Immune Responses to Insulin Aspart and Biphasic Insulin Aspart in People With Type 1 and Type 2 Diabetes

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OBJECTIVE — The antibody responses to a novel rapid-acting insulin analog, insulin aspart (IAsp), and their potential clinical correlates were studied with a specifically developed method in 2,420 people with diabetes treated for up to 1 year with preprandial subcutaneous injections of IAsp.

RESEARCH DESIGN AND METHODS — Circulating insulin antibodies were analyzed by radioimmunoassay with ^{125}I insulin or IAsp tracers and polyethylene glycol precipitation. Four multinational, open, parallel group studies were conducted in Europe and North America, with a total of 1,534 people with diabetes exposed to IAsp and 886 people exposed to human insulin (HI) as meal-related insulin for 6–12 months.

RESULTS — Insulin antibodies specific to HI or IAsp were absent in a majority of patients throughout the 6- to 12-month study periods. A majority of the patients (64–68%) had antibodies cross-reacting between HI and IAsp when entering the studies, with baseline levels (means ± SD of percent bound/total) of 16.6 ± 16.3% in study 1 and 10.3 ± 14.0% in study 4. In all four studies, cross-reactive antibodies increased in patients exposed to IAsp, with a maximum at 3 months, and thereafter there was a decline toward baseline levels at 9–12 months (levels at 3 and 12 months: 22.3 ± 19.7 and 16.8 ± 16.5% in study 1 and 21.5 ± 21.9 and 16.9 ± 17.4% in study 4). Antibody levels showed similar changes in people with type 1 and type 2 diabetes, and there was no consistent relationship between antibody formation and glycemic control or between antibody formation and safety in terms of adverse events.

CONCLUSIONS — Treatment with IAsp is associated with an increase in cross-reactive insulin antibodies, with a subsequent fall toward baseline values, without any indication of clinical relevance because no effect on efficacy or safety could be identified.

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Abbreviations: BIAsp, biphasic insulin aspart; BHI, biphasic human insulin; B/T, bound/total; C_{max}, maximum glucose concentration; HI, human insulin; IAsp, insulin aspart; PEG, polyethylene glycol; t_{max}, time of maximum concentration.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Anti-insulin antibodies are common in people with diabetes treated with subcutaneous human insulin (HI) (1). It has been shown that up to 80% of people treated with subcutaneous insulin may develop anti-insulin antibodies (1). Hypothetically, anti-insulin antibodies could affect the pharmacokinetics of the exogenously administered insulin by several mechanisms, which would either enhance or reduce the pharmacodynamic response. Antibodies could enhance and prolong the pharmacodynamic action by serving as a carrier, or they could reduce insulin action by neutralization. As the insulin-binding sites of the antibodies produced vary from individual to individual, the pharmacodynamic effect could be expected to vary from subject to subject in an unpredictable manner.

Insulin aspart (IAsp) is, together with insulin lispro (2), one of the two available rapid-acting insulin analogs with the advantage of being able to mimic the peripheral insulin response to a meal more closely than soluble HI, even when administered immediately before the meal (3–5). This has been made possible by substitution of aspartic acid for proline in position B28, thereby reducing the tendency to form hexamers (6). Absorption from the subcutaneous tissue is promoted and is no longer limited by dissolution of the hexamers (6,7). Clinical experimental studies have shown lower postprandial glucose with IAsp injected just before a meal than with HI injected 30 min before a meal (5,8). In longer-term studies, IAsp provided glucose control, assessed by HbA_{1c}, that was at least as good or better than that obtained with HI (9–11). IAsp is also in clinical testing in a mixed rapid- and intermediate-acting formulation, biphasic IAsp (BIAsp) (12).

