

# $\beta$ -cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial

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## Summary

**Background** Type 1 diabetes results from autoimmune destruction of insulin-producing pancreatic  $\beta$  cells. The 60 kDa heat-shock protein (hsp60) is one of the known target self antigens. An immunomodulatory peptide from hsp60, p277, arrested  $\beta$ -cell destruction and maintained insulin production in newly diabetic NOD mice. We did a randomised, double-blind, phase II study of peptide treatment in patients with newly diagnosed (<6 months) type 1 diabetes.

**Methods** 35 patients with type 1 diabetes and basal C-peptide concentrations above 0.1 nmol/L were assigned subcutaneous injections of 1 mg p277 and 40 mg mannitol in vegetable oil (DiaPep277; n=18) at entry, 1 month, and 6 months, or three placebo injections (mannitol in vehicle; placebo; n=17). The primary endpoint was glucagon-stimulated C-peptide production. Secondary endpoints were metabolic control and T-cell autoimmunity to hsp60 and to p277 (assayed by cytokine secretion). 31 patients completed 10 months of follow-up and were included in the intention-to-treat analysis.

**Findings** At 10 months, mean C-peptide concentrations had fallen in the placebo group (n=16) but were maintained in the DiaPep277 group (n=15; 0.26 [SD 0.11] vs 0.93 [0.35] nmol/L; p=0.039). Need for exogenous insulin was higher in the placebo than in the DiaPep277 group (0.67 [0.33] vs 0.43 [0.17] U/kg; p=0.042). Haemoglobin A1c concentrations were low (around 7%) in both groups. T-cell reactivity to hsp60 and p277 in the DiaPep277 group showed an enhanced T-helper-2 cytokine phenotype. No adverse effects were noted.

**Interpretation** Although this study was small, treatment of newly diagnosed type 1 diabetes with DiaPep277 seems to preserve endogenous insulin production, perhaps through induction of a shift from T-helper-1 to T-helper-2 cytokines produced by the autoimmune T cells.

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## Introduction

Type 1 diabetes mellitus is caused by the progressive destruction of the insulin-producing  $\beta$  cells through an autoimmune process.<sup>1</sup> Autoimmune destruction remains subclinical until the number of  $\beta$  cells is insufficient to produce the amount of insulin needed to maintain glucose homeostasis. At this point, diabetes becomes apparent. To some degree, insulin replacement repairs the secondary endocrine disease, but it cannot stop autoimmune destruction of  $\beta$  cells. Primary cure of type 1 diabetes would require stopping the autoimmune process in time to rescue the  $\beta$  cells.

The autoimmunity that brings about type 1 diabetes has been studied in NOD mice.<sup>2</sup> Despite the differences between the species, this model has been helpful in raising fundamental questions about human disease.<sup>3</sup> Autoimmune T cells in NOD mice react spontaneously to many different self antigens,<sup>3</sup> one of which is the 60 kDa heat-shock protein (hsp60).<sup>4</sup> The basis for our study was the observation that treatment of NOD mice with a peptide of hsp60, peptide p277, could save residual  $\beta$ -cell function even late in the course of autoimmunity, after the onset of clinical hyperglycaemia.<sup>5</sup> Cessation of  $\beta$ -cell destruction seemed to result from the induction by p277 of a shift in the cytokine profile of hsp60 autoimmunity from a proinflammatory T-helper-1 (Th1) phenotype to an anti-inflammatory T-helper-2 (Th2) phenotype.<sup>6–8</sup> The immunomodulation of hsp60 autoimmunity induced by p277 was specific; Th1 immunity to bacterial antigens was not affected by p277 treatment.<sup>7</sup>

