Proinsulin Immunoreactivity in Recent-Onset IDDM

The significance of insulin antibodies and insulin autoantibodies

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OBJECTIVE — To study the natural history of fasting proinsulin immunoreactivity (PIM) during the first 30 months of IDDM and its relationship to fasting C-peptide and insulin antibodies.

RESEARCH DESIGN AND METHODS — An incidence cohort of 204 consecutive newly diagnosed IDDM patients were followed prospectively, having blood drawn for measurements at diagnosis and at 1, 3, 6, 9, 12, 18, 24, and 30 months. A sensitive enzyme-linked immunosorbent assay was used for the determination of PIM.

RESULTS — All patients had detectable fasting PIM in plasma at diagnosis, with a median value and interquartile range of 3.5 pmol/l (2.2–6.2). The median PIM level increased during the first months of IDDM to reach a peak at 9–12 months (9.9–10.3 pmol/l). PIM then declined gradually to 5.6 pmol/l (1.9–13.5) at 30 months without reaching baseline. PIM at each time point was widely scattered in a skewed log-normal distribution without signs of bimodality. After the onset of insulin treatment, median insulin antibody level increased and declined in a similar pattern. Both PIM and antibody level were significantly higher in children and adolescents compared with adults. However, stepwise multiple regression analysis showed that age was only of minor importance for the PIM variation during the study period. Insulin antibody level and fasting C-peptide were the major determinants at 3–30 months, accounting for ~40% of the variation (R^2). Blood glucose was of minor importance, and insulin dose, HbA_{1c}, and BMI were of no importance. The correlation between fasting PIM and fasting C-peptide improved (R^2 doubled) if the insulin antibody level was accounted for. Further, the slope of the correlation curve between PIM and C-peptide increased threefold when antibody binding was >4%. At diagnosis, insulin autoantibodies could be detected in 19% of the patients. Their presence predicted higher proinsulin at 1–3 months, a higher insulin dose the 1st year, and higher levels of insulin antibodies later in the study.

CONCLUSIONS — Circulating insulin antibodies may affect the level of PIM in IDDM, probably by adding a pool of IgG-bound PIM thereby increasing half-life and plasma concentration. This may explain why C-peptide and PIM levels do not change in concert during the 1st years of IDDM. Unlike C-peptide, PIM can not therefore quantitate β-cell secretion unless the presence of insulin antibodies is ruled out.

Fasting proinsulin immunoreactivity (PIM) in plasma is elevated in recent-onset IDDM patients (1,2), both absolute and relative to C-peptide (1). During the 1st year of IDDM, the plasma level of PIM seems to run through a pattern of changes, different from that of C-peptide. Instead of peaking at 3 months, PIM continues to be elevated 3–9 months after diagnosis (1). Treatment with cyclosporin, an inhibitor of the immune system, resulted in higher C-peptide levels during remission (3), whereas PIM was unchanged from baseline (1). In the Canadian-European–controlled trial (1,3) designed to study the effect of cyclosporin, we did not find PIM related to any other of the studied parameters but C-peptide (unpublished data). Thus, we suggested that the changes of the ratio of PIM–to–C-peptide during the 1st year of IDDM reflected alterations of β-cell function per se (1). However, the changes in insulin-antibody binding during the 1st year of insulin treatment, with or without cyclosporin, had a striking similarity to the changes in PIM (1,4), suggesting the presence of a relationship between circulating insulin antibodies and PIM levels.

We therefore studied the natural history of PIM and its possible relationship to C-peptide and insulin antibodies during the 1st 30 months of IDDM in a cohort of 204 patients.

RESEARCH DESIGN AND METHODS

Patients

Newly diagnosed diabetic patients consecutively admitted to Steno Memorial Hospital between 1 September 1979 and 31 August 1984 who fulfilled clinical criteria of IDDM at diagnosis and clinical and C-peptide criteria at the 5-year re-evaluation participated in the study (n = 204). A detailed description of the study exclusions has recently been published (5). The subjects represent a sample of the newly diagnosed IDDM patients of the nonmigrating white population of Denmark (6).

Protocol

Patients were treated with purified porcine insulin (Velosulin and Insulatard, Nordisk, Gentofte A/S) throughout the study. Blood was drawn after an 8-h overnight fast to measure fasting C-peptide, insulin antibodies, blood glucose (BG), and HbA_{1c} at diagnosis and at 1, 3, 6, 12, 18, 24, and 30 months. Additional fasting

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BG, blood glucose; ELISA, enzyme-linked immunosorbent assay; MCR, metabolic clearance rate; PIM, proinsulin immunoreactivity.

plasma samples from each time point were kept at −20°C for later determination of proinsulin immunoreactivity. Comparing measurements of the annual cohorts ruled out a time-dependent trend in the results.

