

ORIGINAL ARTICLE

GAD Treatment and Insulin Secretion in Recent-Onset Type 1 Diabetes

Johnny Ludvigsson, M.D., Ph.D., Maria Faresjö, Ph.D., Maria Hjorth, M.Sc.,
Stina Axelsson, M.Sc., Mikael Chéramy, M.Sc., Mikael Pihl, M.Sc.,
Outi Vaarala, M.D., Ph.D., Gun Forsander, M.D., Ph.D.,
Sten Ivarsson, M.D., Ph.D., Calle Johansson, M.D., Agne Lindh, M.D.,
Nils-Östen Nilsson, M.D., Jan Åman, M.D., Ph.D., Eva Örtqvist, M.D., Ph.D.,
Peter Zerhouni, M.Sc., and Rosaura Casas, Ph.D.

ABSTRACT

BACKGROUND

The 65-kD isoform of glutamic acid decarboxylase (GAD) is a major autoantigen in patients with type 1 diabetes mellitus. This trial assessed the ability of alum-formulated GAD (GAD-alum) to reverse recent-onset type 1 diabetes in patients 10 to 18 years of age.

METHODS

We randomly assigned 70 patients with type 1 diabetes who had fasting C-peptide levels above 0.1 nmol per liter (0.3 ng per milliliter) and GAD autoantibodies, recruited within 18 months after receiving the diagnosis of diabetes, to receive subcutaneous injections of 20 μ g of GAD-alum (35 patients) or placebo (alum alone, 35 patients) on study days 1 and 30. At day 1 and months 3, 9, 15, 21, and 30, patients underwent a mixed-meal tolerance test to stimulate residual insulin secretion (measured as the C-peptide level). The effect of GAD-alum on the immune system was also studied.

RESULTS

Insulin secretion gradually decreased in both study groups. The study treatment had no significant effect on change in fasting C-peptide level after 15 months (the primary end point). Fasting C-peptide levels declined from baseline levels significantly less over 30 months in the GAD-alum group than in the placebo group (-0.21 vs. -0.27 nmol per liter [-0.62 vs. -0.81 ng per milliliter], $P=0.045$), as did stimulated secretion measured as the area under the curve (-0.72 vs. -1.02 nmol per liter per 2 hours [-2.20 vs. -3.08 ng per milliliter per 2 hours], $P=0.04$). No protective effect was seen in patients treated 6 months or more after receiving the diagnosis. Adverse events appeared to be mild and similar in frequency between the two groups. The GAD-alum treatment induced a GAD-specific immune response.

CONCLUSIONS

GAD-alum may contribute to the preservation of residual insulin secretion in patients with recent-onset type 1 diabetes, although it did not change the insulin requirement. (ClinicalTrials.gov number, NCT00435981.)

From Linköping University, Linköping (J.L., M.F., M.H., S.A., M.C., M.P., O.V., R.C.), the Queen Silvia Children's Hospital, Gothenburg (G.F.), Malmö University Hospital, Malmö (S.I.), Regional Hospital Ryhov, Jönköping (C.J.), Southern Älvsborg Hospital, Borås (A.L.), Halmstad County Hospital, Halmstad (N.-Ö.N.), Örebro University Hospital, Örebro (J.Å.), Astrid Lindgrens Children's Hospital, Karolinska University Hospital, Stockholm (E.Ö.), and Diamed Medical, Stockholm (P.Z.) — all in Sweden; and the National Public Health Institute, Helsinki (O.V.). Address reprint requests to Dr. Ludvigsson at the Division of Pediatrics, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, SE-58185 Linköping, Sweden, or at johnny.ludvigsson@lio.se.

This article (10.1056/NEJMoa0804328) was published at www.nejm.org on October 8, 2008.

N Engl J Med 2008;359.

Copyright © 2008 Massachusetts Medical Society.

TYPE 1 DIABETES MELLITUS IS AN AUTO-immune disease¹ that causes substantial morbidity and mortality.^{2,3} Even modest residual insulin secretion, with stimulated C-peptide levels above 0.2 nmol per liter (0.6 ng per milliliter), has been reported to provide clinically meaningful benefits in terms of reducing long-term complications.⁴ However, most attempts to preserve residual beta-cell function have achieved minimal benefits or have been associated with adverse effects.⁵⁻¹⁴ Treatment with anti-CD3 monoclonal antibodies appears promising, although many patients in whom this approach has been used have had therapy-related adverse events.^{15,16}

As an alternative to immunosuppression, autoantigens may be used to induce immunologic tolerance.¹⁷ Insulin and the 65-kD isoform of glutamic acid decarboxylase (GAD) are major autoantigens in patients with type 1 diabetes^{18,19} and have been tested in immunomodulation experiments.²⁰ Data from studies of nonobese diabetic (NOD) mice have indicated that GAD prevents type 1 diabetes.^{21,22} A dose-finding study in patients with latent autoimmune diabetes in adults (LADA) indicated that a primary injection and a booster injection of 20 μ g each of recombinant human GAD in a standard vaccine formulation with alum (GAD-alum) might preserve residual insulin secretion without serious adverse effects.²³

In the current study, we administered GAD-alum to young patients with type 1 diabetes of recent onset to see whether treatment with the autoantigen would reduce or halt the loss of residual insulin secretion. Here, we report results from the 15-month study period followed by 15 months of further observation.

METHODS

This study was approved by the research ethics committee at Linköping University, Linköping, Sweden, and by the regulatory authorities in Sweden. Patients provided written informed assent, and their parents provided written informed consent.

STUDY DESIGN

The first author designed the study and recruited study sites and patients, oversaw the data analyses, wrote the manuscript, and vouches for the completeness and accuracy of the data and the analy-

ses. The sponsor, Diamyd Medical, provided the study drug, was involved in the design of the study, held the data, performed the statistical analyses, and oversaw the conduct of the trial.