Any substance not normally found in the human body may serve as an antigen. This includes insulin analogs. For IAsp, there are three types of antibodies of interest, namely antibodies specific to HI (versus IAsp), antibodies specific to IAsp (versus HI), and antibodies cross-reactive to both IAsp and HI. A method was de-

Table 1—*Radioimmunoassay series*

Series	Assay mixture	Result represent the sum of
A	Sample + phosphate buffer + insulin tracer	Background, human insulin–specific and cross-reacting antibodies
B	Sample + cold insulin + insulin tracer	Background
C	Sample + cold IAsp + insulin tracer	Background and human insulin–specific antibodies
D	Sample + cold IAsp + IAsp tracer	Background
E	Sample + cold insulin + IAsp tracer	Background and IAsp antibodies

veloped to measure antibody-binding capacity because this is assumed to reflect the overall insulin antibody concentration. The present investigation was performed to clarify the role of insulin antibodies in diabetic individuals treated with IAsp with regard to clinical efficacy and safety. A large sample size minimized the risk of drawing incorrect conclusions. Furthermore, because antibodies were collected for various time periods, data from several large-scale multinational studies were compared to maximize validity in the findings.

RESEARCH DESIGN AND METHODS

— Insulin antibodies were measured in four clinical studies with IAsp (9–11) or BIAsp (12). The four trials had a similar design and similar end points and were multicenter, randomized, open-labeled, parallel-group studies conducted in a total of 200 centers in North America (76 sites) and northern Europe (124 sites). The studies were approved by national regulatory agencies and local ethics committees and were performed in accordance with good clinical research practice. Written informed consent was obtained from all patients.

Patients

Of the four studies, studies 1 and 2 included 1,954 patients with type 1 diabetes, whereas study 3 included 182 patients with type 2 diabetes. They were randomized to IAsp (n = 1,396) or HI (n = 740) in a mealtime plus basal regimen, with NPH as basal insulin. The fourth study (12) included 294 patients with either type 1 diabetes (n = 104) or type 2 diabetes (n = 190) randomized to treatment with twice-daily BIAsp 30 (n = 143) or biphasic HI (BHI) 30 (n = 151). A total of 10 patients withdrew before starting treatment, so in total, 1,534 patients were exposed to IAsp/BIAsp 30, and 886 were exposed to HI/BHI 30.

The patients recruited were adult men and women with type 1 (study 1 and 2) or type 2 diabetes (study 3) by World Health Organization criteria (13), with a duration of diabetes of ≥2 years, and were treated with insulin for at least 1 year. For inclusion, BMI was <35.0 kg/m^2 and HbA_{1c} was ≤11.0% (reference value <6.0%). People with active proliferative retinopathy or nephropathy (serum creatinine >150 μmol/l), recurrent severe hypoglycemia, significant cardiovascular disease, or systemic corticosteroid treatment, or who required >1.4 units · kg^{-1} · day^{-1} insulin or were pregnant or abusing drugs, were excluded from the trials. Study 4 included patients with either type of diabetes who were ≥35 years with a BMI up to 40.0 kg/m^2.

Sampling for insulin antibodies was performed at 0, 3, 6, 9, and 12 months in study 1; at 0 and 6 months in study 2; at 0, 3, and 6 months in study 3; and at 0, 3, 6, and 12 months in study 4. Sampling for eight-point self-monitored blood glucose concentrations was performed at 6 months in studies 1–3 and at 3 months in study 4.

Antibody assay

Serum samples for antibody determinations were collected in the fasting state and kept at −20°C until shipment and thereafter stored at −20°C until analysis. All samples were obtained from fasting individuals to minimize interference from administered HI and IAsp. The antibody levels were shown to be stable for up to at least five freeze/thaw cycles, and a review of other control data showed stability up to at least 5 years at −20°C.

The antibody determinations were performed in a subtraction radioimmunoassay (as described below) that allowed determination of three subgroups of antibodies: antibodies cross-reacting between HI and IAsp, antibodies specific for HI (versus IAsp), and antibodies specific for IAsp (versus HI). In principle, antibodies in the samples were detected via their ability to bind to the tracers (^{125}I-labeled HI or IAsp) and the ability of polyethylene glycol (PEG) precipitation to separate antibody-bound tracer from unbound tracer. Each sample was analyzed in five different ways, all in duplicate. The series are described in Table 1. The results were expressed as the percent bound radioactivity relative to the total amount of radioactivity present. Results were given to one decimal place. For each sample, the following was calculated: *1*) the amount of insulin-specific antibodies (series C–B); and *2*) the amount of IAsp-specific antibodies (series E–D); *3*) the amount of cross-reacting antibodies series (A–C). The tracers used were HI tracer [^{125}I-(Tyr-A14) HI] (1) and IAsp tracer [^{125}I-(Tyr-A14) IAsp], both with specific activities of 30 mCi/mg and made by Novo Nordisk (14). Nonradioactive HI and IAsp were obtained from Novo Nordisk.