Assays

PIM (intact proinsulin and the four conversion intermediates) was determined with a two-site sandwich enzyme-linked immunosorbent assay (ELISA). The assay is based on an anti–C-peptide and anti-insulin monoclonal antibody (7). Detection limit in plasma is 0.25 pmol/l, and interassay coefficient of variation is 4.7–8.7%. The reactivity of the conversion intermediates is 65–99% of intact proinsulin on a molar basis. C-peptide was determined by a radioimmunoassay method using the polyclonal antibody M1230 (8,9). Detection limit is 60 pmol/l. Cross-reactivity with proinsulin is 10–15% on a molar basis. HbA_{1c} was determined as described by Svendsen et al. (10) until July 1986, then by high-performance liquid chromatography (DIAMAT, Bio-Rad, Richmond, CA). The normal range was 4.1–6.4 and 4.3–6.2%, respectively. The between-methods correlation was strong ($r = 0.98$, $n = 194$). Insulin-antibody binding was measured by a fluid phase assay, using monoiodinated insulin and polyethylene glycol 6,000 to separate bound from free radiolabel. Binding is expressed as the ratio (%) between bound and free ^{125}I (11). The detection limit was selected as the nonspecific binding + 4 SD.

Table 1—*Characteristics at diagnosis of a cohort of 204 IDDM patients*

	Men	Women	Total
n	123	81	204
Age (years)	26 (18–36)	28 (20–37)	27.5 (19–37)
BMI (kg/m^2)	20.5 (19.0–22.8)*	19.7 (18.0–21.0)*	20.2 (18.3–22.2)
HbA_{1c} (%)	11.5 (10.2–12.9)	12.2 (10.7–13.0)	11.6 (10.3–12.9)
PIM (pmol/l)	3.2 (1.8–6.4)	3.8 (2.8–5.8)	3.5 (2.2–6.2)
C-peptide (pmol/l)	150 (130–230)*	180 (140–260)*	170 (110–250)
Number with insulin autoantibodies	11/84	14/50	25/134
Insulin dosage at admission (U/kg)	0.34 (0.24–0.42)*	0.42 (0.32–0.59)*	0.36 (0.27–0.51)

Data are medians (interquartile ranges). *$P < 0.05$.

Laboratory study

The recovery of proinsulin was studied by adding 10 and 20 pmol of intact proinsulin to 10 selected samples containing varying levels of insulin antibodies.

Statistical analysis

Values are medians and interquartile ranges. The level of significance between groups was evaluated using two-tailed Mann-Whitney *U* or Kruskall-Wallis tests. Changes within groups were evaluated with Wilcoxon's test. A number of multiple regression analyses with stepwise variable selection were performed with PIM or PIM-to-C-peptide ratio at each time point as the dependent variables. R^2 and *P* values are given in the tables to describe the relationships. As a level of significance, 5% was chosen.

RESULTS — The characteristics of the participating subjects are indicated in Table 1. All patients had detectable (>0.25 pmol/l) fasting PIM at the time of diagnosis with a median value of 3.5 pmol/l (2.2–6.2). During the following months, median PIM increased significantly ($P <$ 0.01, 0.001, 0.001, and 0.01 [6 vs. 12 months] for each step, respectively) to reach a peak at 9–12 months (Fig. 1). It then declined gradually ($P < 0.01$ for each step) to 5.6 pmol/l (1.6–13.5) at 30 months, without reaching its baseline level ($P < 0.05$). PIM levels at diagnosis and at the subsequent time points were widely scattered in skewed log-normal distributions as indicated in Fig. 1. No