At eight pediatric clinics in Sweden, 118 patients who were between 10 and 18 years of age who had presented with type 1 diabetes within the previous 18 months were screened for the presence of GAD autoantibodies and fasting C-peptide levels above 0.1 nmol per liter (0.3 ng per milliliter). A total of 70 patients were eligible and were randomly assigned to a double-blind treatment with either 20 μ g of GAD-alum (Diamyd, Diamyd Medical) (35 patients) or placebo (the same formulation without the recombinant human GAD — i.e., alum alone) (35 patients). The pharmacy at the Malmö University Hospital in Malmö, Sweden, prepackaged identical-appearing ampules containing either GAD-alum or placebo in identical per-patient boxes. The boxes were labeled with treatment numbers, from 1 to 70, according to a computer-generated randomization list produced by Clinical Data Care in Lund, Sweden. A block size of 10 was used, but block size was not revealed until unblinding. There was no stratification. When a study site identified an eligible patient and an appointment for the first injection had been made, the study site ordered a treatment box. The central pharmacy sent treatment boxes in consecutive order as ordered by the study sites. Thus, the treatment was assigned in random order over the total study population and was blinded to all but the pharmacy and the provider of the randomization list during the main study period.

All patients were treated with multiple daily injections of insulin with a target glycated hemoglobin level of less than 6.5%. At baseline, two patients in the placebo group but no patients in the GAD-alum group were using an insulin pump. The trial aimed to evaluate the safety as well as the efficacy of GAD-alum treatment as compared with placebo in preserving residual insulin secretion. The prespecified primary efficacy end point was the change between baseline and month 15 in the fasting C-peptide level, and the prespecified secondary efficacy end points were changes between baseline and various prespecified time points, up to month 30, in fasting and stimulated C-peptide levels and glycated hemoglobin values. Other end points prespecified for formal analysis were insulin requirement, fasting plasma glucose

level, fasting C-peptide:plasma glucose ratio, and GAD autoantibody titer.

Each patient received a subcutaneous primary injection of either GAD-alum or placebo on day 1, followed by a boost 1 month later. Patients remained in the clinic for observation for 3 hours after injection.

On day 1 and at months 3, 9, 15, 21, and 30, a 2-hour mixed-meal tolerance test was performed in accordance with a European study on estimation of beta-cell function.²⁴ This test consists of the ingestion, within a 5-minute period, of 6 ml of a liquid meal (Sustacal) per kilogram of body weight (maximum, 360 ml). The meal test was performed in the morning (between 7 and 10 a.m.) after an overnight fast, in which no food or drink (with the exception of water) and no smoking occurred after 10 p.m. the preceding day. The patients took no short-acting insulin for at least 6 hours before the test; however, patients receiving a continuous subcutaneous infusion of insulin continued it at the normal basal rate but received no additional boluses for at least 6 hours before the test.

After completion of the main study period (15 months), the treatment code was revealed to the statistician, the programmer of the SAS statistical package (version 8.2 for Windows), and the sponsor, and the data were analyzed. The analyses included measurements of C-peptide levels, glycated hemoglobin levels, insulin requirement (units per kilogram of body weight and 24-hour requirement), plasma glucose levels, and GAD autoantibody titers.

With unblinding of the data only to those listed in the preceding paragraph, the study continued for an extension period of 15 months. Data through the end of the study, at the month 30 follow-up, are included in the current report. A final analysis after month 30 was prespecified in the protocol.

The T-cell studies reported here were performed under a separate investigator-initiated protocol. Informed consent was obtained for this protocol as described above for the main protocol.

LABORATORY TESTS

Laboratory analyses were performed at Linköping University in Linköping, Sweden. C-peptide levels were measured in serum samples with the use of a time-resolved fluoroimmunoassay (AutoDELFIA C-peptide kit, Wallac) as the study progressed. Results for each assay were validated with the

inclusion of a C-peptide control module containing a high-level control, a medium-level control, and a low-level control (Immulate, Diagnostic Products Corp.). A software program (1224 MultiCalc, Wallac) was used for automatic measurements and calculation of results; measurements were expressed in nanomoles per liter.

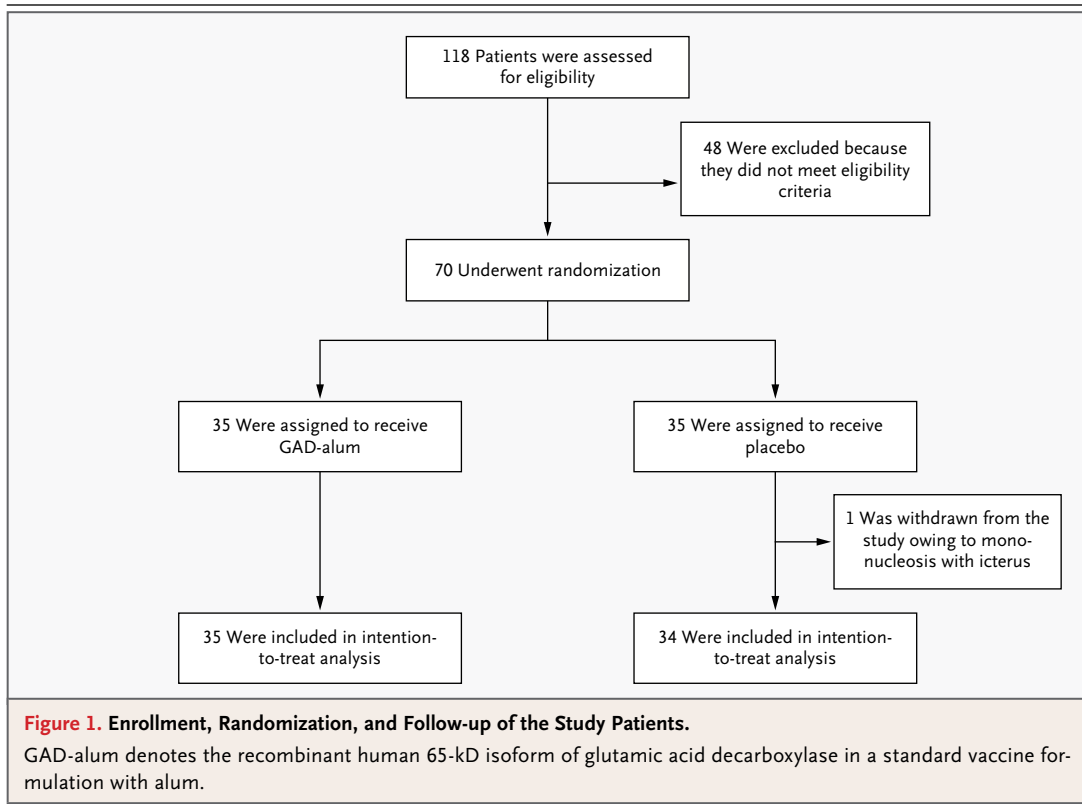
The type of HLA-DQ-A1* and -B1* alleles was determined by means of polymerase-chain-reaction (PCR) amplification of exon 2 sequences and hybridization with allele-specific probes detected with the use of a time-resolved fluorescence immunoassay, as previously described.²⁵ As detailed in a population-based Swedish case-control study,²⁶ the patients were then categorized as high risk, moderate risk, or low risk, on the basis of HLA typing.

