Evidence That Nasal Insulin Induces Immune Tolerance to Insulin in Adults With Autoimmune Diabetes

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OBJECTIVE—Insulin in pancreatic β-cells is a target of autoimmunity in type 1 diabetes (T1D). In the NOD mouse model of T1D, oral or nasal administration of insulin induces immune tolerance to insulin and protects against autoimmune diabetes. Evidence for tolerance to mucosally administered insulin or other autoantigens is poorly documented in humans. Adults with recent-onset T1D in whom the disease process is subacute afford an opportunity to determine whether mucosal insulin induces tolerance to insulin subsequently injected for treatment.

RESEARCH DESIGN AND METHODS—We randomized 52 adults with recent-onset, noninsulin-requiring T1D to nasal insulin or placebo for 12 months. Fasting blood glucose and serum C-peptide, glucagon-stimulated serum C-peptide, and serum antibodies to islet antigens were monitored three times monthly for 24 months. An enhanced ELISpot assay was used to measure the T-cell response to human proinsulin.

RESULTS—β-Cell function declined by 35% overall, and 23 of 52 participants (44%) progressed to insulin treatment. Metabolic parameters remained similar between nasal insulin and placebo groups, but the insulin antibody response to injected insulin was significantly blunted in a sustained manner in those who had received nasal insulin. In a small cohort, the interferon-γ response of blood T-cells to proinsulin was suppressed after nasal insulin.

CONCLUSIONS—Although nasal insulin did not retard loss of residual β-cell function in adults with established T1D, evidence that it induced immune tolerance to insulin provides a rationale for its application to prevent diabetes in at-risk individuals.

Type 1 diabetes (T1D) is an autoimmune disease that destroys insulin-producing β-cells in the islets of the pancreas. Studies in the NOD mouse model of spontaneous T1D provide compelling evidence that insulin is a prime autoantigen that drives T-cell-mediated destruction of β-cells (1–3). Insulin is also a major target of the autoimmune response against β-cells in children with T1D (4,5). Ideally, autoimmune diseases would be prevented by restoring immune tolerance to the autoantigens that are postulated to drive pathogenic immune responses. In rodent models of autoimmune disease, exposure of the mucosal immune system to soluble autoantigens has been shown to induce disease-protective immune tolerance associated with regulatory T-cells (6), for example, in the NOD mouse after oral (7,8) or aerosol (9) insulin. The potential of mucosal insulin as an immunotherapeutic agent to prevent T1D in humans would be supported by evidence that it induces immune tolerance to insulin.

Several studies have examined the effects of mucosal insulin in T1D. Two trials of oral insulin after the clinical onset of diabetes failed to demonstrate protection against loss of residual β-cell function (10,11). These used a very small dose of insulin (7.5 mg daily for 1 year) relative to that which protected NOD mice and did not document immune responses to oral insulin to demonstrate bioavailability. Moreover, it can be argued that even if protective immunity had been induced by oral insulin it might be ineffective in clinical, end-stage disease. The Diabetes Prevention Trial-Type 1 (DPT-1) studied asymptomatic at-risk, islet autoantibody-positive, first-degree T1D relatives (12), using the same low dose of oral insulin. Although not a prespecified aim, oral insulin was found to significantly increase disease-free survival in participants who had circulating autoantibodies to insulin at entry. Oral insulin is rapidly degraded in the stomach, and its bioavailability in the upper small intestine is unpredictable (13). On the other hand, insulin administered nasally is intact on immediate contact with the nasopharyngeal mucosa. In asymptomatic children and young adults with islet autoantibodies at moderate risk for T1D, nasal insulin induced an increase in antibody and a decrease in T-cell proliferative responses to insulin ex vivo (14), consistent with an immune-tolerizing effect, as observed after aerosol insulin in NOD mice (9). Subsequently, a randomized trial of nasal insulin administered daily to islet autoantibody-positive children less than 3 years of age at very high risk for T1D found no effect on progression to diabetes (15), but evidence for an effect of nasal insulin on immune function was not reported.

