Pancreatic β-Cell Function and Immune Responses to Insulin After Administration of Intranasal Insulin to Humans At Risk for Type 1 Diabetes

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OBJECTIVE — Mucosal administration of insulin retards development of autoimmune diabetes in the nonobese diabetic mouse model. We conducted a double-blind crossover study in humans at risk for type 1 diabetes to determine if intranasal insulin was safe, in particular did not accelerate β-cell destruction, and could induce immune effects consistent with mucosal tolerance.

RESEARCH DESIGN AND METHODS — A total of 38 individuals, median age 10.8 years, with antibodies to one or more pancreatic islet antigens (insulin, GAD65, or tyrosine phosphatase-like insulinoma antigen 2) were randomized to treatment with intranasal insulin (1.6 mg) or a carrier solution, daily for 10 days and then 2 days a week for 6 months, before crossover. The primary outcome was β-cell function measured as first-phase insulin response (FPIR) to intravenous glucose at 0, 6, and 12 months and then yearly; the secondary outcome was immunity to islet antigens, measured monthly for 12 months.

RESULTS — No local or systemic adverse effects were observed. Diabetes developed in 12 participants with negligible β-cell function at entry after a median of 1.1 year. Of the remaining 26, the majority had antibodies to two or three islet antigens and FPIR greater than the first percentile at entry, as well as β-cell function that generally remained stable over a median follow-up of 3.0 years. Intranasal insulin was associated with an increase in antibody and a decrease in T-cell responses to insulin.

CONCLUSIONS — Results from this pilot study suggest that intranasal insulin does not accelerate loss of β-cell function in individuals at risk for type 1 diabetes and induces immune changes consistent with mucosal tolerance to insulin. These findings justify a formal trial to determine if intranasal insulin is immunotherapeutic and retards progression to clinical diabetes.

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Type 1 diabetes is an autoimmune disease in which T-cells mediate destruction of insulin-secreting β-cells in the pancreatic islets. Asymptomatic individuals with preclinical type 1 diabetes can be identified by the presence of circulating antibodies (Abs) to insulin, GAD 65-kDa isoform, and tyrosine phosphatase-like insulinoma antigen 2 (IA2) (1–3). Insulin is the only self-antigen specific for β-cells, and several lines of evidence indicate that it may play a major role in driving autoimmune β-cell destruction (4–7). In experimental rodent models, administration of self-antigens to mucosa-associated lymphoid tissues can induce immune tolerance and prevent autoimmune disease (8,9). In the nonobese diabetic (NOD) mouse, a spontaneous model of autoimmune type 1 diabetes, oral (10) or naso-respiratory (11) insulin induces regulatory, diabetes-protective T-cells. After naso-respiratory insulin, there was a decrease in T-cell and an increase in Ab responses to insulin (11), conforming to the shift from T-helper (Th)-1 cellular immunity to Th2 humoral immunity that is associated with protection against diabetes in this rodent model (12). In human volunteers, oral (13) or intranasal (14) administration of the experimental antigen, keyhole limpet hemocyanin (KLH), induced similar shifts in T-cell and Ab responses to KLH. Mucosa-mediated immune responses to potentially therapeutic self-antigens have not, however, been documented in humans.

Enthusiasm to translate therapeutic effects of mucosal tolerance from rodents to humans has been tempered by failure to demonstrate clinical benefit in the following trials of oral antigens: myelin basic protein in multiple sclerosis (15), collagen in rheumatoid arthritis (16,17), and insulin in recently diagnosed (18,19) type 1 diabetes. These trials did not report evidence for an immune effect; therefore, it is not clear that the dose of oral antigen used was in fact immunogenic. A randomized trial of oral insulin completed in islet Ab–positive individuals at risk for type 1 diabetes was reported orally by Dr. Jay Skyler (Chairman, Diabetes Prevention Trial–Type 1 [DPT-1] Study Group) at the American Diabetes Association 63rd Scientific Meeting in 2003 but has not been formally published. Compared with the oral route, naso-respiratory administration has the advantage that anti-

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Abbreviations: Ab, antibody; DPT-1, Diabetes Prevention Trial–Type 1; FPIR, first-phase insulin response; IA2, tyrosine phosphatase-like insulinoma antigen 2; I/P, period 1 = I (insulin) and period 2 = P (placebo); IQR, interquartile range; KLH, keyhole limpet hemocyanin; P/I, period 1 = P (placebo) and period 2 = I (insulin); Th, T-helper.

