in the HI group developed very low levels of HPI binding (1.2 and 1.9%). Binding of HI (>2.4%) was seen in both treatment groups; however, the prevalence of HI binding was less in the HPI group at 6 mo (39 of 60 in HI group vs. 20 of 51 in HPI group, $P = .008$). Concomitant treatment with regular HI did not affect the prevalence or level of binding of HPI or HI. We conclude that human proinsulin is a weak immunogen when used as the principal insulin agonist and may reduce both the formation of anti-HI antibodies and the need for concomitant therapy with regular HI. *Diabetes* 37:276–80, 1988

In the β-cell, proinsulin and its degradation products constitute ~6% of insulin-related peptides (1,2). Patients treated with conventionally purified pancreatic beef and pork insulins regularly develop proinsulin-specific antibodies as well as antibodies to other pancreatic peptides (3). We recently observed that proinsulin-specific antibodies often are the dominant antibodies in patients treated with conventionally purified animal insulin preparations (4). However, such proinsulin-specific antibodies are seldom detectable during treatment with more purified animal insulins (proinsulin content <30 ppm) (5). Kapp and Strayer (6) have postulated that proinsulin might augment the immune response to the insulin molecule itself when proinsulin and insulin are administered jointly. Development of recombinant DNA methods to produce human proinsulin (HPI) (7) has allowed us to undertake a clinical trial comparing the effects of HPI and NPH human insulin (HI) in patients with insulin-dependent (type I) and non-insulin-dependent (type II) diabetes mellitus.

In undertaking this study, we were concerned that in HPI-treated individuals proinsulin-specific antibodies would develop in significant concentrations and that the development of anti-human insulin antibodies would be enhanced over that seen in patients treated only with HI. This study concerns the development of anti-proinsulin and anti-insulin antibodies in insulin-naive patients. In contrast to previous studies, which used animal proinsulins, we found that HPI is a weak immunogen and may be much less immunogenic than repository HI.

MATERIALS AND METHODS

Clinical trial. An open-label 24-mo multicenter study was carried out to compare the effects of HPI and HI in patients with type I and type II diabetes. Written informed consent was obtained before entry. Protocols were reviewed by human experimentation committees in individual participating institutions and conform to the principles outlined in the Declaration of Helsinki. Insulin agonist therapy was begun on clinical grounds. Inclusion criteria were age >18 yr and fasting overnight glucose level >200 mg/dl or 2-h postprandial glucose levels >300 mg/dl. Patients were excluded if they were treated with more than seven doses of NPH or regular insulin over the 7 days before entry, had severe diabetic retinopathy, had a life-threatening complicating disease, had

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TABLE 1
Proinsulin- and insulin-specific antibody method—additions to incubation tubes

	Charcoal extract† (μl)	Buffer‡ (μl)	^{125}I-labeled antigen§ (μl)	Antigen excess (μl)
Proinsulin-specific antibodies*				
Buffer tube	120	100	80 (0.06 ng) HPI	
Insulin excess	120		80 (0.06 ng) HPI	100 (2 μg) HI
Proinsulin excess	120		80 (0.06 ng) HPI	100 (3 μg) HPI
Insulin-specific antibodies				
Maximum binding	120	100	80 (0.04 ng) HI	100
Insulin excess	120		80 (0.04 ng) HI	100 (2 μg) HI

HPI, human proinsulin; HI, human insulin.
*For given species, species-specific antigens are used. Total-count tubes containing only labeled antigen prepared at time of labeled-antigen addition.
†Sera diluted 1:4; therefore 120 μl extract contain equivalent of 30 μl serum.
‡Buffer used is 0.04 sodium phosphate, pH 7.4, containing 0.1% ovine albumin and 0.025% Merthiolate. (Radioactive and nonradioactive antigen also diluted with this buffer.)
§Radiolabeled antigens purified by HPLC to obtain monoiodinated A14-tyrosine radiotracers.

serum creatinine >2 mg/dl, or if women participants wanted to become pregnant during the study. Participants were categorized as having type I diabetes based on onset of disease before 41 yr of age and a body mass index of <23.4 in men and <27.0 in women (equivalent to <116% of ideal body wt; 8), and all others were categorized as having type II. We have found that these criteria differentiated individuals less likely (type II) or more likely (type I) to develop an antibody response to insulin but did not differentiate the occasional patient who developed type II diabetes before 41 yr of age or the patient >41 yr old with type I diabetes (9). Patients were randomized to a particular therapy after entry by means of a central randomization table (Lilly, Indianapolis, IN).