On day 1 of the analysis, equal volumes of sample (50 μl), tracer, and cold insulin/buffer were mixed and incubated overnight at 4°C. On day 2, a PEG 6,000 molecular weight solution was added to a final concentration at 12.5% vol/vol. After mixing and centrifugation, the supernatant was discarded, the pellet was washed with 12.5% PEG, and the radioactivity of the pellets was counted.

Validation of the analysis showed that the intra-assay variation was <5% for medium and high antibody responses and up to 11% for low antibody responses. Day-to-day variation was always <15%. As long as the day-to-day variation was <15%, it was deemed acceptable to analyze different samples from one patient on different days.

The normal ranges for the three populations of antibodies were defined as the upper limits of the 90% CI of the 95th percentile (15). In 150 plasma samples from healthy volunteers, the upper normal range was found to be 0.5% bound/total (B/T) for HI-specific antibodies, 4.6% B/T for IAsp-specific antibodies, and 1.0% B/T for cross-reacting antibodies. Because it was considered that any development of antibodies would be of interest, irrespective of whether it oc-

Table 2—*Clinical characteristics of the patients in the four studies at the time of study entry*

	Study 1 (type 1 diabetes)		Study 2 (type 1 diabetes)		Study 3 (type 2 diabetes)		Study 4 (type 1 and 2 diabetes)	
	IAsp	HI	IAsp	HI	IAsp	HI	BIAsp	BHI
Total number exposed	596	286	707	358	91	91	140	151
Age (years)	39 ± 10	40 ± 12	38 ± 11	38 ± 12	57 ± 10	58 ± 10	55 ± 14	58 ± 13
Sex (%M)	51	53	55	56	63	60	58	53
Ethnic group (Europid, %)	94	93	99	99	76	76	100	99
BMI (kg/m^2)	26 ± 4	26 ± 3	25 ± 3	25 ± 3	30 ± 4	30 ± 4	27 ± 4	27 ± 4
Duration of diabetes (years)	16 ± 10	16 ± 9	15 ± 10	15 ± 10	13 ± 8	13 ± 8	15 ± 10	15 ± 10
HbA_{1c} (%)	8.1 ± 1.2	8.1 ± 1.3	8.0 ± 1.2	8.0 ± 1.2	8.1 ± 1.2	7.9 ± 1.1	8.2 ± 1.2	8.2 ± 1.3
Insulin dose (units/kg)								
Mealtime (units/kg)	0.42 ± 0.14	0.42 ± 0.16	0.40 ± 0.15	0.41 ± 0.17	0.38 ± 0.20	0.39 ± 0.18	0.61 ± 0.24*	0.60 ± 0.22*
Basal (units/kg)	0.24 ± 0.12	0.25 ± 0.14	0.29 ± 0.12	0.29 ± 0.12	0.23 ± 0.13	0.22 ± 0.12		

Data are means ± SD or %, unless otherwise indicated. Normal HbA_{1c} <6.0%. *Premixed insulin (combined mealtime and basal insulin).

curred below, across, or above the limit of the normal range, all results are included in the calculations presented here.

Statistical methods

All statistical analyses of efficacy were based on the intention-to-treat population. The two treatments were compared with respect to the change from baseline to the end of the 6- or 12-month treatment period in IAsp-specific, HI-specific, and cross-reactive antibodies to either insulin, using ANOVA with the baseline value as a covariate.