Glycated hemoglobin was analyzed by an immunologic method and calibrated against the Swedish national standard Mono-S. Levels were continuously checked against the External Quality Assurance in Laboratory Medicine in Sweden reference standard.

Serum GAD autoantibody titers were estimated in duplicate by means of a radiobinding assay using 35S-labeled recombinant human GAD produced through *in vitro* transcription and translation (with a pEx9 vector). Sepharose protein A was used to separate free GAD from antibody-bound, labeled GAD. Interassay variation was 10% for the negative control and 8% for the positive control. A diabetes autoantibody standardization program (DASP) in which we participated has shown that this assay has a sensitivity of 76 to 80% and specificity of 96 to 99%.

Peripheral-blood mononuclear cells (PBMCs) were isolated from sodium-heparinized venous-blood samples through Ficoll-Paque density-gradient centrifugation (Pharmacia Biotech). One million PBMCs diluted in AIM V medium supplemented with 20 μ M β -mercaptoethanol were cultured with GAD antigen (Diamyd Diagnostics) or phytohemagglutinin for purposes of mitogenic stimulation (Sigma) at a final concentration of 5 μ g per milliliter at 37°C, in 5% carbon dioxide. Additional wells with medium alone were used as negative controls. After 72 hours, the PBMCs were separated from the supernatant, and both cells and cell supernatant were frozen at -70°C until used for real-time reverse-transcription PCR and multiplex fluorochrome analysis.

The cytokines interleukins 5, 6, 10, 12 (p70),



13, and 17, tumor necrosis factor α (TNF- α), and interferon- γ were measured in the cell supernatant from each sample with the use of a Bio-Plex Human Cytokine 8-Plex Panel and a Bio-Plex Cytokine Reagent Kit according to the manufacturer's instructions (Bio-Rad Laboratories). A Luminex 100 instrument (involving xMAP technology, Luminex) was used for quantification of each specific reaction. The median fluorescence intensities were analyzed with the use of STarStation software, version 2.0 (AppliedCytometry). A five-parameter curve fit was applied to each standard. The antigen-induced cytokine levels were calculated by subtracting the spontaneous cytokine secretion for each sample from the response induced by the antigen.

Total RNA was isolated from PBMCs according to the RNeasy 96 spin protocol (Qiagen Sciences) and was quantified through optical densitometry at 260 nm. Using equal amounts of total RNA (7 ng per microliter), we synthesized complementary DNA (cDNA) by using the High Capacity cDNA Archive Kit (Applied Biosystems). Reverse transcription of total RNA to cDNA was performed with the use of the GeneAmp PCR System 2700

(Applied Biosystems). The FAM-labeled primer-probes Hs00203958 and Hs00171257 (Applied Biosystems) were used to estimate transcription levels of the transcription factor forkhead box P3 (FOXP3) and transforming growth factor β (TGF- β), respectively. VIC-labeled primer-probes were used to estimate transcription levels of the endogenous control (18s) ribosomal RNA (Applied Biosystems). Relative transcription was estimated with the use of the comparative cycle-threshold (Ct) method (protocol P/N 4303859B, Applied Biosystems).

STATISTICAL ANALYSIS

Results from a study of latent autoimmune diabetes in adult patients²³ suggested that including 35 patients in each study group would provide the study with a statistical power of 80 to 90% for assessing differences in C-peptide levels, with a significance level of 5%, assuming a mean (\pm SD) difference in fasting C-peptide levels of 0.12 ± 0.15 nmol per liter (0.36 ± 0.45 ng per milliliter) between the two groups. Data management and statistical analysis of clinical data were performed by the contract research organization Trial Form Support in Lund, Sweden, whose services were pro-

Table 1. Baseline Characteristics of the Patients, According to Study Group.*

Characteristic	GAD-Alum (N = 35)	Placebo (N = 34)
Age — yr	13.8±2.3	12.8±1.9
Time since diabetes diagnosis — mo	9.9±5.3	8.8±5.4
BMI†	19.5±2.4	20.5±3.2
Sex — no. (%)		
Female	23 (66)	18 (53)
Male	12 (34)	16 (47)
HLA classification — no. (%)		
High risk	18 (51)	16 (47)
Moderate risk	9 (26)	7 (21)
Low risk	8 (23)	11 (32)
Tanner genital development stage at screening — no. (%)‡		
1	4 (11)	7 (21)
2–3	8 (23)	10 (29)
4–5	23 (66)	17 (50)
Fasting C-peptide — nmol/liter	0.33±0.19	0.35±0.23
Stimulated C-peptide AUC — nmol/liter/2 hr	1.24±0.57	1.41±0.87
Glycated hemoglobin — %	6.3±1.3	6.2±1.0
Insulin dose — U/kg of body weight/24 hr	0.66±0.30	0.66±0.28
Plasma glucose before MMTT — mmol/liter	9.4±4.0	8.8±3.3
Fasting C-peptide:plasma glucose ratio — ×10 ⁻¹²	40±23	45±29
Median GAD autoantibody titer — U/ml	601	861

* Plus-minus values are means ±SD. AUC denotes area under the curve, GAD the 65-kD isoform of glutamic acid decarboxylase, GAD-alum recombinant human GAD in a standard vaccine formulation with alum, and MMTT mixed-meal tolerance test. To convert values for C-peptide to nanograms per milliliter, divide by 0.33. To convert values for glucose to milligrams per deciliter, divide by 0.05551.

† The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters.