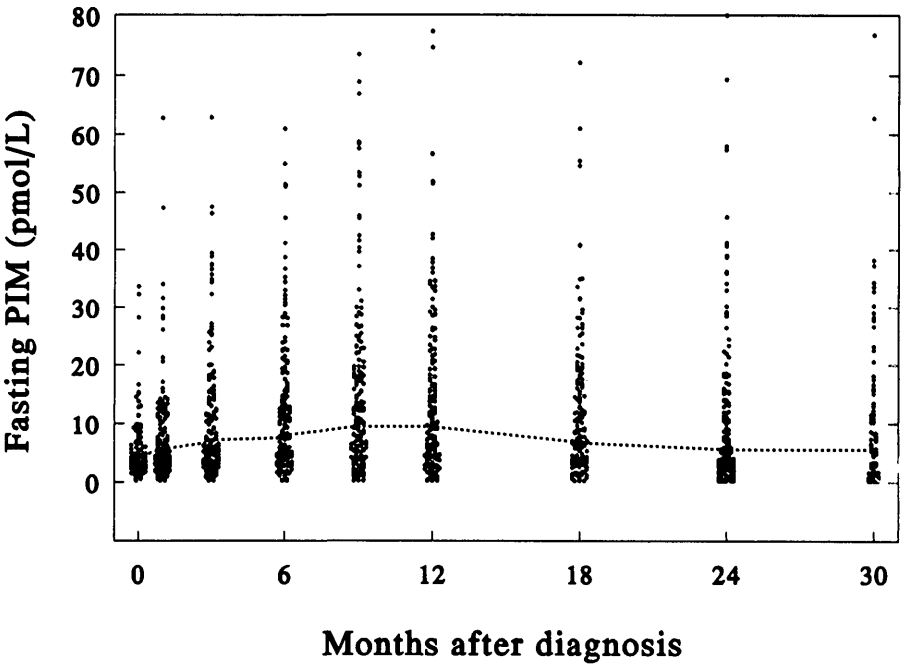

Figure 1—*Fasting PIM during the 1st 30 months of IDDM in 204 consecutive subjects. The median values are connected with a dotted line.*

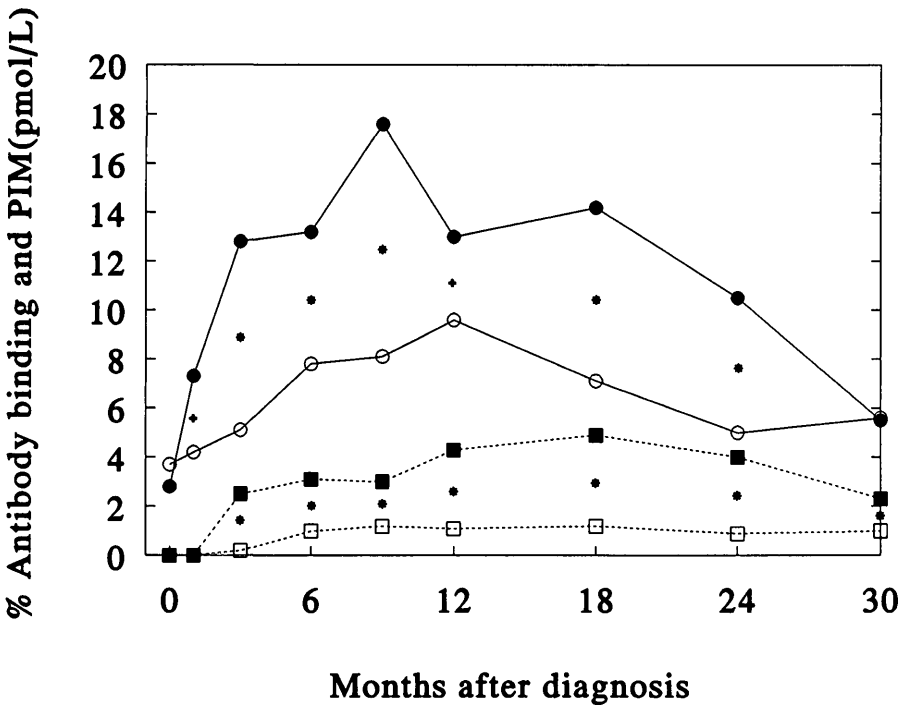

Figure 2—*Median fasting PIM (——) and median insulin-antibody binding (%) (– – – –) during the 1st 30 months of IDDM in 51 children and adolescents (● and ■) compared with 151 adults (○ and □). Values were significantly higher in children and adolescents compared with adults (Mann-Whitney U test): *P < 0.01, +P < 0.05.*

signs of bimodality could be demonstrated. A similar result was achieved by looking at the individual areas under PIM curves.