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

Table 1—*Characteristics of participants at entry and their status at follow-up, ordered by randomization*

Sex	Age (years)	HLA-DR		FPIR (mU/l)	Islet Abs*			Study arm†	Status‡	Follow-up (years)§
					IAbs	GAD65Abs	IA2Abs			
F	10.0	3	4	28	785	43	47	I/P	D	0.33
F	32.3	3	13	40	205	16	0.2	I/P	D	0.75
M	18.0	4	4	36	1	54	0.8	I/P	D	0.75
F	9.5	3	4	18	365	66	61	I/P	D	0.92
M	12.7	3	4	50	21	3.6	85	I/P	D	1.75
M	10.7	3	4	43	205	69	78	I/P	D	2.08
M	8.9	11	6	40	740	36	0.5	I/P	ND	1.92
M	14.5	1	2	252	1	10	0	I/P	ND	1.92
F	18.7	3	4	55	110	60	0	I/P	ND	2.17
F	7.6	4	4	127	205	56	0	I/P	ND	2.17
M	11.8	3	4	91	66	67	0	I/P	ND	2.50
M	19.5	3	3	65	12	12	2.0	I/P	ND	2.50
F	6.2	3	4	21	22	49	0	I/P	ND	3.00
M	6.3	3	3	130	170	24	0	I/P	ND	3.08
M	14.0	4	4	72	175	45	0	I/P	ND	3.17
M	14.7	4	2	161	200	83	0	I/P	ND	3.33
M	9.8	4	6	76	65	66	85	I/P	ND	3.33
F	10.9	3	4	93	89	93	31	I/P	ND	3.50
F	15.4	4	4	121	425	46	28	I/P	ND	3.75
M	15.3	4	4	234	115	46	85	I/P	ND	3.75
F	14.4	3	4	5	1,400	3.3	8.1	P/I	D	0.58
M	10.4	3	4	56	26	63	41	P/I	D	0.75
F	6.3	3	8	12	86	54	7.0	P/I	D	1.25
F	8.5	8	4	35	1,900	55	98	P/I	D	2.08
M	4.6	1	4	20	360	6.2	58	P/I	D	3.50
M	16.1	3	13	42	23	42	87	P/I	D	3.50
M	11.8	4	4	76	212	72	1.1	P/I	ND	1.58
M	6.1	3	4	203	106	61	0	P/I	ND	2.25
M	8.8	4	8	41	400	7.2	0	P/I	ND	2.33
M	8.8	3	4	83	12	9.3	33	P/I	ND	2.33
F	10.5	4	13	197	101	42	0	P/I	ND	2.42
M	19.8	3	3	112	40	55	8.5	P/I	ND	2.75
F	5.8	10	13	96	108	12	46	P/I	ND	3.00
F	21.8	3	4	101	89	64	0	P/I	ND	3.17
M	7.2	3	4	75	89	71	0	P/I	ND	3.17
F	11.2	4	4	101	61	65	0	P/I	ND	3.25
F	7.7	3	4	104	31	51	92	P/I	ND	3.50
M	19.6	3	3	146	25	7.4	80	P/I	ND	3.75

*Islet Ab control reference ranges: insulin ≤35 nU/ml; GAD65 ≤5 units/ml; IA2 ≤3 units/ml. †Randomisation to insulin (I) followed by placebo (P) at crossover (I/P), or to placebo followed by insulin at crossover (P/I). ‡ND, did not develop diabetes; D, developed diabetes (follow-up ceased at diagnosis). §Follow-up was from the start of the 12-month study. HLA-DR, HLA D locus related.

gen is delivered directly in an undegraded form to the mucosa and in some cases has been shown to be more effective (20). Nevertheless, it is debatable whether mucosa-mediated immunoregulation would counteract pathogenic immunity in end-stage autoimmune disease, and the preferred candidates for such immunoregulatory therapy are asymptomatic individuals with preclinical autoimmune disease. Therefore, with the aim of establishing the potential efficacy of mucosal antigen for preventing autoimmune disease, we conducted a randomized, double-blind, crossover study in individuals at risk of type 1 diabetes to determine if intranasal insulin was safe and could induce changes in immunity to insulin consistent with mucosal tolerance. In the absence of absorption-promoting agents, intranasal insulin has no systemic metabolic effects (21–23). However, in regard to safety, it is necessary to ensure that mucosal administration of insulin to individuals at risk for type 1 diabetes does not activate pathogenic immunity and accelerate β-cell destruction.

RESEARCH DESIGN AND METHODS

— A total of 38 individuals at risk for type 1 diabetes (16 females and 22 males, median age 10.8 years) (Table 1) were recruited by invitation from the Melbourne Pre-Diabetes Family Study (24), with informed consent and Human Ethics Committee approval. All were first-degree relatives of someone with type 1 diabetes and had circulating Abs to at least one of the following islet antigens: insulin, GAD65, or IA2. Of the participants, 4 had one Ab, 22 had two Abs, and 12 had three Abs. The risk for type 1 diabetes increases with the number of antigen specificities (1–3). All subjects were included in the intention-to-treat analysis.