‡ The Tanner stage of genital development can range from 1 to 5, with an increasing score indicating more developed genitalia.

cured by the sponsor and were overseen by the first author. A prespecified analysis of covariance (ANCOVA) model was used, in which the change from baseline was taken as the response variable, the study treatment as the explanatory variable, and the baseline value as a covariate. Age, sex, duration of diabetes at baseline, GAD autoantibody titer, and HLA type were identified in advance as factors to use in additional exploratory analyses and were used to prespecify subgroups. Descriptive statistics regarding change in fasting and stimulated C-peptide levels for these subgroups was foreseen, but no formal analysis was planned in the protocol.

ANCOVA involves the assumption of normally distributed response data and homogeneity of

variances. In our study, given the number of patients and the lack of findings regarding variance heterogeneity, the ANCOVA models used are statistically valid. Missing data were not replaced. For no analysis was there more than one subject with missing data.

In all tests, the null hypothesis was that there was no difference between active treatment and placebo. Two-sided tests were used for all hypotheses, and the P values are presented with 95% confidence intervals. Since there was only one primary analysis, the P values were not adjusted for multiplicity.²⁷ The hypothesis of no difference between the study groups was assessed at months 15 and 30 of follow-up. In the graphs, for completeness, all significant P values are shown. The

data management and statistical analysis of the GAD-autoantibody and T-cell data were performed at the University of Linköping in Linköping, Sweden.

RESULTS

RECRUITMENT AND RANDOMIZATION OF PATIENTS

Seventy of the 118 patients screened were eligible. Twenty-eight patients screened were negative for GAD autoantibodies, 17 did not meet the C-peptide criterion, 2 failed to meet both of these criteria, and 1 was excluded owing to epilepsy. Screening took place over a 2-week period in January and February 2005. The first injection of GAD-alum or placebo took place in February 2005, and the last patient completed the 30-month visit in October 2007.

All but one patient received two doses of either GAD-alum or placebo (Fig. 1). One patient (a girl in the placebo group) was withdrawn from the study after 1 week, owing to confirmed infectious mononucleosis with icterus; she received only one injection. A total of 69 patients, 35 in the GAD-alum group and 34 in the placebo group, were included in the intention-to-treat analysis.

BASELINE CHARACTERISTICS OF PATIENTS

Data for the two study groups were similar at baseline, defined as the day of the first injection, before injection (Table 1). The distribution of HLA genotypes did not differ between the GAD-alum

group and the placebo group (Table 1). In accordance with accepted procedures, the baseline data were not compared statistically between the two groups.²⁸

SAFETY

There were seven serious adverse events in five patients in the GAD-alum group and five serious adverse events in four patients in the placebo group (Table 2). The serious adverse events in the GAD-alum–treated patients were knee trauma, ketoacidosis, lower-limb fracture, and diarrhea, and one patient had ketoacidosis, high glycated hemoglobin, and streptococcal tonsillitis. The serious adverse events in the placebo group were mononucleosis, ankle fracture, cessation of insulin use, and two episodes of hypoglycemia with seizure in one patient. No serious adverse events occurring by month 30 were judged to be related to the study treatment.

The frequency and pattern of adverse events during the main study period were similar between the two study groups. Twenty-nine patients in the GAD-alum group reported 70 adverse events, and 25 patients in the placebo group reported 56 adverse events. The most commonly reported adverse events were upper respiratory tract infection, nasopharyngitis, gastroenteritis, and headache. In two patients, both in the GAD-alum group, the adverse events were judged as possibly related to the study treatment; mild hypoglycemia developed in one patient and moderate hypoglycemia in an-

Table 2. Serious Adverse Events between Baseline and Month 30.*

Event	Onset (Time since First Injection)	Outcome at Month 30	Assessment by Investigator and Safety Committee	Study Group
Mononucleosis	1 wk	Recovered	Not related to study treatment	Placebo†
Ankle fracture	8 mo	Recovered	Not related to study treatment	Placebo
Knee trauma	9 mo	Recovered	Not related to study treatment	GAD-alum
Cessation of insulin administration	10 mo	Recovered	Not related to study treatment	Placebo
Ketoacidosis	16 mo	Recovered	Not related to study treatment	GAD-alum
Hypoglycemia with seizure (two episodes in one patient)	18 mo and 19 mo	Recovered (from both episodes)	Neither episode related to study treatment	Placebo
Ketoacidosis, high glycated hemoglobin level, and streptococcal tonsillitis (all in one patient)	18 mo, 18 mo, and 26 mo, respectively	Recovered (from all three events)	None of the three events related to study treatment	GAD-alum
Lower-limb fracture	23 mo	Recovered	Not related to study treatment	GAD-alum
Diarrhea	30 mo	Recovered	Not related to study treatment	GAD-alum

* GAD-alum denotes the recombinant human 65-kD isoform of glutamic acid decarboxylase in a standard vaccine formulation with alum.

† The patient in the placebo group who had mononucleosis did not receive a second injection and was withdrawn from the study.