Materials for treatment include a recombinant DNA HPI (40 U or 10 mg/ml) and NPH and regular formulations of recombinant DNA HI (100 U/ml). HI is produced by an A- and B-chain combination technique and therefore contains no human proinsulin (10). None of the study insulins was administered by continuous subcutaneous insulin infusion. In case of urgent indications, study HI or HPI can be temporarily administered intravenously. Investigators were encouraged to avoid the use of regular HI in the two groups unless in their judgment regular HI was clinically indicated to achieve better glycemic control. No other prospective guidelines were provided for the use of regular insulin. Dosage guidelines were given concerning HPI and the two insulin formulations; these suggested the use of HPI as an intermediate-duration insulin (11). Data on the use of combined regular HI and HPI are also provided.

All patients underwent baseline evaluations for insulin and proinsulin antibodies 1–30 days before initiation of insulin agonist therapy. A second baseline sample for antibody measurements was obtained just before initiation of therapy. In the case of acutely ill patients in whom delaying therapy would be detrimental, two baseline samples could be collected 1–2 h before beginning therapy. Sera for the antibody measurements were obtained at baseline and after 2, 3, 4, 6, 8, 10, 12, 18, and 24 mo.

Laboratory methods. Proinsulin and insulin antibodies were assessed with a modification of our published techniques (5). The ^{125}I-labeled (A14) HI and ^{125}I-labeled (79) HPI (iodinated on the same position in HI and HPI) were prepared according to published methods (12). HI and HPI were provided by Lilly. Additions to the incubation tubes are summarized in Table 1. The sequence of steps in the assay is shown in Fig. 1. All determinations were performed in duplicate. To exclude previous animal insulin therapy, in addition to history, the patients' pretreatment sera were studied for the presence of beef and human proinsulin-specific and insulin-specific antibodies. After baseline, only the presence of antibodies to the human antigens was assessed. We define binding attributable to HPI-specific antibodies as that binding of ^{125}I-HPI that can be blocked by HPI but not HI. Such binding is calculated as (percent bound in maximum-binding tube minus percent bound in proinsulin-excess tube) minus (percent bound in maximum-binding tube minus percent bound insulin-excess tube) equals percent proinsulin-specific binding.

In all studies done in our laboratory to date, HPI and HI blocked ^{125}I-insulin binding equally on a molar basis (data not shown). In the analyses presented in this study, we did not repeat the studies in which insulin binding is blocked by HPI, but theoretically an occasional serum could be encountered in which proinsulin blocks insulin binding less well than insulin itself. We assume that blocking of insulin binding by proinsulin is due to portions of the insulin molecule common to both proinsulin and insulin. Insulin-specific binding, i.e., binding of ^{125}I-HI that can be blocked either by HI or

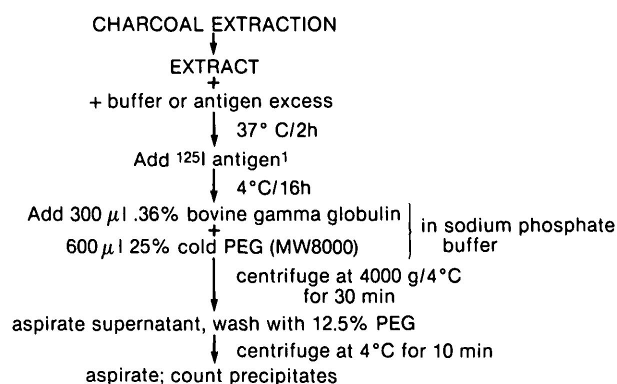

FIG. 1. Sequence of assay steps. All tubes are prepared in duplicate.

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HPI, is defined as percent bound in maximum-binding tube minus percent bound in insulin excess tube equals percent insulin-specific binding.