Treatment, assignment, and compliance

Humulin (a gift from Eli Lilly, Australia) or carrier solution (placebo) was transferred under sterile conditions into 15-ml brown glass bottles (fitted with plastic pump spray nozzles) by the Clinical Trials Service, Pharmacy Department, Royal Melbourne Hospital. Humulin contains pure recombinant human insulin at 4 mg/ml in a carrier of water with 1.6 mg/ml glycerol and 0.25/ml *m*-cresol preservative. There is no evidence that either glycerol or *m*-cresol is immunogenic or alters immune responses. Randomization from computer-generated numbers was performed in blocks of four by the Clinical Trials Service. Two 100-μl spray doses per nostril, equivalent to 20 units or 800 μg insulin per nostril (total 1.6 mg), were self-administered daily for 10 consecutive days and then on each weekend day. Participants and, if appropriate, their parent(s) were instructed in the use of the nasal spray before the study, and their technique was checked during the study. The dose schedule was based on the following considerations. In the NOD mouse, an antidiabetic effect of aerosolized insulin was observed after daily administration for up to 10 days and then

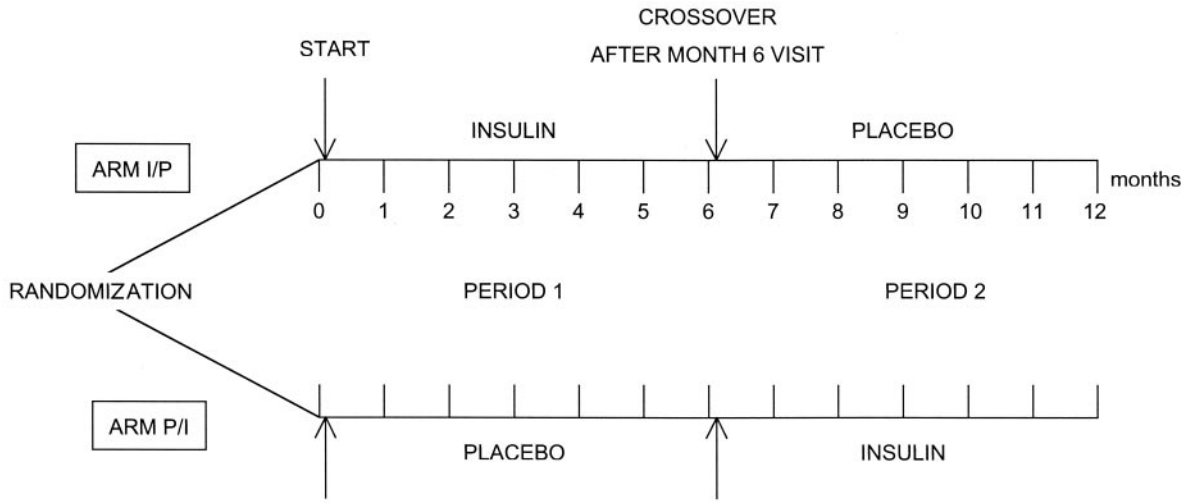

Figure 1—*Study design protocol.*

once or twice weekly (11). In adult humans, Waldo et al. (14) found that 100 mg KLH in a 1-ml nasal spray given on four occasions 2 weeks apart increased Ab and decreased T-cell proliferative responses to KLH. These investigators suggested that Ab sensitization might be avoided with a lower dose. The concentration of insulin in commercially available preparations is fixed at 4 mg/ml, and there is a practical limit to the volume that can be administered intranasally, particularly in children. We also reasoned that a lower spray volume with a proportionately lower dose of antigen would be delivered more selectively to the nasopharyngeal lymphoid tissue, which is relatively rich in T-cells compared with B-cells, with less spillover into the respiratory tract and esophagus. Finally, although each insulin dose was less than the dose of KLH used by Waldo et al. (14), it was administered on more occasions over a 6-month period. It was in an acceptable volume for children. Compliance was based on monthly interview and monitoring of the unused solution volume. Participants or their parents were instructed to report any symptoms and any local effect associated with spray use. They were also instructed to report any malfunction or breakage of the spray bottle pump in order to receive an immediate replacement.

Outcome measures and study protocol

The primary objective was to determine if intranasal insulin was safe and, in particular, did not accelerate β-cell destruction as determined by serial measurement of first-phase insulin response (FPIR) to intravenous glucose. The secondary objective was to determine if intranasal insulin induced changes in immunity to insulin consistent with the induction of mucosal tolerance.

A crossover design (Fig. 1) was used to measure treatment effect as well as "period" and treatment "carry-over" (i.e., residual) effects. Participants were randomized to two arms (two 6-month treatment periods): in the I/P arm, period 1 = I (insulin) and period 2 = P (placebo); in the P/I arm, period 1 = P and period 2 = I. This design allowed treatment effects to be measured serially in the same individuals in a crossover comparison of periods 1 and 2, as well as between different individuals in a parallel across-arm comparison of insulin versus placebo in period 1.

FPIR was measured at randomization and at 6 and 12 months. After the initial visit, participants were seen monthly at visits 1–6 in period 1 and visits 7–12 in period 2, when venous blood was taken for measurement of blood glucose; plasma insulin, GAD65, and IA2 Abs; and T-cell proliferative responses in the absence of antigen and in the presence of tetanus toxoid or denatured human insulin. Treatment was started at the initial visit after blood sampling and crossed over after the month 6 visit (Fig. 1). After completion of the 12-month study, participants were followed regularly and, where possible, retested at least yearly. The diagnosis of diabetes was based on American Diabetes Association criteria (25).