Based on the antibody data recorded in the phase III trials, the potential effect of insulin antibodies on insulin action was assessed by means of three correlation analyses:

- Partial correlations between change in HbA_{1c} from baseline to 6 months of treatment and change in insulin antibodies (IAsp-specific, HI-specific, and cross-reacting with IAsp and HI), conditioned on change in basal as well as meal-related insulin doses (study 1–3) or change in breakfast and dinner insulin doses (study 4).
- Partial correlations between change in basal and meal-related insulin doses (study 1–3) or breakfast and dinner-related insulin doses (study 4) during the treatment period and change in insulin antibodies (IAsp-specific, HI-specific, and cross-reacting), conditioned on changes in HbA_{1c}.
- Partial correlations between self-monitored prebreakfast morning glucose levels and cross-reacting insulin antibodies at 6 months (study 1–3) or at 3 months (study 4; only collected at this time point), conditioned on the following: meal and basal insulin dose at baseline and after 6 months (study 1–3), breakfast and dinner insulin dose at baseline and after 3 months (study 4), baseline self-monitored prebreakfast glucose, and baseline insulin antibodies.

Spearman rank correlations were used because of their robustness to assumptions about the distributions of the variables.

A meal test was performed in study 1 after 9–11 months of treatment in 107 IAsp-treated patients. To determine whether there were any linear dependencies with glycodynamic parameters, regression analyses were performed between cross-reactive antibody levels and maximum glucose concentration (C_{max}), time of maximum concentration (t_{max}), and glucose excursion during the first 4 h, while adjusting for insulin dose and meal energy content.

Cross-reacting insulin antibody levels were categorized into three intervals: ≤5, 5–25, and >25%. These categories were arbitrarily selected before release of the data and did not reflect any known potentially clinical significant levels of antibodies, since it was not known what levels of antibodies might be potentially clinically significant.

RESULTS — The studies included a large number of predominantly adult Caucasian subjects reflecting the population of insulin-treated adult type 1 and type 2 diabetic patients across Europe and North America (Table 2) (9–12).

Antibodies specific to IAsp

Antibodies specific to IAsp were rare. Mean IAsp-specific antibody levels remained undetectable in most patients throughout the studies, with a mean below the upper normal limit (Table 3).

Antibodies specific to HI

Likewise, antibodies specific to HI remained undetectable in a majority of patients throughout the studies (Table 3).

Antibodies cross-reacting to IAsp and HI

A majority of patients had cross-reactive antibodies at baseline. For example, 64% of patients in study 1 had a cross-reactive antibody level of >5% at baseline (Table 4). These antibodies increased significantly in all four studies in patients treated with IAsp and both in type 1 and type 2 diabetic patients (Table 3). The maximum level was similar in all studies and was found after the first 3 months of treatment, after which mean levels decreased toward baseline (Fig. 1). In the 12-month study with IAsp (study 1) (Table 3), the level of cross-reactive antibodies had returned to baseline by 9 months (Fig. 1). The change in cross-reactive antibodies from baseline to 12 months did not differ significantly between treatment with IAsp and HI, the point estimate being 0.72% (95% CI −0.70 to 2.13).

In the 12-month study with the biphasic formulation of IAsp, BIAsp 30 (study 4) (Table 3), there was an initial significant 11.2% increase in cross-reactive antibodies followed by a decrease from 3 months onwards. The 4.6% absolute decrease between months 3 and 12

Table 3—*Serum levels of antibodies (% B/T) specific to IAsp, specific to HI, and cross-reactive to IAsp and HI in four studies in patients with diabetes (studies 1-4) (9,10,11,12) assessed during the first treatment year*