Reproducibility of measurements between assays was assessed with nine sera known to be positive for beef insulin antibodies over a broad range of binding (16–74% binding in maximum-binding tubes). Sera were studied in duplicate on four different occasions with fresh charcoal extraction each time. The pooled standard deviations, taking into account within- and between-sample variation, with ^{125}I-labeled beef proinsulin, were: maximum-binding tube, 3.98%; insulin-excess tube, 2.94%; and proinsulin-excess tube, 0.85%. Overall, the mean (±SE) coefficients of variation were 8.8 ± 2, 12.9 ± 1.8, and 13.1 ± 1.7%, respectively. The within-assay coefficient of variation for maximum binding, with a single serum analyzed eight times, was 2.0%; for the addition of proinsulin it was 2.7%.

Statistical methods. Data are given, unless otherwise specified, as means ± SE. Comparisons between groups were performed with Student's *t* test for continuous variables and χ^2-tests for discrete variables. Analysis of variance was used to calculate pooled standard deviations and estimate reliability of measurements with the assay techniques. Percent binding of ^{125}I-HI or ^{125}I-HPI over time was compared between groups with repeated measures analysis of variance (13). Insulin dosage requirements were compared with analysis of covariance (13).

RESULTS

Characteristics and baseline data. A description of the study population is shown in Table 2. The study population was primarily patients with type II diabetes. A similar proportion of patients with type I diabetes was included in each treatment group. Except for a small difference in mean body mass index (HI group < HPI group, $P < .05$), the groups were equivalent.

Regular HI was used in both treatment groups as the investigators deemed clinically necessary to achieve glycemic control. During 6 mo of therapy, 36 of 61 patients in the HI group and 16 of 53 in the HPI group were treated with regular HI. At the 6-mo visit, 32 of 61 patients in the HI group and 16 of 53 patients in the HPI group were treated with regular HI (P = .022). Thus, during HPI therapy regular HI was added less often than during NPH HI therapy. After 6 mo of therapy, the HbA_{1c} levels (Smith Kline, Van Nuys, CA; normal range 3.6–4.9% by HPLC) were 5.2 ± 1.1 versus 5.3 ± 1.3% (mean ± SD) in the HI and HPI groups, respectively. When insulin dose was compared between these groups, it was necessary to use analysis of covariance because of baseline differences in weight and possible individual differences of glycemic control. This was especially notable in one individual in the HI group whose dosage requirements of 256 U/day were explainable based on marked obesity (body wt 350 lb). After 6 mo, the adjusted mean dosage of long-acting insulin agonist was 36 U NPH HI versus 28 U HPI (P = .025) and 10 U regular HI versus 2 U regular HI (P = .002) in the HI and HPI groups. Regular insulin was added in a similar proportion of patients with type I and type II diabetes in both treatment groups, and the use of regular insulin could not be ascribed to type of diabetes in this study.

TABLE 2
Population description

	Treatment		
	Insulin	Proinsulin	*P*
n	61	53	
Age (yr)	49.0 ± 1.6	48.1 ± 1.8	NS
Body mass index (kg/m²)	26.5 ± 0.9	29.1 ± 1.0	<.05
Sex (M/F)	34/27	28/25	NS
Race (White/Black)	44/17	40/13	NS
Age at diabetes onset (yr)	45.8 ± 1.5	43.5 ± 1.5	NS
Duration of diabetes (yr)	3.1 ± 0.5	4.6 ± 0.7	NS
HbA_{1c} (%)	8.7 ± 0.3	8.5 ± 0.3	NS
Type of diabetes (type I/type II)	13/48	8/45	NS

Data are means ± SE where appropriate.

TABLE 3
Prevalance of significant binding of ^{125}I-labeled human proinsulin

	Treatment			
	Insulin		Proinsulin	
Time (mo)	*n*	Binding (%)	*n*	Binding (%)
2	0 of 60		2 of 51	2.4, 1.5
3	0 of 58		2 of 50	2.4, 2.6
4	1 of 60	1.2	6 of 50	1.3, 1.4, 1.5, 2.0, 2.7, 5.0
6	1 of 60	1.9	7 of 51	1.5, 1.5, 2.0, 2.2, 2.6, 3.5, 4.7

Significant binding of ^{125}I-labeled human proinsulin is >1.2%.