PIM reached significantly higher levels in the 51 children and adolescents (≤18 years old) compared with the 153 adults (Fig. 2). Likewise, larger insulin-antibody binding could be demonstrated in the younger age-groups (Fig. 2). To study determinants of PIM variation, we performed multiple regression analyses with stepwise variable selection on each time point, with fasting PIM as the dependent variable and age, fasting C-peptide, insulin antibody level, insulin dose, BMI, HbA_{1c}, and fasting BG as individually independent variables. R^2 and P values from these analyses are indicated in Table 2. PIM and C-peptide were closely related at diagnosis and remained so throughout the study. Insulin antibody binding was also closely correlated to PIM but only after 3 months of insulin treatment (Table 2). Taken together, ~20–50% of the variation in PIM could be explained by fasting C-peptide and insulin antibody level. Age and BG were only of minor importance (Table 2), and the other parameters were of no importance. In both young (≤18 years old) and adult subjects, multiple regression analyses gave rise to similar results.

Simple regression analyses of PIM versus insulin antibody level showed a fit to a linear model, with the best correlation at 12 months (R^2 0.23, $P < 0.001$). This correlation was neither affected by the exclusion of 38 subjects without antibodies ($R^2 = 0.21$, $P < 0.001$) nor the exclusion of 29 subjects with antibody levels >8% ($R^2 = 0.10$, $P < 0.001$). Further, the incremental change of PIM from diagnosis to 3 months was significantly correlated to the increment of the respective insulin antibody levels ($R^2 = 0.26$, $P < 0.001$).

To highlight a possible effect of insulin antibodies on the relationship between PIM and C-peptide, we divided the material after the level of insulin-antibody binding at 12 months of IDDM and compared the correlations between PIM and C-peptide (Table 3). First, the correlations were stronger in each subgroup compared with the total group. Second, the slope of the curve was threefold higher when the antibody binding was

Table 2—*Multiple regression analyses with stepwise selection on the relationship of fasting PIM to fasting C-peptide, insulin-antibody binding, fasting BG, and age at diagnosis in 204 recent-onset IDDM patients*

	Fasting C-peptide		Insulin-antibody binding		Fasting BG		Age at diagnosis	
	R^2	P	R^2	P	R^2	P	R^2	P
PIM at								
Diagnosis	0.43	<0.001	<0.01	NS	<0.01	NS	<0.01	NS
1 month	0.23	<0.001	<0.01	NS	0.05	<0.01	0.06	<0.01
3 months	0.19	<0.001	0.21	<0.001	0.05	0.01	0.07	0.001
6 months	0.12	<0.001	0.03	0.01	0.08	<0.01	0.09	<0.001
9 months	0.16	<0.001	0.25	<0.001	<0.01	NS	0.05	0.001
12 months	0.22	<0.001	0.24	<0.001	<0.01	NS	0.02	<0.05
18 months	0.16	<0.001	0.16	<0.001	<0.01	NS	0.03	0.01
24 months	0.18	<0.001	0.23	<0.001	<0.01	NS	<0.01	NS
30 months	0.16	<0.001	0.13	<0.001	0.04	<0.05	<0.01	NS

Data are R^2 and P values for multiple regression analyses with stepwise selection. Fasting PIM from diagnosis to 30 months is the dependent variable. Fasting C-peptide, insulin-antibody binding, fasting BG, age at diagnosis, insulin dose per kilogram BMI, and HbA_{1c} at each time point were all tested as independent variables. Only parameters of importance for the variation are indicated.

Table 3—*Fasting PIM versus fasting C-peptide on different levels of insulin-antibody binding in 182 of 204 IDDM patients 1 year after diagnosis*

Insulin-antibody binding (%)	*n*	R^2	Equation	*P*
0	37	0.40	$y = -2.3 + 50x$	<0.001
0.1–2.0	66	0.30	$y = 1.5 + 50x$	<0.001
2.1–4.0	25	0.25	$y = 7.3 + 62x$	0.01
>4.0	54	0.35	$y = 3.0 + 166x$	<0.001
All	182	0.14	$y = 2.7 + 77x$	<0.001

R^2 and *P* values are for simple regressions with PIM as a dependent and C-peptide as an independent variable.

>4%. The 1-year incremental changes of median fasting PIM in these subgroups were 2.7, 6.1, 9.2, and 14.0 pmol/l, respectively with increasing levels of antibodies ($P < 0.001$). The corresponding median ratios of PIM to C-peptide were 0.7, 2.5, 3.0, and 8.2% ($P < 0.001$).

To highlight the validity of the immunochemically determined proinsulin concentrations in samples with endogenous antibodies, we studied the recovery of proinsulin in the assay by adding 10 and 20 pmol proinsulin (Novo Nordisk A/S) to 10 selected samples with increasing levels of insulin-antibody binding (0, 0, 1.7, 2.2, 3.7, 4.0, 7.4, 8.6, 16.3, and 16.9%). Recovery tended to decrease with increasing level of antibodies (the recovery of 20 pmol versus insulin-antibody binding: $r = -0.62$, $P = 0.056$). However, it remained within reasonable limits: 62–105% and 69–101% for the recovery of 10 and 20 pmol, respectively.