	Baseline		3 Months		6 Months		9 Months		12 Months	
	n	Mean	*n*	Mean	*n*	Mean	*n*	Mean	*n*	Mean
IAsp or BIAsp 30 treatment group										
IAsp-specific										
Study 1	577	0.9 ± 2.8	504	1.1 ± 3.6	542	1.2 ± 3.6	438	1.1 ± 3.6	464	0.9 ± 3.2
Study 2	701	1.1 ± 3.5			676	1.2 ± 3.3				
Study 3	89	1.6 ± 4.2	84	1.2 ± 3.5	87	1.6 ± 4.2				
Study 4	135	1.0 ± 2.5	128	1.0 ± 2.6	99	1.2 ± 2.7			87	0.8 ± 1.9
HI-specific										
Study 1	577	0.8 ± 2.3	504	0.9 ± 2.2	542	0.8 ± 2.1	438	0.7 ± 1.7	464	0.7 ± 1.9
Study 2	701	0.7 ± 3.2			676	0.6 ± 3.1				
Study 3	89	0.2 ± 0.7	84	0.3 ± 0.6	87	0.2 ± 0.6				
Study 4	135	0.5 ± 3.4	128	0.6 ± 3.5	99	0.1 ± 1.3			87	0.2 ± 0.8
Cross-reactive										
Study 1	577	16.6 ± 16.3	504	22.3 ± 19.7*	542	19.6 ± 17.7*	438	17.7 ± 17.2	464	16.8 ± 16.5
Study 2	701	12.2 ± 14.0			676	16.9 ± 15.8*				
Study 3	89	11.0 ± 16.6	84	13.9 ± 18.8	87	14.1 ± 17.5*				
Study 4	135	10.3 ± 14.0	128	21.5 ± 21.9*	99	19.7 ± 18.7*			87	16.9 ± 17.4*
HI or BHI 30 treatment group										
Cross-reactive										
Study 1	277	16.7 ± 16.5	239	17.3 ± 17.3	253	15.9 ± 16.5	197	17.0 ± 17.4	201	16.5 ± 16.5
Study 2	354	11.0 ± 13.2			340	11.0 ± 13.3				
Study 3	90	9.7 ± 14.7	80	9.3 ± 13.5	82	8.1 ± 13.1				
Study 4	145	10.1 ± 15.4	138	10.8 ± 16.2	99	12.2 ± 17.4			93	11.8 ± 16.9

Means data are means ± SD, with *n* = number of patients with antibody measurements at the specified time point. *Significant difference from baseline.

observed in the BIAsp 30 group was significant (95% CI −8.8 to −0.5) (Table 3). Although cross-reactive antibodies did not return to baseline levels, the absolute values were similar to those found in the 12-month study on IAsp (Fig. 1).

Correlation with clinical efficacy

There was no correlation between absolute levels of antibodies and HbA_{1c} at baseline. There was a positive correlation between meal-related daily dose of insulin and cross-reactive antibodies at baseline in three of four studies (correlation coefficient of 0.11 [$P < 0.01$], 0.16 [$P < 0.001$], 0.16 [NS], and 0.30 [$P < 0.05$] in the respective studies) and a significant correlation to daily basal insulin dose in two of four studies (correlation coefficient of 0.16 [$P < 0.001$] in study 2 and 0.34 [$P < 0.001$] in study 4).

There were no consistent correlations between baseline characteristics (HbA_{1c}, duration of diabetes, and number of daily basal injections) or demographic variables (sex, age, and BMI) and changes in cross-reactive antibody levels (data not shown).

In study 2, a weak inverse relationship between change in cross-reactive antibodies and HbA_{1c} was noted at 6 months (Table 5). However, this was not confirmed in the other studies. Furthermore, there were no consistent correlations between change in insulin dose and changes in cross-reactive antibody levels at 6 months in any of the studies (Table 6). However, in study 3, an inverse relationship between cross-reactive antibodies and change in meal-related insulin dose was observed.

Correlation with glucodynamic parameters in a meal test

There was no correlation in a linear regression analysis between cross-reactive antibodies and the glucose C_{max} (slope 0.00 [95% CI −0.04 to 0.04]), glucose t_{max} (slope −0.10 [−0.46 to 0.26]), or glucose excursion during the first 4 h (slope 0.03 [−0.03 to 0.09]).

There were no consistent correlations between fasting self-monitored blood glucose at 3 or 6 months and cross-reactive antibody levels in studies 1–4 (Table 5). Likewise, no significant correlations could be demonstrated between the average daily prandial increase in blood glucose at 3 months and cross-reactive antibody levels (data not shown).