Two of 113 subjects had HI antibodies at baseline; none had proinsulin antibodies. Both were young men (29 and 31 yr old) with no previous insulin therapy and the sudden onset of type I diabetes. They were both subsequently assigned to the insulin therapy group. One of these positive individuals, because of high binding (12.4%), was excluded from calculation of 99% confidence intervals for binding in untreated patients. The other positive individual had 6% binding and was included. These two patients were included in all other analyses. Sera were considered positive for specific antibody binding when percent bound (99% confidence intervals in parentheses) >2.0% for beef proinsulin (−1.1 to 2.0%), >2.1% for beef insulin (−1.0 to 2.1%), >1.2% for HPI (−1.0 to 1.2%), and >2.4% for HI (−1.4 to 2.4%).

Prevalence of HPI-specific antibodies. Mean percent HPI binding did not increase in either treatment group. However, a small number of individuals developed binding of HPI. All patients with significant binding are shown in Table 3. In the HI group, 2 individuals developed significantly elevated levels of HPI binding: 1 patient at 4 mo and 1 at 6 mo. The individual who became positive at 4 mo was no longer positive at 6 mo. Data for the other positive individual beyond 6 mo are not available. This small variation in measurement of antigen binding is expected when large numbers of samples are being assayed. In the HPI treatment group, 8 patients developed statistically significant binding of HPI (transient in 1). However, the highest level observed in any

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individual was 5%, and only 7 of 51 individuals had significant binding at 6 mo of therapy. Concomitant treatment with regular insulin did not affect the development of anti-HPI antibodies at any visit. In the HI group, 1 patient treated with regular HI and 1 patient treated only with NPH HI developed trace amounts of anti-HPI antibodies at 4 and 6 mo, respectively. Similarly, the proportion of individuals developing anti-HPI antibodies in the HPI group was similar with and without the addition of regular HI. At 6 mo, 3 of 15 patients treated with HPI plus regular HI and 4 of 36 patients treated only with HPI developed anti-HPI antibodies ($P = .66$).

Comparison of ^{125}I-HI binding and prevalence of significant binding. Significant binding of HI developed in patients from both groups. At 2, 3, 4, and 6 mo, mean binding in the HI group was 2.3, 4.6, 5.7, and 6.4% and in the HPI group was 1.8, 2.8, 4.2, and 4.4%. Both groups showed an increase over time ($P < .001$). Differences between groups were not significant; however, the prevalence of significant antibody binding in the two groups, shown in Table 4, was significantly different. Binding of ^{125}I-HI was more prevalent in the HI than the HPI group at 3, 4, and 6 mo ($P = .025$, .002, and .008). Concomitant treatment with regular HI does not affect the mean binding or prevalence of significant binding. At 6 mo, in the HI group, 19 of 30 individuals treated with regular HI and 20 of 30 individuals treated only with NPH HI developed anti-HI antibodies ($P = .99$). In the HPI group, 8 of 15 patients treated with regular HI and 12 of 36 patients treated only with HPI developed anti-HI antibodies ($P = .22$).

Relationship between antibody levels and insulin dosage. Stepwise multiple regression was performed with insulin dosage as the dependent variable and age, weight, body mass index, treatment group, and insulin and proinsulin antibody levels available as independent variables. Total dosage (all $P < .001$) and dosage of long-acting agonist ($P <$ .026, .008, and .001, respectively) were significantly associated with age, body mass index, and treatment (HI or HPI). Older patients and those treated with HPI were treated with lower dosages, whereas heavier subjects required more insulin. Antibody levels could not be shown to affect dosage.

Relationship of type of diabetes and development of antibodies to insulin or proinsulin. Development of anti-HI antibodies in both treatment groups was seen in similar proportions of patients with type I and type II diabetes. Proinsulin binding was seen in 3 of 7 patients with type I diabetes in the HPI group and 4 of 40 patients with type II diabetes ($P = .045$). Therefore, we could not confirm differences between type I and type II diabetes with regard to insulin antibodies, but we could confirm differences between type I and type II diabetes with the development of proinsulin antibodies. The number of patients analyzed was small, and one should not generalize from these data.