β-Cell function

FPIR was measured as the sum of plasma insulin levels 1 and 3 min after the intravenous injection of 0.5 g glucose/kg body wt (26). Previously, we reported that when performed by a single operator as in this study, the within-subject reproducibility of FPIR expressed as a mean coefficient of variation was 11.4% (range 5.2–19.2) (27,28). The first percentile cutoff for FPIR in healthy prepubescent children and young adults is ~50 mU/l (29,30). The cutoff increases transiently to ~75 mU/l in late puberty (Tanner stages IV–V). Insulin was measured with an IMX kit (Abbott Laboratories, Abbott Park, IL).

Immune parameters

Islet antibodies were measured by liquid-phase precipitation assays as previously described (1,2,24). The assays have had optimal sensitivity, specificity, validity, and consistency in International Workshops and Standardization Programs conducted by the Immunology of Diabetes Society (e.g., the study by Verge et al. [2]). The specificity and sensitivity of our assays in the 2003 Diabetes Antibody Standardization Program were as follows: insulin Abs, 95 and 26%; GAD65 Abs, 97 and 80%; IA2 Abs, 100 and 68%. The thresholds for positivity determined by receiver operator characteristic analysis of 246 control subjects and 135 patients with newly diagnosed type 1 diabetes were as follows: 35 nU/ml insulin Abs, 5

units/ml GAD65 Abs, and 3 units/ml IA2 Abs.

Proliferation of peripheral blood T-cells in the absence or presence of tetanus toxoid (20 Lyons flocculating units/ml) or denatured human insulin (50 μg/ml) was measured as counts per minute (cpm) ^{3}H-thymidine uptake on samples collected between 8:30 and 10:00 A.M., as previously described (31). Because the distribution of responses between replicates is not Gaussian, data were expressed as the medians of sextuplicates. Results were then expressed as a stimulation index, which is the ratio of median values in the presence and absence of antigen. Preservative-free tetanus toxoid (CSL, Melbourne, Australia) was used as a control antigen. Hormonally active native insulin may suppress the function of antigen-presenting cells or T-cells (32). Therefore, insulin was denatured by heating a 1-mg/ml solution of human insulin in 0.01 mol/l HCl containing 100 mmol/l dithiothreitol at 90°C for 30 min, followed by dialysis against sterile PBS. The endotoxin concentration in a 1-mg/ml solution of denatured insulin, measured by Limulus lysate bioassay (BioWhittaker, Walkersville, MD), was <3 ng/ml.

Sample size and statistical analyses

There were no available data on the effect of intranasal insulin on metabolic or immune parameters in humans with which to estimate sample size. As an alternative, we used in-house measurements of insulin Abs in newly diagnosed patients treated with subcutaneous insulin. We estimated a sample size of 22 patients per group for a two-sided test at a power of 80% and a level of significance of 5% to detect a 50% difference in the rate of change of insulin Abs (nU/ml per month). Given the lack of previous data on which to base sample size and the crossover design of the study, the consultant statistician's advice was to perform a blinded interim analysis after approximately half this number of participants had completed the study. This analysis was performed independently by Dr. Jane Mathews, Statistical Centre, Peter MacCallum Cancer Institute, Melbourne.

Immune parameters were analyzed for two time periods: period 1 after randomization and before crossover (monthly visits 1–6) and period 2 after crossover (monthly visits 7–12) (Fig. 1). In each period, parameters measured monthly in each participant were summarized as follows: *1*) the rate of change, or slope, derived from the linear regression of monthly values and *2*) the median of the monthly values. Two comparisons were made by nonparametric Mann-Whitney tests (two-tailed). First, a parallel across-arm comparison of I (insulin) versus P (placebo) in period 1 and, second, a comparison of periods 1 and 2 within each arm, for "treatment" (I vs. P), "period," and "carry-over" effects. The period effect was tested by comparing the difference in a parameter between periods 1 and 2 for participants randomized to one arm with that between periods 2 and 1 for participants randomized to the other arm. A treatment carry-over effect was tested by comparing the difference between I and P in period 1 with the difference between I and P in period 2, according to Jones and Kenward (33). Group results were expressed as the median and interquartile range (IQR) and significance was taken as $P < 0.05$. The raw data are available on request.

RESULTS — The characteristics at entry and status at follow-up of 20 participants randomized to the I/P arm and 18 to the P/I arm are tabulated (Table 1). No adverse local or general effects were reported. Most participants initially noted the odor of the spray (because of the preservative), but this did not affect compliance. By interview and spray bottle inspection, compliance was 100%. Four participants reported breakage of the plastic pump during operation on one to three occasions. In these cases, a replacement bottle pump was provided within 3 days, and that dose was repeated. A blinded interim analysis performed after 24 participants had completed the 12-month study revealed no change in FPIR but a highly significant difference in insulin Abs between the two periods in each arm. Because there had been no change in β-cell function but a difference in insulin Abs, the ethics committee considered that the primary and secondary objectives had been met. A further 14 participants who had been entered by this stage went on to complete the study, making a total of 38 participants finally analyzed.