Association between adverse events and antibodies

The increase in cross-reactive antibodies with IAsp did not produce any clinical adverse effects. There were no reports of allergic reactions or other adverse events that could be specifically linked to insulin antibody formation. Of 1,534 patients treated with IAsp or BIAsp, 21 had potentially allergic symptoms assessed as not unlikely to have been related to the study drug, compared with 9 of 866 patients treated with HI or BHI. Three patients withdrew, two treated with IAsp and one with HI (all because of rash). Main reactions were injection site reactions (nine on IAsp, five on HI), rash (five on IAsp, two on HI), and singular cases of injection site pain (one in each group), hot flushes (one in each group), allergic reaction (one on IAsp), allergy (one on IAsp), eczema (one on IAsp), edema (one on IAsp), pruritus (one on IAsp), and purpura (one on

Table 4—*Categorization by insulin antibody level (5, 5–25 and >25%) of IAsp-specific, HI-specific, and cross-reactive antibodies during 12 months of treatment in 884 patients with type 1 diabetes (study 1) (9)*

Time and antibody level (%)	IAsp			HI		
	Antibodies specific to:			Antibodies specific to:		
	IAsp	HI	Cross-reacting	IAsp	HI	Cross-reacting
Baseline						
n	484	484	484	217	217	217
≤5	459 (94.8)	467 (96.5)	174 (36.0)	205 (94.5)	209 (96.3)	69 (31.8)
5–25	24 (5.0)	16 (3.3)	180 (37.2)	12 (5.5)	8 (3.7)	95 (43.8)
>25	1 (0.2)	1 (0.2)	130 (26.9)	0 (0.0)	0 (0.0)	53 (24.4)
Month 3						
n	458	458	458	207	207	207
≤5	428 (93.4)	442 (96.5)	128 (27.9)	194 (93.7)	199 (96.1)	68 (32.9)
5–25	28 (6.1)	16 (3.5)	156 (34.1)	13 (6.3)	8 (3.9)	85 (41.1)
>25	2 (0.4)	0 (0.0)	174 (38.0)	0 (0.0)	0 (0.0)	54 (26.1)
Month 6						
n	490	490	490	219	219	219
≤5	457 (93.3)	473 (96.5)	138 (28.2)	205 (93.6)	211 (96.3)	81 (37.0)
5–25	31 (6.3)	17 (3.5)	200 (40.8)	14 (6.4)	8 (3.7)	88 (40.2)
>25	2 (0.4)	0 (0.0)	152 (31.0)	0 (0.0)	0 (0.0)	50 (22.8)
Month 9						
n	438	438	438	197	197	197
≤5	409 (93.4)	425 (97.0)	132 (30.1)	185 (93.9)	189 (95.9)	62 (31.5)
5–25	28 (6.4)	13 (3.0)	186 (42.5)	12 (6.1)	8 (4.1)	82 (41.6)
>25	1 (0.2)	0 (0.0)	120 (27.4)	0 (0.0)	0 (0.0)	53 (26.9)
Month 12						
n	464	464	464	201	201	201
≤5	438 (94.4)	450 (97.0)	147 (31.7)	192 (95.5)	193 (96.0)	67 (33.3)
5–25	25 (5.4)	14 (3.0)	192 (41.4)	9 (4.5)	7 (3.5)	80 (39.8)
>25	1 (0.2)	0 (0.0)	125 (26.9)	0 (0.0)	1 (0.5)	54 (26.9)

Data are *n* (%), with *n* = number of patients with antibody measurements at the specified timepoint. Only patients completing the 12 months observation period are included in the present table.

IAsp). There were no cases with injection site atrophy or hypertrophy reported. Mean antibody levels in potentially allergic patients did not evolve differently than in other patients (in the subpopulation of 19 IAsp-treated patients, cross-reactive antibody levels were 26 ± 21 at baseline and 28 ± 22 at 6 months). There were no significant correlations between major hypoglycemia episodes and levels of cross-reactive antibodies at 3 or 6 months.

In the subgroup of patients with large increases in antibody levels (>25% increase), there was also no increase in adverse events. Thus, there was no difference in study drop-out rate (and hence no selection bias in the data presented), effect on glucose control, or adverse events reported in this subgroup of patients compared with other patients.