A clear demonstration in humans of immune tolerance induced by nasal insulin would provide a rationale for further trials in at-risk individuals selected on the basis of immune status, disease stage, and risk. Compared with children with classic T1D, adults with T1D have greater residual β-cell function at diagnosis and in most cases do not initially require insulin for treatment (16). This affords an opportunity to evaluate whether nasal insulin has a tolerizing effect on immune responses to insulin subsequently injected for treatment, analogous to “antigen rechallenge” in animal models. We therefore conducted a randomized trial to determine the effect of nasal insulin on immune and metabolic parameters in adults with recent-onset T1D.
**RESULTS**

Characteristics of participants in the nasal insulin and placebo arms did not differ at baseline (Table 1). Blood glucose control (median HbA1c, 6.6%) was satisfactory overall, but participants were overweight (median BMI, 27.0 kg/m²). All had circulating autoantibodies to GADA, 16 of 52 participants (31%) had autoantibodies to insulinoma antigen-2 (IA2A), and five of 52 participants (10%) had autoantibodies to insulin (IAA). HLA class II DR3 or 4 risk alleles for T1D were present in 47 of 52 participants (90%).

**Metabolic parameters.** Glucagon-stimulated serum C-peptide, a measure of β-cell function, decreased overall by 35% over 24 months, from a baseline median of 0.43 nmol/L (interquartile range 0.24–0.62) to a median of 0.28 nmol/L [0.06–0.40] (P = 0.012). Likewise, fasting serum C-peptide decreased by 53% from a baseline median of 0.75 nmol/L [0.48–0.98] to 0.35 nmol/L [0.23–0.95] (P = 0.043). The decline in β-cell function did not differ between the nasal insulin and placebo groups (Fig. 1A and B). At 24 months, median stimulated serum C-peptide in the nasal insulin group was 0.27 [0.06–0.60] nM compared with 0.29 [0.06–0.48] nM in the placebo group (P = 0.86). The β-cell function was also assessed from the slopes of stimulated serum C-peptide during the study (nasal insulin −0.011 vs. nasal placebo −0.018; P = 0.99), confirming there was no difference between the nasal insulin and placebo groups.

Blood glucose control did not change over the course of the study; median HbA1c, at baseline was 6.6% [5.8–7.6] compared with 6.9% [6.3–7.6] at 24 months (P = 0.64, Kruskal-Wallis test). Fasting plasma glucose and HbA1c did not differ between the nasal insulin and nasal placebo groups (Fig. 1C and D). Glycemia control was attributable to close monitoring and prompt escalation of oral hypoglycemic drugs or rapid transition to treatment with
subcutaneous insulin to meet control targets. Progression to insulin treatment over 24 months occurred in 23 of 52 participants (44%), being similar in the nasal insulin (12/26) and placebo (11/26) groups (log-rank test \( P = 0.88 \); Fig. 2A).

**Immune parameters.** Concentrations of GADA and IA2A were similar at baseline in the nasal insulin and placebo groups and remained unchanged throughout the study (Fig. 1E and F). At baseline, only three participants in the nasal insulin group and two participants in the control group had detectable IAA (Table 2). After treatment with s.c. insulin, the induced IA response (Table 2) was significantly blunted in participants who had received nasal insulin. This is illustrated in several ways (Figs. 2 and 3).