β-Cell function

A total of 12 participants, 6 randomized to either arm, developed diabetes a median of 1.1 (range 0.33–3.6) years from entry. They were similar in age and number of islet Abs to the 26 who did not develop diabetes and had similar changes in Ab and T-cell responses to insulin (Fisher's exact tests). However, their FPIR at entry was significantly lower than in those who did not develop diabetes (median [IQR] 35.5 [19.0–42.5] vs. 98.5 [73.5–138] mU/l, $P < 0.0001$, Mann-Whitney test). A total of 14 participants had an FPIR at entry less than or equal to the first percentile, and of these 11 developed diabetes. Of the other 24 participants with an FPIR at entry >50 mU/l, only one with a borderline FPIR of 56 mU/l developed diabetes. FPIR at entry was similar between the randomized groups. There was no difference in FPIR at entry and after 12 months (median 98.5 vs. 107 mU/l) in the 26 participants who did not develop diabetes (Fig. 2). An isolated fall in FPIR at 12 months in two participants was associated with documented hemolysis of the plasma samples, a condition known to artifactually lower insulin values in the assay. With yearly follow-up for a median 3.0 years, FPIR was remeasured at least once in 21 of the individuals who remained nondiabetic (Fig. 2). Comparing the last measurement with that at entry revealed no change in 12, an increase >50% in 6, and a decrease >50% in 3.

Immune parameters

Administration of intranasal insulin was followed by an increase in circulating insulin Abs (Fig. 3). The increase in insulin Abs was associated with a decrease in T-cell responses to insulin, shown for the monthly rate of change or slope of these parameters for each participant (Fig. 4). The difference in insulin Abs ($nU \cdot ml^{-1} \cdot month^{-1}$) was highly significant for the parallel arm comparison of insulin versus placebo in period 1 (median [IQR] 7.4 [1.7 to 35] vs. −6.3 [−22 to 6.7], $P = 0.003$) and for the within-arm crossover comparisons between the periods (I/P arm: 7.4 [1.7 to 35] vs. −6.8 [−35 to 2.7], $P = 0.004$; P/I arm: −6.3 [−22 to 6.7] vs. 26 [13 to 125]), $P < 0.0001$). There was no period effect, but the influence of intranasal insulin on insulin Abs was associated with a carry-over effect from the insulin into the placebo period ($P = 0.02$). Similar findings were obtained when, for each participant, the median of monthly values in each 6-month period rather than the monthly rate of

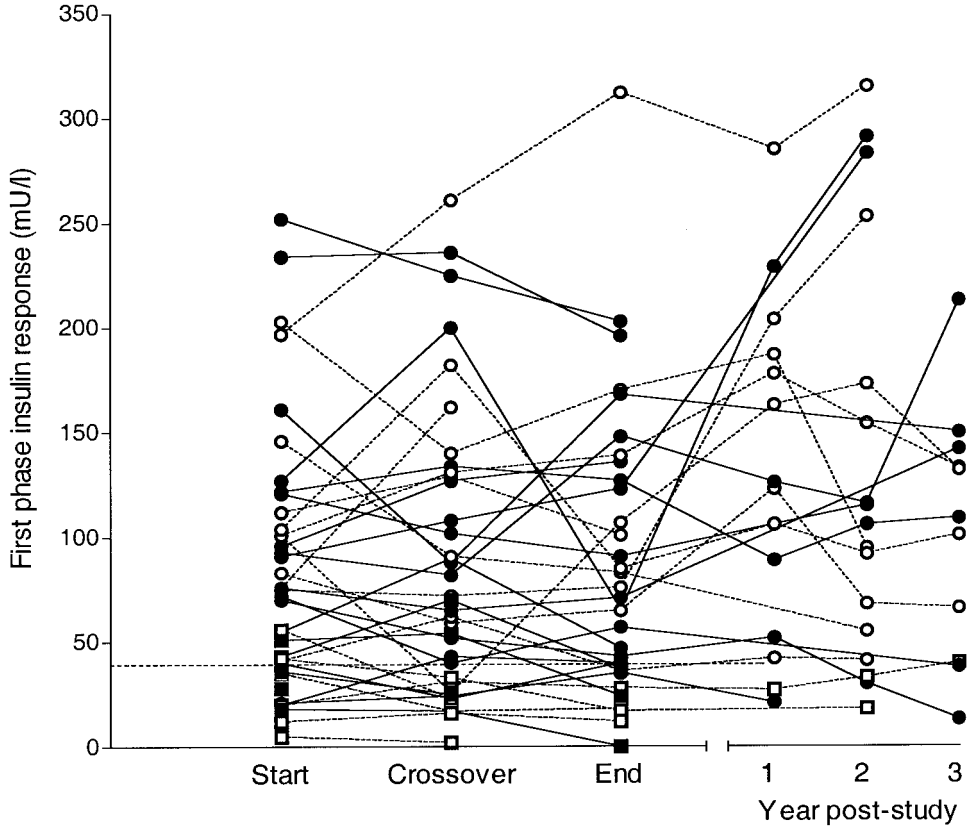

Figure 2—*β-Cell function in study participants. FPIR measured at the start, crossover, and end of the study and during follow-up. Circles and squares denote, respectively, participants who remained diabetes-free or who developed diabetes. Closed symbols joined by solid lines are participants randomized to the I/P arm; open symbols joined by dotted lines are those randomized to the P/I arm.*

change of insulin Ab values was used for comparison (data not shown).