TABLE 4
Prevalence of significant binding of ^{125}I-labeled human insulin

	Treatment				
	Insulin (n)		Proinsulin (n)		
Time (mo)	>2.4%	≤2.4%	>2.4%	≤2.4%	P
2	16	44	10	41	.501
3	25	33	11	39	.025
4	37	24	15	35	.002
6	39	21	20	31	.008

DISCUSSION

Studies in normal individuals and patients with diabetes have suggested that HPI would be useful alone or as a complement to HI therapy (11,14–16). In these studies, HPI was given in pharmacologic amounts, with resulting concentrations many times greater than those seen during an overnight fast (17). Although HPI has less biologic activity than HI, the slower rate of clearance of HPI (⅓ to ⅕ that of HI) allows HPI to persist at concentrations that limit substrate availability and depress glucose production but do not stimulate peripheral glucose disposal (14,18).

Beef and pork proinsulins are potent immunogens when patients are treated with insulins containing 2–4% of proinsulin (3,19–24). Reduction in the immunogenicity of therapeutic animal insulins has been attributed to almost total removal of proinsulin (to <10 ppm) and the increasing use of porcine and human insulins (both semisynthetic and recombinant DNA origin; 3,6,25). The HPI differs substantially from animal proinsulins in the connecting peptide portions of these molecules (26). Therefore, homologous HPI might be predicted to differ substantially in immunogenicity from beef and pork proinsulins. However, if HPI were as highly immunogenic as beef and pork proinsulins, its use as a therapeutic agent might have to be limited.

Reeves and Douglas (24) have shown that proinsulin antibody responses are increased in patients treated with combined soluble and repository beef and pork insulins. These responses were not shown to be related to differences in animal proinsulin, which was present in trace amounts in the treatment preparations. However, in this study of patients treated with predominantly HPI plus regular HI versus those treated with HPI alone, neither proinsulin-specific nor insulin-specific antibody responses differed. Thus, in contrast to previously cited studies, the concomitant use of soluble HI did not enhance the immune response to HPI or repository HI.

The differences in usage of regular HI between treatment groups resulted in virtually identical HbA_{1c} levels. Patients in the HPI group were in general heavier yet achieved similar glycemic control with a lower dosage of long-acting agonist and less frequent use of regular HI. However, uniform criteria for the use of regular HI were not provided prospectively to investigators. These differences in dose and use of long-acting insulin may be attributable in part to pharmacokinetic differences between long-acting insulin agonists.

One possible explanation for the very low immunogenic potential of HPI is that it is due to the magnitude of the immunogenic load presented to the patient's immune system. In our earlier studies, treatment with HI produced by A- and B-chain combination technique produced maximal immune responses after 6 mo of therapy (27). Thus, HI remains immunogenic when it is used as the sole insulin agonist. We believe that one possible explanation for the low immunogenicity of HPI is that HPI is not a repository preparation and, like soluble HI, is rapidly absorbed from subcutaneous injection sites (28). In the case of beef and pork

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insulins, repository preparations have been shown to be more immunogenic than soluble insulin (29). We recently obtained data that confirm these findings for NPH HI versus soluble HI (9).

We unexpectedly found that significant increases in ^{125}I-insulin binding occurred less often in HPI-treated than in HI-treated groups. However, these data also suggest that HI-specific antibodies developed more often than HPI-specific antibodies, even when predominantly or solely HPI therapy is used. Of the 20 individuals in the HPI-treated group developing such an insulin antibody response, only 6 had significant HPI-specific responses. One of the 8 HPI-treated individuals who developed HPI-specific antibodies did not develop anti-HI antibodies. Thus, development of insulin and proinsulin antibodies is discordant. Proinsulin therapy may indirectly reduce the insulin immune response by making it possible to achieve repository activity with a soluble insulin agonist or may evoke suppression of immune responses through stimulation of T-lymphocyte suppressor substances (30).

HPI, unlike its beef and pork counterparts, has a low immunogenic potential. Decreased rather than increased insulin antibody responses were observed in patients treated with HPI in contrast with those treated with HI. The weak immunogenic properties of HPI, its prolonged activity when given in soluble form, and its possible suppression of the insulin immune response make this insulin agonist a potential substitute for repository insulins.

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