First, IA concentration is plotted for each participant in the nasal insulin (12/26) and placebo (11/26) groups (log-rank test \( P = 0.88 \); Fig. 2A). The IA concentration in participants who had received nasal insulin remained significantly lower in those from the nasal insulin group than in the placebo group (4.1 vs. 12.2 units/mL, \( P = 0.001 \)). By 24 months, IA concentration in participants receiving s.c. insulin was significantly lower in the 12 participants who had received nasal insulin (4.7 [2.3–11] units/mL) than in the 11 participants who had received nasal placebo (17 [6.0–32] units/mL) (\( P = 0.019 \)) (Fig. 2E). The IA concentration in participants who had received nasal insulin remained suppressed to at least 12 months after commencing daily s. c. insulin treatment (Fig. 3). The relationship between s.c. insulin dose (shown in Table 2 as total daily dose) and the IA response was examined. The median dose of s.c. insulin in the nasal insulin group (26 units; interquartile range 20–34) was not significantly different from that in the placebo group (34 units; interquartile range 22–50) (\( P = 0.34 \)). In the nasal insulin group only, insulin dose and IA concentration were significantly correlated at 3 months (\( r = 0.81, P = 0.001 \) and 6 months (\( r = 0.64, P = 0.024 \)) after starting s.c. insulin, but in each case significance depended on the single highest paired values.

IFN-\( \gamma \) ELISpot responses to proinsulin and a control antigen, tetanus toxoid, were measured on frozen-thawed PBMCs available from five participants in each group at baseline and 3 months (Fig. 4). No participant required
s.c. insulin by 3 months. Responses to tetanus toxoid were similar between the groups at baseline and 3 months. However, responses to proinsulin decreased significantly by 3 months in participants who received nasal insulin ($P = 0.03$; paired, one-tailed $t$ test) but not placebo ($P = 0.31$).

**DISCUSSION**

Mucosa-mediated immune tolerance in humans was first demonstrated to the experimental antigen, KLH, given orally (25) or nasally (26) to healthy volunteers. After oral KLH, T-cell but not B-cell (antibody) responses to rechallenge with s.c. KLH were suppressed. After nasal KLH,
both T- and B-cell responses to s.c. KLH were suppressed, suggesting that tolerance induction via the nasal route may be more effective. Despite these findings, and a wealth of evidence for tolerogenic protective effects of oral or nasal autoantigen in mouse models of autoimmune disease, reviewed by Harrison and Hafler (6), the therapeutic promise of mucosa-mediated tolerance for human autoimmune disease has failed to meet expectations. Part of

FIG. 2. A: Progression to treatment with s.c. insulin, plotted as Kaplan-Meier survival curves (nasal insulin participants, solid line; nasal placebo participants, dashed line). B: IA concentrations in nasal placebo participants at 3-month study intervals. C: IA concentrations in nasal insulin participants at 3-month study intervals. D: Median IA concentrations for all participants at 3-month study intervals (includes values before and after commencement of s.c. insulin). ○, Nasal placebo and ●, nasal insulin participants. Upper quartile ranges (divided by 5 to allow fit) are shown as vertical lines. E: Median IA concentrations in nasal placebo (○) and nasal insulin (●) participants by time after commencement of s.c. insulin. Upper quartile ranges are shown as vertical lines.
the explanation may be that earlier human studies were conducted in advanced disease. In addition, evidence that administered autoantigen was bioavailable and elicited immune responses consistent with tolerance has been lacking. Our finding that the antibody response to s.c. insulin was suppressed by prior treatment with nasal insulin is the first evidence for immune tolerance induction to an autoantigen demonstrated by rechallenge in humans.

**TABLE 2**

<table>
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<th>Participant</th>
<th>Baseline</th>
<th>3.0</th>
<th>6.0</th>
<th>9.0</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
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</tr>
</thead>
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<td>Nasal insulin</td>
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<td>0.0</td>
<td>0.5</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Nasal placebo</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*s.c. insulin dose (units). NA, not assayed.
Tolerance was robust because the suppressive effect was sustained in the majority of individuals despite ongoing daily insulin injections. Differences between the nasal insulin and placebo groups could not be attributed to the doses of s.c. insulin required. Tolerance to exogenous insulin does not necessarily equate with tolerance to endogenous insulin, i.e., with suppression of autoimmune responses to insulin. This is implied, however, by evidence for tolerance at the T-cell level, namely, suppression of IFN-γ responses to proinsulin after nasal insulin in a small cohort, which must be qualified by the lack of T-cell data across the study. Proinsulin rather than insulin was used for the ELISpot assay because the serum-free medium contained a high concentration of insulin, but T-cells recognize epitopes in proinsulin that are not present in insulin (1,27).