The maximum levels of insulin Abs after intranasal insulin, in relation to pretreatment levels, are summarized in Table 2. During period 1, 11 of 20 participants in the I/P arm compared with 2 of 18 participants in the P/I arm had an increase in insulin Abs >100 nU/ml within 4 months of starting treatment. There were strong positive relationships between levels of insulin Abs in participants before and after intranasal insulin. Relating the median level in the placebo period with the corresponding median level in the insulin period of the P/I arm yielded a correlation coefficient (r) of 0.79 ($P = 0.0001$, two-tailed Spearman test). The slope of this relationship by least squares linear regression was 1.5, indicating that responses were proportionally stronger with increasing pretreatment levels of insulin (auto) Ab. There was also a significant correlation between the levels of median insulin Ab in the insulin and placebo periods of the I/P arm ($r = 0.94$, $P < 0.0001$). The levels of GAD65 and IA2 Abs did not change significantly throughout the study.

Basal T-cell proliferation in the absence of antigen was not different for any of the comparisons. Thus, basal counts per minute (median cpm) expressed as the median (IQR) for each period were 778 (445–1,140) and 698 (485–1,326) for the I/P arm and 822 (502–1,289) and 908 (643–1,319) for the P/I arm. T-cell proliferation indexes to tetanus (cpm tetanus/cpm basal), expressed as monthly rates of change (I/P arm: −3.48 [−8.38 to 0.59] vs. −0.92 [−3.94 to 3.03]; P/I arm: −6.00 [−7.91 to 0.305] vs. −1.18 [−6.35 to 1.62]) or medians (data not shown) for each period, were also similar in both arms.

T-cell proliferation indexes to insulin,

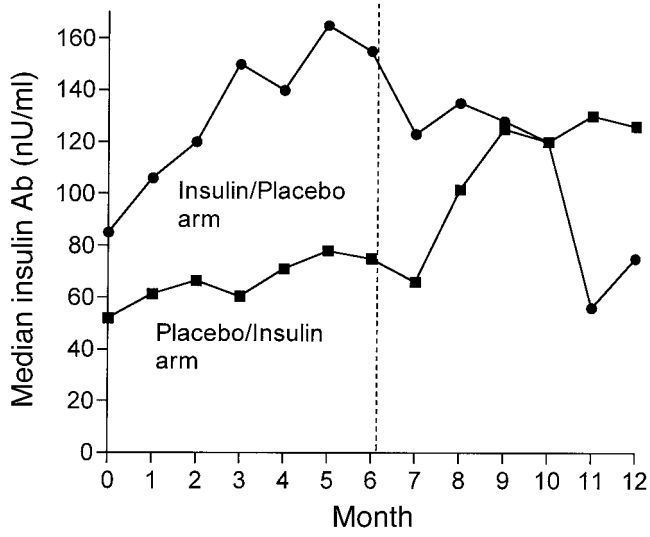

Figure 3—*Monthly insulin Abs (median for participants in either arm) during the study.*