Of the patients, 19% already had insulin autoantibodies at the time of diagnosis. They reached a higher level of PIM and insulin-antibody binding after 3 months and needed a larger amount of insulin to achieve similar mean HbA_{1c} during the study period as compared with subjects without autoantibodies. Fasting C-peptide tended to be lower among subjects with autoantibodies but the difference was not significant ($P = 0.06$ at diagnosis). The prevalences of insulin antibodies at 1 year were similar in the two groups: 96 and 74%, respectively (NS).

CONCLUSIONS— We have described the natural course of fasting PIM after onset of IDDM in an unselected cohort of patients. We have previously demonstrated homogeneity in the pattern of increase and decline of β-cell function, measured as fasting C-peptide (5). The present study of PIM in the same IDDM population showed a similar homogeneity, at least during the first 30 months of the disease.

Compared with the patients 5–6 years younger in the placebo group of the Canadian-European cyclosporin trial (1), we found a fasting PIM within the normal range at diagnosis and a later peak of PIM during remission and relapse, 9–12 months against 3–9 months. The demonstration of an age-dependent difference in the pattern of change of PIM and the fact that neither of the subjects had started insulin treatment at entry of this study could explain the difference. Changing from an ELISA method using polyclonal antibodies with a detection limit of 1.2 pmol/l (12) to a more sensitive ELISA using two monoclonal antibodies was of no significance. The correlation between the old and the present assay was: $r = 0.98$, $P < 0.001$, present = 0.99 × old + 0.11 pmol/l.

The present study showed the same similarity between level of PIM and insulin antibodies during the 1st year, as demonstrated in the cyclosporin trial (1,4). Multiple regression analyses indicated that antibody binding was an equally important determinant of PIM variation as C-peptide after the onset of insulin treatment (Table 2). The relationship between PIM and C-peptide was affected by the insulin-antibody binding, especially when it exceeded 4%. In the laboratory study, the recovery of proinsulin tended to be influenced by the antibody level (0–16%) in the samples but not by the amount of intact proinsulin added (10–20 pmol/l).

The increased concentrations of PIM could be explained by an increased secretion or a decreased metabolic clearance rate (MCR). Because the pattern of PIM and C-peptide changes during remission and relapse are not in concert with each other, changes in MCR is a likely explanation. PIM bound to circulating IgG antibodies would have a lower MCR causing an increase in PIM levels. Proinsulin has been shown by others to cross-react with insulin antibodies (13), and the recovery experiment suggests that antibody-bound PIM was measured along with free PIM in our assay. The presence of proinsulin autoantibodies, which may or may not be detected in the present insulin antibody assay, can add further to the variation of the detected PIM levels and may therefore have weakened the correlation to insulin-antibody binding (13). It therefore does not seem justified to suggest that the changes of the ratio of PIM to C-peptide during remission and relapse in IDDM patients reflects differential alteration in β-cell secretion (1).

With the present tracer-binding technique, we found insulin autoantibodies at diagnosis in a frequency comparable with others (14). In accordance with the findings of Ludvigsson et al. (15), the presence of autoantibodies was of no predictive value on C-peptide levels. Insulin dose per kilogram was correlated to insulin-antibody binding throughout the study (12 months: $r = 0.38$, $P < 0.001$). Thus, the larger insulin requirements in subjects with autoantibodies at diagnosis could be related to the higher levels of insulin antibodies reached by these subjects. The mechanism may be a reduced bioavailability of the circulating IgG-bound insulin. However, after the major shift to human insulin, these problems with purified animal insulin is of minor interest (16–18). The prevalence of insulin antibodies after onset of insulin treatment was independent of the presence of autoantibodies at diagnosis as described by others (15).

We have described the natural course of fasting PIM after the onset of IDDM. Although significantly correlated during the 1st years of IDDM, PIM and C-peptide did not change in concert during remission and relapse. A significant correlation between PIM and circulating insulin antibodies in this period suggest that the explanation for this phenomenon is a changed metabolic clearance rate of PIM probably by adding a pool of IgG-bound PIM and thereby increasing its half-life and plasma concentration rather than being a reflection of a differential al-

teration in β-cell secretion. Unlike C-peptide, PIM levels can therefore not quantitate β-cell secretion unless the presence of insulin antibodies is ruled out.

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