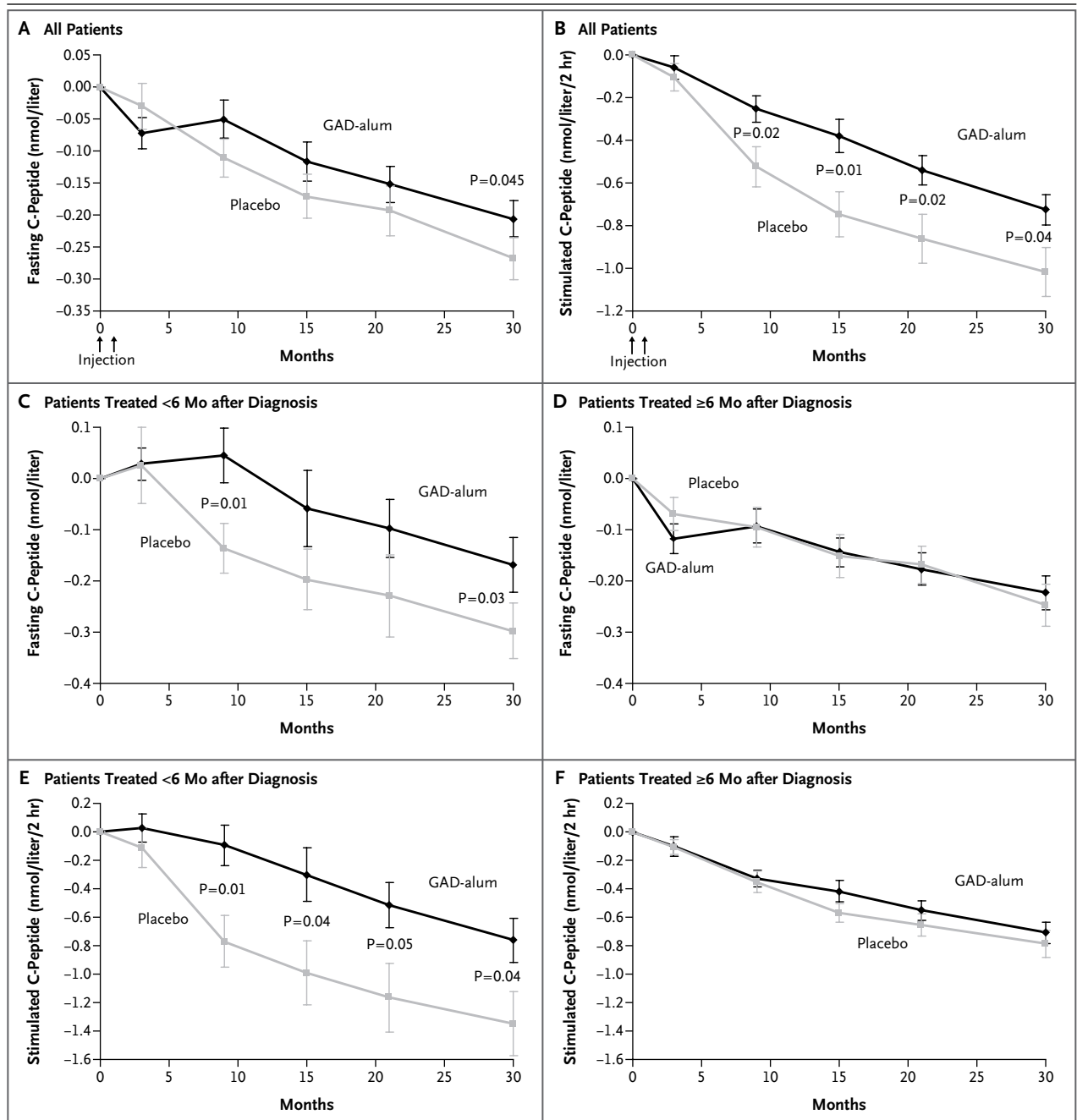


Figure 2. Mean Changes from Baseline Levels of Fasting and Stimulated C-Peptide, According to Treatment Group and Time of Treatment Relative to Diagnosis.

Mean changes from baseline in fasting (Panel A) and stimulated (Panel B) C-peptide levels are given for all patients included in intention-to-treat analyses in the group receiving the recombinant human 65-kD isoform of glutamic acid decarboxylase in a standard vaccine formulation with alum (GAD-alum, 35 patients) and in the group receiving placebo (34 patients). Mean changes from baseline in fasting (Panel C) and stimulated (Panel E) C-peptide levels are also shown for patients treated less than 6 months after receiving the diagnosis of diabetes (11 patients in the GAD-alum group and 14 patients in the placebo group). Finally, mean changes from baseline in fasting (Panel D) and stimulated (Panel F) C-peptide levels are shown for those treated 6 months or more after diagnosis (24 patients in the GAD-alum group and 20 patients in the placebo group). Stimulated C-peptide level was measured on the basis of areas under the curve in response to the mixed-meal tolerance test. I bars indicate standard errors. To convert values for C-peptide to nanograms per milliliter, divide by 0.33.

Table 3. Prespecified Efficacy End Points.*

End Point	Absolute Value at Mo 30			Change from Baseline to Mo 15			Change from Baseline to Mo 30		
	GAD-Alum (N=35)	Placebo (N=34)	P Value from ANCOVA	GAD-Alum (N=35)	Placebo (N=34)	Treatment Effect (95% CI)	GAD-Alum (N=35)	Placebo (N=34)	Treatment Effect (95% CI)
Fasting C-peptide — nmol/liter	0.13±0.11	0.09±0.10	0.28	-0.12±0.18	-0.17±0.20	0.04 (-0.04 to 0.12)	-0.21±0.17	-0.27±0.19	0.04 (0.00 to 0.09)
Stimulated C-peptide AUC — nmol/liter/2 hr	0.51±0.50	0.40±0.46	0.01	-0.38±0.46	-0.75±0.61	0.30 (0.07 to 0.54)	-0.72±0.42	-1.02±0.67	0.19 (0.01 to 0.37)
Glycated hemoglobin — %	7.4±1.8	7.1±1.2	0.57	0.3±1.3	0.5±1.5	-0.2 (-0.7 to 0.4)	1.1±1.8	0.9±1.3	0.2 (-0.5 to 0.9)
Insulin dose — U/kg of body weight/24 hr	0.96±0.39	0.96±0.25	0.19	0.15±0.22	0.22±0.29	-0.08 (-0.19 to 0.04)	0.30±0.37	0.30±0.30	0.00 (-0.14 to 0.14)
Plasma glucose before MMTT — mmol/liter	9.7±4.5	9.6±4.4	0.57	0.1±5.8	1.3±5.8	-0.6 (-2.8 to 1.6)	0.4±5.8	0.7±5.6	0.2 (-2.0 to 2.3)
Fasting C-peptide:plasma glucose ratio — ×10 ⁻¹²	15±14	10±12	0.26	-15±20	-23±24	5 (-4 to 15)	-25±19	-35±23	6 (1 to 11)

* Plus-minus values are means ±SD. Treatment effect was estimated using the least-square-means method with the baseline value as a covariate. ANCOVA denotes analysis of covariance, AUC area under the curve, GAD-alum the recombinant human 65-kD isoform of glutamic acid decarboxylase in a standard vaccine formulation with alum, and MMTT mixed-meal tolerance test. To convert values for C-peptide to nanograms per milliliter, divide by 0.33. To convert values for glucose to milligrams per deciliter, divide by 0.05551.