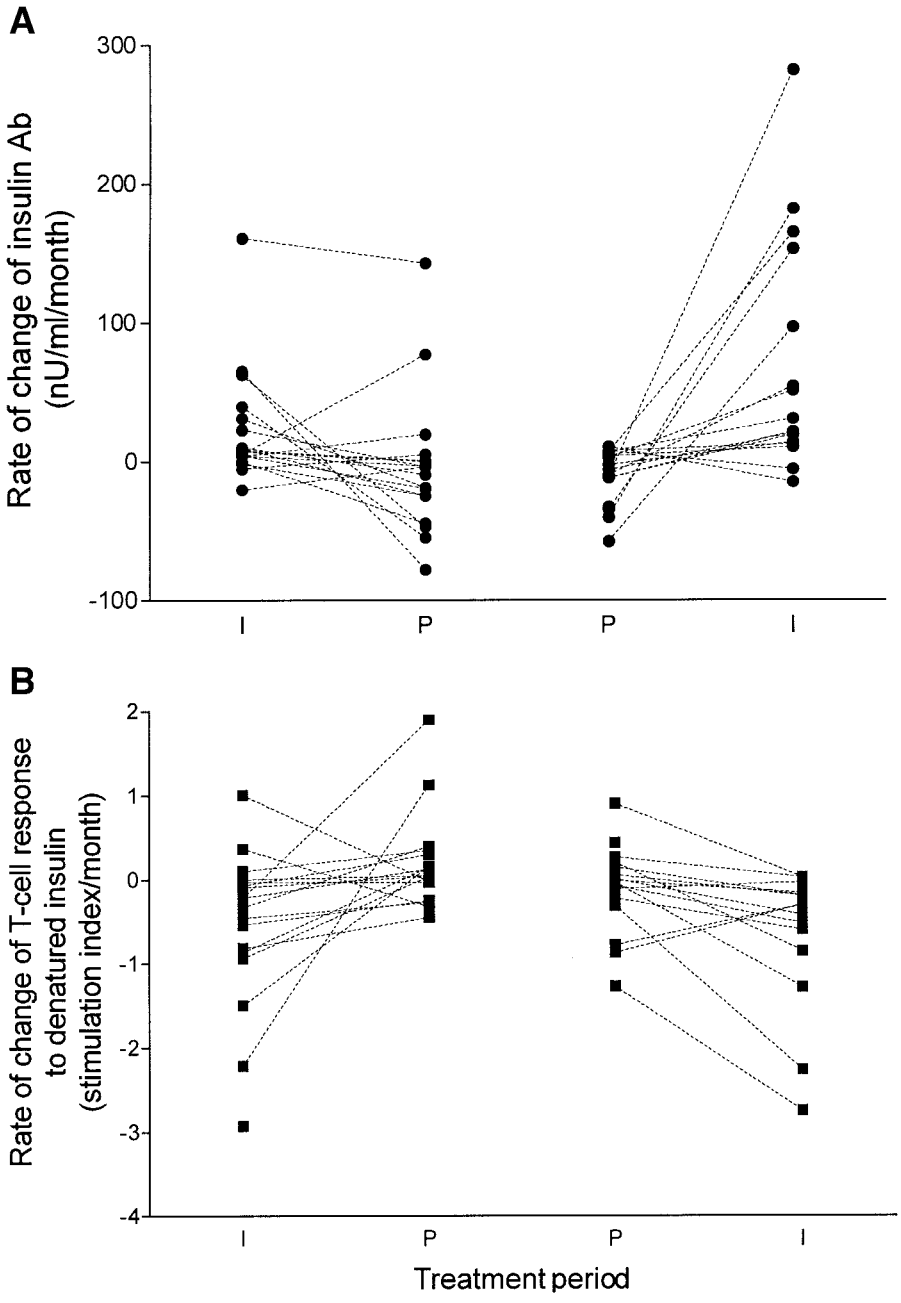

Figure 4—*Ab and T-cell responses to insulin in individual participants. The monthly rate of change of either insulin Ab* (A) *or the T-cell response to denatured insulin* (B) *was determined from serial measurements in each participant in the insulin (I)/placebo (P) and P/I arms. Dotted lines link each subject in the two treatment periods.*

expressed as monthly rate of change (Fig. 4), were not significantly different in the parallel arm comparison of insulin and placebo in the first period (median [IQR]) (−0.28 [−0.91 to −0.02] vs. −0.03 [−0.27 to 0.18], $P = 0.09$). However, in the within-arm crossover comparisons, intranasal insulin was associated with significant decreases in T-cell proliferation to insulin (I/P arm: −0.28 [−0.9 to −0.02] vs. −0.09 [−0.14 to 0.33], $P = 0.003$; P/I arm: −0.03 [−0.27 to 0.18] vs. −0.30 [−0.73 to 0.10], $P = 0.02$). There was no effect of period and no carry-over effect. Using the median of the monthly values in each 6-month period, T-cell proliferation to insulin during intranasal insulin was significantly decreased in the parallel arm comparison of insulin and placebo in period 1 (1.20 [0.79–2.13] vs. 1.85 [1.35–3.50], $P = 0.03$) and in the crossover comparison in the P/I arm (1.85 [1.35–3.50] vs. 1.13 [0.65–1.60], $P = 0.008$). There was also a significant difference in the crossover comparison in the I/P arm (1.20 [0.79–2.13] vs. 0.60 [0.33–0.88], $P = 0.0006$) associated with a carry-over effect of intranasal insulin to suppress the T-cell response into the placebo period ($P = 0.002$). Again, there was no effect of period. No relationships were found between immune responses and HLA D locus–related status, FPIR, or diabetes development or between immune or metabolic responses and insulin dose per kilogram.

Table 2—*Maximum insulin Ab level (nU/ml) level after intranasal insulin*

		After intranasal insulin		
	n	<35	35–100	>100
Pretreatment*				
<35	10	1	5	4
35–100	12	0	2	10
>100	16	0	0	16†

*Value at the initial visit in the I/P arm or the median value in the placebo period of the P/I arm. †Twelve increased by >100 nU/ml.