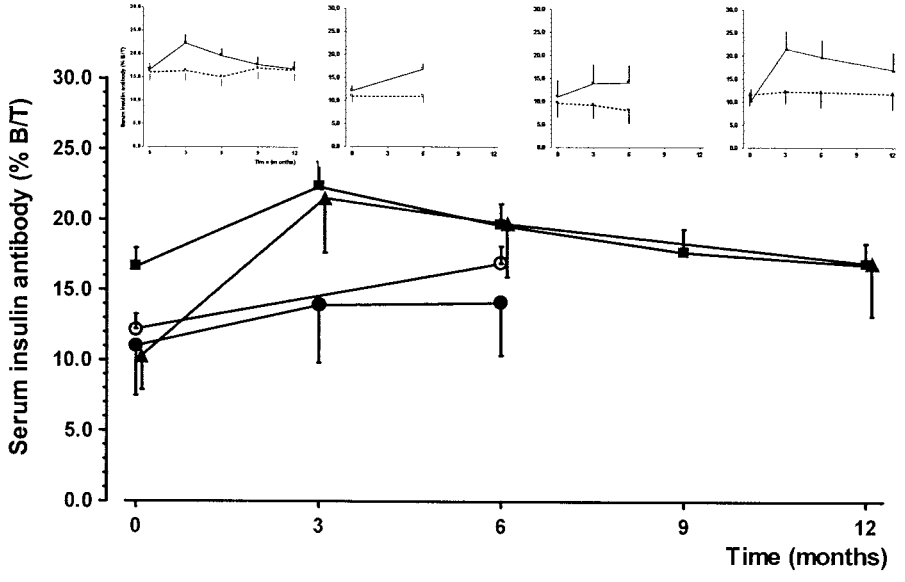

Figure 1—*Cross-reactive antibodies over time in the four studies in patients treated with insulin aspart. ■, study 1; ○, study 2; ●, study 3; ▲, study 4. Inserts show each individual study, comparing insulin aspart (full lines) with HI (hatched lines). Data are means ± 2 SEM.*

CONCLUSIONS — Most changes to molecular structure showing even a slight difference to endogenous substances in

Table 5—*Partial Spearman rank correlations between changes in insulin antibodies and HbA_{1c} after 6 months treatment, conditioned on change in total daily insulin dose, or self-monitored morning fasting blood glucose levels at 6 months (in studies 1–3) and at 3 months (in study 4)*

	Study 1	Study 2	Study 3	Study 4
Change in antibodies and HbA_{1C}				
IAsp-specific	0.017	0.011	0.144	0.191
HI-specific	−0.016	−0.014	0.076	−0.091
Cross-reacting	−0.074	−0.115*	−0.148	0.142
Change in antibodies and fasting blood glucose				
IAsp-specific	0.074	−0.026	0.097	0.097
HI-specific	0.024	0.041	0.079	−0.087
Cross-reacting	0.035	−0.053	−0.222	−0.220†

In the partial correlations the following variables have been conditioned on meal and basal insulin dose (in study 1–3) or breakfast and dinner insulin dose (in study 4) at baseline and after 6 months, baseline self-monitored fasting glucose, and baseline insulin antibodies. *$P < 0.01$; †$P < 0.05$.

the human body will evoke an immune response. In the case of insulin, this has been thoroughly examined because early insulin preparations were of beef or pork origin. Beef insulin differs from HI by three amino acids, whereas porcine insulin differs by one amino acid at position B30. IAsp differs from HI by one amino acid at position B28 and was as immunogenic as porcine insulin and less immunogenic than bovine insulin in a transgenic mouse model (16). When studying experiences with beef and pork insulin, it has to be kept in mind that the first such preparations contained insulin-like contaminants that could also be immunogenic. Although case reports occasionally have pointed at insulin antibody levels as a possible factor in some adverse events, such as hypoglycemia unawareness, firm scientifically valid evidence for this observation has not emerged. Considering the amount of exposure, the conclusion is that porcine monocomponent insulin is as safe as HI (1,17,18).

The antigenicity obtained with insulin analogs such as IAsp may be compared with the antigenicity of porcine monocomponent insulin (16). Because HI and IAsp are homologous except for one peripherally located amino acid, the vast majority of antibodies will be cross-reactive. Soluble IAsp did not produce any increase in IAsp-specific antibodies. However, a majority of patients had cross-reactive antibodies already before exposure to IAsp. An increase in insulin antibodies has been observed in previous studies of subcutaneous administration of HI (18,1). It is not known whether this is an effect of the disease per se or in part induced by the subcutaneous injections of insulin. The baseline levels of cross-reactive antibodies in our studies reflect this previous influence of disease and/or long-term injections of subcutaneous HI. In this context, it is of interest to note that the North American patients with type 1 diabetes in study 1 had higher insulin antibody levels at baseline than European type 1 diabetic patients with matching demographics in study 2, a finding for which we have no explanation. The type 2 patients had slightly lower insulin antibody levels, which seems reasonable considering the shorter time treated with insulin and the higher age, because it is known that elderly people have a less responsive immune system (19). Because all antibody levels were assayed by one common laboratory in all four studies, differences in methodology cannot explain differences between studies.