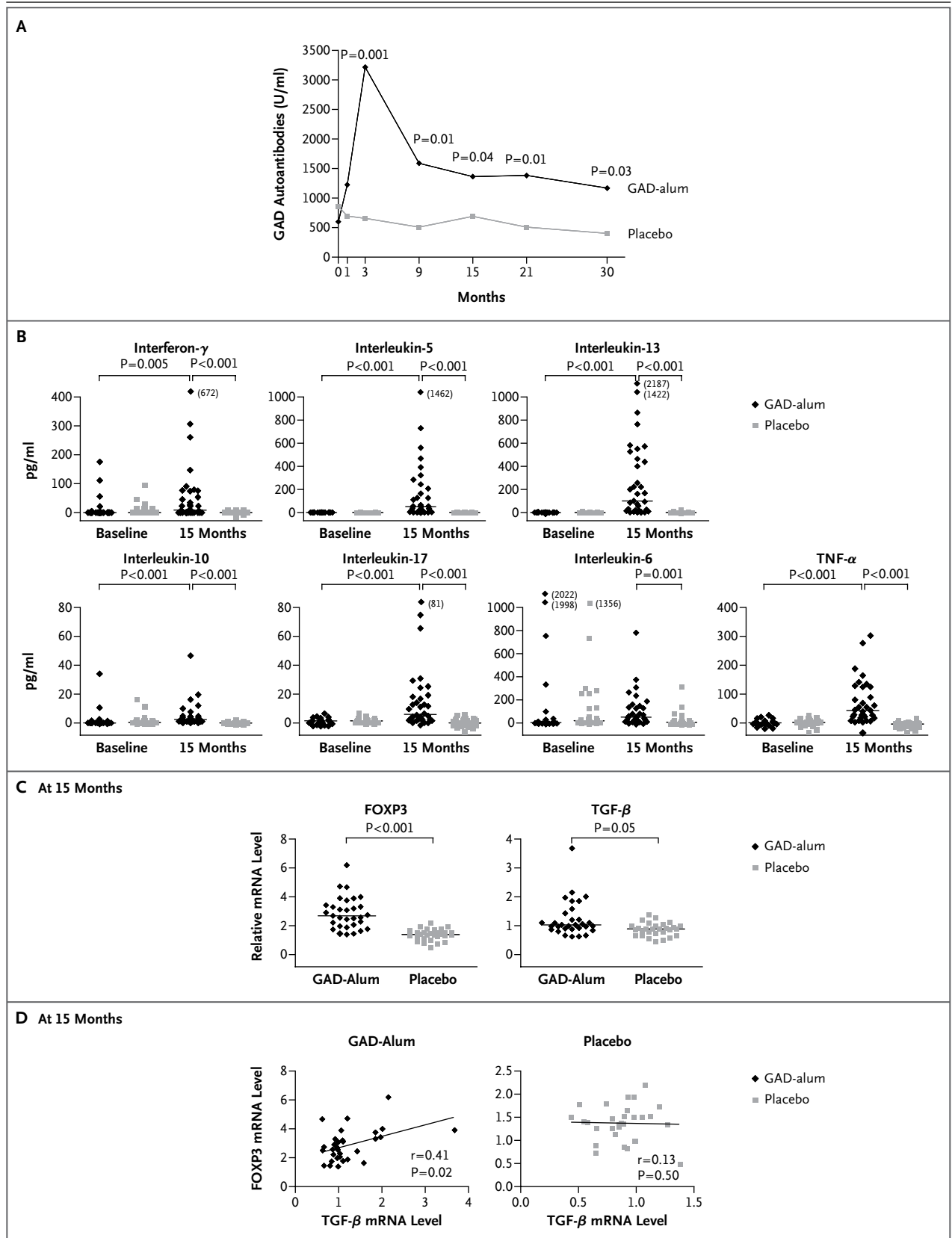
Figure 3 (facing page). Effects on the Immune System.

Panel A shows the median titers of autoantibodies against the 65-kD isoform of glutamic acid decarboxylase (GAD), from baseline to month 30, in the group receiving recombinant human GAD in a standard vaccine formulation with alum (GAD-alum) (35 patients) and in the group receiving placebo (34 patients). Panel B shows GAD-induced secretion of cytokines in peripheral-blood mononuclear cells (PBMCs) at baseline and at 15 months. The outlier values exceed the plotted values and are thus given in parentheses. Panel C shows the GAD-induced messenger RNA (mRNA) levels for the transcription factor forkhead box P3 (FOXP3) and transforming growth factor β (TGF- β) in PBMCs at 15 months. The levels are a measure of the relative transcription, based on the comparative cycle-threshold (Ct) method. Panel D shows correlations between relative levels of mRNA transcripts of FOXP3 and TGF- β in PBMC at 15 months (Spearman's rank correlation). In Panels B and C, individual data points are shown along with medians, indicated by horizontal lines. P values in Panels A, B, and C were calculated with the use of the Mann-Whitney U-test with Bonferroni correction. In Panel B, data were available at baseline and at 15 months for 27 patients and 33 patients, respectively, in the GAD-alum group and 26 patients and 29 patients, respectively, in the placebo group. In Panels C and D, data were available for 31 patients in the GAD-alum group and 29 patients in the placebo group. TNF- α denotes tumor necrosis factor α .

other. There were equal numbers of mild skin reactions (erythema, edema, and tenderness) at the injection site in the GAD-alum group and the placebo group. None required treatment or led to refusal of the second injection. Neurologic assessment was performed because of concern that treatment with GAD-alum might induce stiff-person syndrome; there were no notable neurologic differences between the two study groups.

PRESPECIFIED EFFICACY END POINTS

Both study groups showed a progressive decrease from the baseline level in both fasting and stimulated C-peptide secretion, indicating a gradual loss of beta-cell function (Fig. 2A and 2B). There was no significant effect of the study treatment on the change in fasting C-peptide level between baseline and month 15 (primary end point). There was a significant effect of the study treatment on the change in fasting C-peptide level by month 30 (P=0.045) (Fig. 2A and Table 3) and on the change in the C-peptide:plasma glucose ratio (P=0.02) (Table 3). Stimulated C-peptide secretion, as measured by the area under the curve, decreased significantly less in the GAD-alum



group than in the placebo group, both by month 15 ($P=0.01$) and by month 30 ($P=0.04$) (Fig. 2B and Table 3).