CONCLUSIONS — Intranasal insulin was well tolerated and had no apparent adverse effects. It was associated with an overall increase in Abs and a decrease in T-cell responses to insulin. The same immune changes were observed after naso-respiratory insulin in NOD mice (11). In the only other studies in humans in which immune markers were documented in response to mucosal antigen, oral (13) or intranasal (14) administration of the experimental antigen, KLH, similarly increased Ab and decreased T-cell responses to KLH. The changes we observed in participants were insulin specific, because no changes were detected in basal or tetanus toxoid–stimulated T-cell responses or in Abs to GAD65 and IA2 after intranasal insulin. The changes in immunity to insulin conform to the pattern of Th1 to Th2 immune deviation that has been associated with mucosal tolerance (8,9) and with protection from diabetes in the NOD mouse (12). Although the directions of the immune responses to

insulin were similar overall within treatment periods, there were large interindividual differences and some exceptions (Fig. 4). The reasons are likely to be several. First, although there was no apparent relationship between dose per kilogram and immune or metabolic effects, the possibility that responses were dose related cannot be excluded and would need to be addressed by dose-ranging studies. Second, although all participants appeared to be compliant, variations in the technique of administering nasal insulin could lead to differences in bioavailability and consequent immune responses. Third, there is likely to be genetic heterogeneity in priming to insulin as an autoantigen, as suggested by our finding of a strong correlation between pretreatment insulin (auto) Ab levels and the insulin Ab response to intranasal insulin. Although we found no association with specific HLA alleles in this study, insulin immunity may be controlled by other genetic loci, for example, *IDDM2*, which appears to regulate the level of proinsulin gene transcription in the thymus and therefore in all likelihood the degree of immune tolerance to insulin (34). A further factor in relation to the T-cell studies is the well-recognized difficulty of detecting responses to insulin and other islet antigens with sensitivity and precision (35). For the in vitro T-cell studies, we used denatured insulin to avoid a possible effect of the hormone to suppress immune function (32) and an assay protocol that allowed us previously to demonstrate proliferation to islet antigen peptide epitopes (31). However, the magnitude of the responses was low, underscoring the need for improved means of assaying antigen-specific T-cells. There were discrepancies between results for the parallel arm and carry-over analyses depending on whether monthly rates of change or medians for each period were used, but the crossover analyses with either parameter were consistent in showing that T-cell proliferation to insulin varied according to treatment period.

Insulin auto-Abs are a risk marker for type 1 diabetes (1–3) and the increase in insulin Abs after intranasal insulin raises concern that this treatment might accelerate diabetes development. However, an increase in insulin Abs was not associated with deterioration of β-cell function in the 24 participants with an FPIR greater than the first percentile at entry, excluding the 1 with a borderline FPIR of 56 mU/l who developed diabetes during the study. On the contrary, the remarkable stability of FPIR on follow-up in these participants, most of whom had Abs to at least two islet antigens and were therefore at significant risk (1–3), contrasts with the decline in FPIR over a similar period that we have observed in comparable individuals from the Melbourne Pre-Diabetes Family Study (24; L.C.H., P.G.C., unpublished data). Furthermore, in the recently reported DPT-1, a trial of oral insulin in islet Ab–positive relatives with FPIR greater than the first percentile, the observed and predicted progression to diabetes over 5 years in the control (and treated) groups was 36% (J. Skyler, personal communication). All participants in the current study received treatment with intranasal insulin for 6 months, and the study was not designed to answer whether intranasal insulin could prevent development of clinical diabetes. However, on the basis of our own and other (36) natural history studies, and the findings of DPT-1, we were surprised that only one of the participants with Abs to two or three islet antigens and FPIR greater than the first percentile progressed to diabetes during follow-up. Individuals who did develop diabetes had similar immune profiles to those who remained asymptomatic but were distinguished by very low FPIR at entry, which is a recognized antecedent of symptomatic disease (37,38).

Trials of oral insulin in individuals with recently diagnosed type 1 diabetes (18,19) showed no apparent clinical benefit but did not report treatment effects on surrogate markers of potential efficacy. Therefore, it is not possible to know if oral insulin was bioavailable and immunogenic at the level of the mucosa. We chose to evaluate the nasal delivery because it has several potential advantages over the oral route. Insulin is delivered in an undegraded form directly to the nasopharyngeal mucosa, whereas oral insulin is subject to degradation in the stomach. Possibly related to this is the fact that the dose of nasal insulin required to induce regulatory T-cells and protect against diabetes development in the NOD mouse (11) is lower than that of oral insulin (10) and therefore may be more readily extrapolated to humans. In addition, immune tolerance has been observed after nasal but not oral delivery of the identical peptide (20). We found that intranasal insulin induced immune effects but had no apparent effect to accelerate diabetes development. This is, to our knowledge, the first demonstration in humans of an effect of mucosally administered autoantigen on potential surrogate disease markers. Although these immune effects are consistent with the induction of mucosal tolerance and are in accord with the literature (11,13,14), they do not constitute proof of immune tolerance to insulin. The ultimate demonstration of this in humans would be the ability of intranasal insulin to prevent or delay diabetes onset. We propose that the present study provides a rationale to determine in a formal trial whether intranasal insulin is immunotherapeutic and retards β-cell destruction and progression to clinical diabetes.

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