There are several possible reasons why immune tolerance to insulin induced by nasal insulin might not have translated into suppression of immunity to other islet autoantigens or protection against ongoing loss of β-cell function in adults with T1D. First, on the basis of the prevalence of autoantibodies, insulin does not seem to be a major autoantigen in this population (16), and protection may require induction of tolerance to other islet autoantigens. On the other hand, although the mechanism of nasal insulin-induced tolerance in humans remains to be defined, studies in mice (6) show that regulatory T-cells induced by mucosal administration of a single autoantigen can have a bystander effect to suppress T-cell responses to other autoantigens presented in the same microenvironment, e.g., pancreas draining lymph nodes. T-cell responses to GAD were suppressed by aerosol insulin in the NOD mouse (9), but evidence is lacking that bystander suppression induced by one antigen modifies ongoing antibody responses to other autoantigens. Whether T-cell tolerance involves induction of insulin-specific regulatory T-cells or the deletion or anergy of insulin-specific pathogenic T-cells, the outcome may be impaired T-cell “help” for IA production by B-cells. Techniques for reliably identifying and characterizing autoantigen-specific T-cells...
in human blood are emerging, and our findings are an impetus for their application. Second, β-cell function declined by more than 30% over 24 months with more than 40% of participants becoming insulin-dependent, indicating progressive loss of β-cell function to end-stage disease. Trials of oral insulin in adults with recent-onset T1D (10,11), which did not document immune outcomes, also found no effect on residual β-cell function. If the balance between pathogenic and protective immunity determines clinical outcome, then autoantigen-specific vaccination should be most effective before or soon after the onset of subclinical disease. Indeed, studies in animal models show no evidence that this approach is protective by the time clinical disease ensues. Third, it is possible that immune tolerance to insulin, even if induced early in the disease process, may be protective only in individuals with preexisting autoimmunity to insulin, as seen in the DPT-1 oral insulin trial (12). Finally, although administration of nasal insulin was associated with suppression of the antibody response to injected insulin, destruction of β-cells is primarily T-cell mediated. Further studies are required not only to determine whether nasal insulin induces insulin-specific regulatory T-cells but also to confirm that, like nasal KLH (26), nasal insulin induces changes in T-cell function to rechallenge indicative of T-cell tolerance.

An important question is whether insulin-induced tolerance would be protective in islet autoantibody-positive children at risk for T1D in whom, in contrast with adults with T1D, insulin seems to be a major autoantigen (4,5). In the DPT-1 randomized controlled trial of oral insulin (12), participating relatives with T1D at entry had an interquartile age of 7–14 years and normal β-cell function. Notably, treatment with oral insulin was associated with slower progression to diabetes in the slightly younger IAA-positive cohort. In the T1D Prediction and Prevention Project randomized trial in Finland (15), nasal insulin had no effect on progression to diabetes in islet autoantibody-positive children less than 3 years of age. Such children are a very high-risk group, and many had low first-phase insulin response to i.v. glucose. Given end-stage β-cell function, they would be less likely to exhibit a clinical response. Again, immune tolerance to insulin was not documented in either trial of oral or nasal insulin in at-risk individuals. A proposed trial (Pre-POINT) (28) aims to address questions of optimal timing, disease stage, dose, and route of administration by intervening with oral or nasal insulin in children genetically predisposed to T1D, before the appearance of islet autoantibodies. The evidence for nasal insulin-induced immune tolerance demonstrated provides a mechanistic rationale for such studies that aim to restore immune tolerance before the onset of islet pathology.

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S.F. designed the research, performed the studies, analyzed the data, and wrote the article. C.P. and S.A.G. performed the studies. E.M. performed the studies and analyzed data. P.G.C. designed the research and performed the studies. L.C.H. designed the research, performed the studies, analyzed data, and wrote the article.

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REFERENCES