The insulin requirement in both study groups increased over the course of the study, as did glycated hemoglobin and plasma glucose levels (Table 3). Glycated hemoglobin values did not differ significantly between the two groups, given the therapeutic target used by physicians (Table 3).

EXPLORATORY ANALYSES OF EFFICACY

The significant effect of the study treatment on the change between baseline and month 30 in fasting and stimulated C-peptide levels remained after adjusting for differences in duration of diabetes, age, sex, and baseline GAD autoantibody levels (data not shown). The subgroups prespecified in the protocol regarding duration of diabetes, age, sex, HLA classification, and baseline GAD autoantibody levels were also investigated for interaction effects (data not shown). Of these, only duration of diabetes had a significant influence on the efficacy of the study treatment ($P=0.05$ for fasting at month 30 and $P=0.03$ for stimulated C-peptide level, measured as area under the curve, at months 15 and 30).

An exploratory formal analysis of the prespecified subgroups regarding duration of diabetes showed that, among patients treated less than 6 months after diagnosis, both fasting and stimulated C-peptide secretion, as measured by area under the curve, decreased significantly less in the GAD-alum group than in the placebo group by month 30 ($P=0.03$ and $P=0.04$, respectively) (Fig. 2C and 2E), whereas no significant difference was observed between the two groups for patients treated 6 months or more after diagnosis (Fig. 2D and 2F). The observed treatment effect among patients treated less than 6 months after diagnosis cannot be attributed to outliers (see the figure in the Supplementary Appendix, available with the full text of this article at www.nejm.org).

EFFECTS ON THE IMMUNE SYSTEM

In the group treated with GAD-alum, GAD autoantibody levels increased rapidly, reached a maximum at 3 months, and then decreased but remained significantly higher than in the placebo group (Fig. 3A).

Spontaneous and phytohemagglutinin-induced secretion of all cytokines was similar in samples from children receiving GAD-alum and those re-

ceiving placebo, both before and 15 months after the first injection (data not shown). Cytokine secretion of interleukins 5, 10, 13, and 17, interferon- γ , and TNF- α , but not of interleukins 6 and 12, increased in response to in vitro stimulation with GAD in the GAD-alum group between baseline and month 15 (Fig. 3B). Fifteen months after the immune intervention, GAD-induced secretion of the cytokines, except interleukin-12, was significantly higher in samples from the GAD-alum group than in samples from the placebo group (Fig. 3B).

Increased GAD-induced expression of FOXP3 and TGF- β was found at month 15 in cells from the GAD-alum group, as compared with the placebo group (Fig. 3C). According to post hoc correlation analyses, the expression of FOXP3 and TGF- β was correlated for the GAD-alum group but not for the placebo group (Fig. 3D).

DISCUSSION

Fasting C-peptide levels in patients with recent-onset type 1 diabetes may decrease quite slowly when fasting glucose levels are kept reasonably low.²⁹ Stimulated C-peptide levels have been considered an end point for assessing the preservation of beta-cell function early in the course of the disease, since the levels correlate with both improved glycemic control and fewer microvascular complications.^{15,16,30} However, we chose fasting C-peptide levels as the primary end point, based on the previous study of GAD-alum use in patients with LADA.²³ There was no significant effect of the GAD-alum treatment on the change in fasting C-peptide level after 15 months (the primary end point), though there was an effect on stimulated C-peptide level. After 30 months, both fasting and stimulated C-peptide levels showed a significantly smaller decline in the GAD-alum group than in the placebo group. However, the apparent protective effect of the GAD-alum treatment on C-peptide secretion was seen only in patients treated less than 6 months after diagnosis.

Our results suggest that two injections of 20 μ g of GAD-alum may contribute to preservation of residual insulin secretion in patients with recent-onset type 1 diabetes. In our patients with newly diagnosed diabetes, the duration and magnitude of the GAD-alum treatment effect seems to be similar to that reported for anti-CD3 treatment,^{15,16} but use of GAD-alum does not appear to be ac-

accompanied by treatment-related adverse events. Residual insulin secretion, especially in the long term, affects important clinical outcomes.⁴ This may be explained by improved overall metabolic control, reduced fluctuation in blood glucose levels and, possibly, increased exposure to C-peptide, which itself may have biologic effects.³¹

During the study, the insulin requirement, plasma glucose, and glycated hemoglobin levels increased in both study groups. As expected, the aggressive treatment to achieve the glycated hemoglobin target resulted in glycated hemoglobin levels in our patients that were similar to those in adolescents in the intensive-treatment group of the Diabetes Control and Complications Trial (DCCT).³²

Minor differences in the demographic characteristics of the two study groups are unlikely to be relevant, in our view. GAD autoantibodies in patients with type 1 diabetes are associated with HLA-DQ-A1*0501-DQB1*02.²⁶ However, the distribution of HLA genotypes in the two study groups were similar and cannot explain the differences in C-peptide responses.

The mechanism by which GAD-alum treatment may alter disease progression in type 1 diabetes is not clear. Since the therapy was otherwise similar in the two groups, differences in preserved beta-cell function would not appear to be related to more intense insulin treatment or better metabolic control in the GAD-alum group. Fasting C-peptide level may be influenced by the actual blood glucose level, but we found significant preservation of the C-peptide level both before and after adjustment for blood glucose level.

Studies in NOD mice have shown that GAD

can induce a potent regulatory response in mice with established autoimmunity and after the onset of type 1 diabetes.^{21,22} A previous dose-finding study revealed that GAD-alum induced an increase in the ratio of CD4+CD25+ T cells to CD4+CD25- T cells at 24 weeks, suggesting a possible effect on regulatory CD4+CD25+ T cells.²³ In our study, GAD-alum induced an antigen-specific T-cell population with a broad range of cytokines involved in the regulatory process of the immune system. The treatment also induced a long-lasting, specific B-cell memory. Thus, modulation of the general memory immune responses to GAD could be responsible for beta-cell preservation.

In conclusion, treatment with GAD-alum had an effect on slowing the loss of residual beta-cell function up to 30 months after intervention and was associated with GAD-specific immune modulation, although it did not change the insulin requirement. Our results provide preliminary proof of concept; large-scale confirmatory studies with GAD-alum are under way in Europe (NCT00723411) and the United States (NCT00751842).