In the present studies, an increase in cross-reactive antibodies was observed, with a subsequent return toward baseline values from 9 months onwards in the study with soluble IAsp. The interesting observation of return baseline values has not been reported for other insulin analogs or for porcine insulin. The difference found in immunogenicity between HI and IAsp was not the result of differences in pH or excipients because these factors were similar between the insulins.

There were no consistent correlations observed between changes in cross-reactive antibody values and insulin dose, HbA_{1c}, blood glucose levels, or adverse event pattern. Increased antibody levels were not linked with increases in insulin doses; in fact, the opposite trend was shown in study 3. The clinical data available for 12 months of exposure demonstrates that the initial increase in cross-reactive insulin antibody levels with IAsp treatment is transient. Any correlation between efficacy and insulin antibodies would have the largest likelihood of presenting itself at a time point when antibodies would be the highest (i.e., at 3–6 months). Because no consistent correlation could be identified, it is unlikely that long-term use of IAsp would impair metabolic control.

The cross-reactive antibody response was similar in type 1 and type 2 diabetic patients as assessed by comparing results of studies 1 and 2 with study 3. This is an important point and interesting observation because the pathogenesis is quite different. Furthermore, the autoimmune response would hypothetically be larger in type 1 diabetes because of a memory T- and B-cell response in people with type 1

Table 6—*Partial Spearman rank correlations between changes in insulin dose and changes in insulin antibodies after 6 months treatment, conditioned on change in HbA_{1c}*

	Study 1		Study 2		Study 3		Study 4	
Antibodies	Meal	Basal	Meal	Basal	Meal	Basal	Breakfast	Dinner
IAsp-specific	−0.059	0.018	0.062	−0.009	−0.120	0.020	0.190	0.156
HI-specific	−0.056	−0.035	−0.065	−0.06	−0.055	0.179	−0.004	0.027
Cross-reacting	−0.027	0.063	−0.032	0.056	−0.222*	0.114	0.149	0.191

*$P < 0.05$.

diabetes and also a higher response in patients with so-called high-responder HLA-haplotypes like HLA-DR3 and -DR4.

Regarding the biphasic formulation, the prolonged release of insulin from subcutaneous depot could be expected to produce an increased antibody response, as could the fact that this formulation provides the full daily insulin dose (i.e., approximately twice as much IAsp). Thus, it is not surprising that study 4 presented with a steeper increase in the levels of cross-reactive antibodies. However, a similar reduction in cross-reactive antibody levels from month 3 onwards was observed with BIAsp 30 compared with IAsp.

Insulin lispro, another rapid-acting insulin analog with amino acid substitutions in the same part of the B chain, also evokes an antibody response to the same extent as IAsp (14% increase in cross-reactive antibodies relative to HI) (20,21). Thus, in a meta-analysis of six studies, cross-reactive insulin antibodies increased from 7.5 ± 2.9 to 8.8 ± 3.5 in 1,811 patients treated with insulin lispro compared with 7.5 ± 2.9 to 7.9 ± 3.2 in those treated with HI. (Note that it not is possible to directly compare the values with those obtained by us because of differences in methodology.) This increase was not reported to be associated with any change in efficacy or safety (22,23). To date, no data have been published on the immunogenicity of biphasic insulin lispro.

In conclusion, treatment with IAsp was found to be associated with an increase in cross-reactive antibody responses, peaking at 3 months and declining subsequently. Such a time profile was demonstrated for both type 1 and type 2 diabetic patients treated with IAsp and also with a soluble formulation and a biphasic formulation of IAsp. No consistent correlation was observed between increases in antibody levels and efficacy or safety. Thus, the increase in antibody levels was without any indication of clinical relevance.

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