Supported by grants from Diamyd Medical and the Swedish Child Diabetes Foundation (Barndiabetesfonden).

Dr. Ludvigsson reports receiving grant support from Diamyd Medical for the mechanistic studies and from NovoNordisk for studies on insulin analogues; and Dr. Örtqvist, lecture fees from Medtronic. Mr. Zerhouni reports being an employee of Diamyd Medical and holding stock options in the company. No other potential conflict of interest relevant to this article was reported.

We thank Lena Berglert, Gosia Smolinska, and Ingela Johansson for their skillful laboratory work; Prof. Åke Lernmark for determination of HLA types and classification of HLA risk groups; Profs. Mark Atkinson, Daniel Kaufman, Åke Lernmark, and David Leslie for their valuable input on a draft of the manuscript; Prof. Lernmark for supplying the pEx9 vector; and John Robertson for oversight of the manufacture of the study medication.

REFERENCES

- Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 2001; 358:221-9.
- The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993;329: 977-86.
- Bojestig M, Arnqvist HJ, Hermansson G, Karlberg BE, Ludvigsson J. Declining incidence of nephropathy in insulin-dependent diabetes mellitus. *N Engl J Med* 1994; 330:15-8. [Erratum, *N Engl J Med* 1994;330: 584.]
- Steffes MW, Sibley S, Jackson M, Thomas W. Beta-cell function and the development of diabetes-related complications in the Diabetes Control and Complications Trial. *Diabetes Care* 2003;26: 832-6.
- Ludvigsson J, Heding L, Liedén G, Marner B, Lernmark A. Plasmapheresis in the initial treatment of insulin-dependent diabetes mellitus in children. *Br Med J (Clin Res Ed)* 1983;286:176-8.
- Dupré J, Stiller CR, Gent M, et al. Clinical trials of cyclosporin in IDDM. *Diabetes Care* 1988;11:Suppl 1:37-44.
- Eisenbarth GS, Srikanta S, Jackson R, et al. Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus. *Diabetes Res* 1985;2:271-6.
- Chase HP, Butler-Simon N, Garg S, McDuffie M, Hoops SL, O'Brien D. A trial of nicotinamide in newly diagnosed patients with type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1990;33: 444-6.
- Pozzilli P, Visalli N, Signore A, et al. Double blind trial of nicotinamide in recent-onset IDDM (the IMDIAB III study). *Diabetologia* 1995;38:848-52.
- Coutant R, Landais P, Rosilio M, et al. Low dose linomide in Type I juvenile diabetes of recent onset: a randomised placebo-controlled double blind trial. *Diabetologia* 1998;41:1040-6.
- Ludvigsson J, Samuelsson U, Johansson C, Stenhammar L. Treatment with antioxidants at onset of type 1 diabetes in children: a randomized, double-blind placebo-controlled study. *Diabetes Metab Res Rev* 2001;17:131-6.

12. Ludvigsson J, Samuelsson U, Ernerudh J, Johansson C, Stenhammar L, Berlin G. Photopheresis at onset of type 1 diabetes: a randomised, double blind, placebo controlled trial. *Arch Dis Child* 2001; 85:149-54.
13. Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. *Lancet* 2001;358: 1749-53.
14. Lazar L, Ofan R, Weintrob N, et al. Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study. *Diabetes Metab Res Rev* 2007;23:286-91.
15. Herold KC, Gitelman SE, Masharani U, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1 (Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes* 2005;54:1763-9.
16. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 2005;352:2598-608.
17. Harrison LC. The prospect of vaccination to prevent type 1 diabetes. *Hum Vaccin* 2005;1:143-50.
18. Jasinski JM, Eisenbarth GS. Insulin as a primary autoantigen for type 1A diabetes. *Clin Dev Immunol* 2005;12:181-6.
19. Baekkeskov S, Nielsen JH, Marner H, Bilde T, Ludvigsson J, Lernmark A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. *Nature* 1982;298: 167-9.
20. Lernmark A, Agardh CD. Immunomodulation with human recombinant autoantigens. *Trends Immunol* 2005;26: 608-12.
21. Tian J, Clare-Salzler M, Herschenfeld A, et al. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 1996;2:1348-53.
22. Tisch R, Liblau RS, Yang XD, Liblau P, McDevitt HO. Induction of GAD65-specific regulatory T cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. *Diabetes* 1998;47:894-9.
23. Agardh CD, Cilio CM, Lethagen A, et al. Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. *J Diabetes Complications* 2005;19:238-46.
24. Greenbaum CJ, Mandrup-Poulsen T, Battelino T, et al. The mixed meal tolerance test versus the glucagon stimulation test for the assessment of beta-cell function in therapeutic trials in type 1 diabetes. *Diabetes Care* 2008 July 15 (Epub ahead of print).
25. Larsson HE, Lynch K, Lernmark B, et al. Diabetes-associated HLA genotypes affect birthweight in the general population. *Diabetologia* 2005;48:1484-91.
26. Graham J, Hagopian WA, Kockum I, et al. Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. *Diabetes* 2002;51: 1346-55.
27. Perneger TV. What's wrong with Bonferroni adjustments. *BMJ* 1998;316: 1236-8.
28. Altman DG, Doré CJ. Randomisation and baseline comparisons in clinical trials. *Lancet* 1990;335:149-53.
29. Daneman D, Clarson C. Residual beta-cell function in children with type 1 diabetes: measurement and impact on glycemic control. *Clin Invest Med* 1987;10: 484-7.
30. Steele C, Hagopian WA, Gitelman S, et al. Insulin secretion in type 1 diabetes. *Diabetes* 2004;53:426-33.
31. Wahren J, Ekberg K, Johansson J, et al. Role of C-peptide in human physiology. *Am J Physiol Endocrinol Metab* 2000; 278:E759-E768.
32. White NH, Cleary PA, Dahms S, et al. Beneficial effects of intensive therapy of diabetes during adolescence: outcomes after the conclusion of the Diabetes Control and Complications Trial (DCCT). *J Pediatr* 2001;139:804-12.

Copyright © 2008 Massachusetts Medical Society.