In view of the observation that patients with type 1 diabetes, like NOD mice, show spontaneous T-cell autoimmunity to hsp60,<sup>9</sup> we set out to test whether the p277 peptide might be used to prevent the autoimmune destruction of  $\beta$  cells in human beings. The ideal candidates for such treatment would be people with preclinical disease who have not yet lost a large proportion of  $\beta$  cells. However, the diagnosis of preclinical type 1 diabetes still has a degree of uncertainty,<sup>10</sup> and a prevention trial would inevitably include individuals who might never develop the disease. Knowing that p277 treatment was effective even in clinically hyperglycaemic NOD mice,<sup>5</sup> we elected to test the treatment in people recently diagnosed as having clinical type 1 diabetes. The primary endpoint of the study at 10 months was preservation of  $\beta$ -cell function detected by a halt in the loss of C-peptide production, and the secondary endpoints were a decreased need for exogenous insulin, the concentration of haemoglobin A1c, and a shift in the cytokine phenotype of the autoimmunity to hsp60 and p277.

## Methods

### Patients and study design

We screened men, aged 16–55 years, who were consecutively diagnosed as having type 1 diabetes at the Endocrine Clinic of the Hadassah University Hospital. Although a phase I study of DiaPep277 injection in 16 adults with long-term diabetes showed no toxic effects at doses up to 2.5 mg per injection (data not shown), we excluded young children and women to obtain additional safety data in adults without a risk of pregnancy. Inclusion criteria were: presentation with acute hyperglycaemia and ketonuria; a body-mass index of 28 kg/m<sup>2</sup> or less; no family history of type 2 diabetes; the presence of autoantibodies to glutamic acid decarboxylase; diabetes of less than 6 months' duration; residual  $\beta$ -cell function detected by a basal C-peptide concentration of more than 0.1 nmol/L; and compliance with diet and insulin treatment with well-controlled diabetes for at least 2 weeks. The patients were free of other diseases, and their informed consent was obtained.

We randomly assigned patients, with masking, to treatment with a preparation of p277 in oil (DiaPep277, Peptor, Rehovot, Israel) or placebo. Randomisation was done by the contract research organisation, in blocks of four. The randomisation list was generated by the computer program RANCODE (version 3.6).

Peptide p277 was synthesised under the regulations of Good Manufacturing Practice by Peptor, 1 mg peptide and 40 mg mannitol (as a filler) in 0.5 mL was administered as DiaPep277 subcutaneously in a vehicle composed of a 10% preparation of a vegetable oil approved for human injection (Lipofundin 10%, B Braun, Melsungen, Germany). The sequence of peptide p277 in DiaPep277 is VLGGGVALLRVIPALDSLTPANED, residues 437–460 of the human hsp60 molecule.<sup>5</sup> To stabilise DiaPep277 without affecting its immunological properties, we substituted valine for the cysteine residues at positions 442 and 447. The placebo was mannitol (40 mg) in the vehicle. Every patient received three injections: at enrolment, 1 month, and 6 months.

We followed up the patients for 10 months to allow time for loss of most of the capacity to produce C-peptide in response to glucagon stimulation. The identity of the groups was unknown to the patients, their physicians, or the staff doing laboratory testing, but it was known to a safety committee, who followed up the patients for adverse effects, and to professional statisticians, who tabulated the data. The study was approved by the institutional review board and by the National Committee for Human Trials of the Israel Ministry of Health.

### Endpoint assessments

To assay the functional  $\beta$ -cell mass, the primary endpoint, C-peptide concentration in the morning, 10–12 h after the last insulin dose, as the fasting basal concentration, and 2 min, 6 min, 10 min, and 20 min after stimulation of the patient with intravenous glucagon (1 mg) was measured by a standard assay.<sup>11</sup> The highest concentration was used as the value for analysis.

The patients' doctors prescribed the amounts of insulin required to control each patient's blood glucose concentration according to accepted standards, and the amount of insulin per kg bodyweight was calculated from the patient's treatment diary. In addition to standard blood tests to detect possible toxic effects, blood samples were tested for haemoglobin A1c (glycosylated haemoglobin) as a measure of the general control of hyperglycaemia.<sup>12</sup>

The cytokine phenotype of the T-cell reactivity to hsp60 and to peptide p277 was measured in vitro with a quantitative ELISpot assay.<sup>13</sup> In this assay, peripheral blood T cells are stimulated by incubation in vitro with the antigen (10  $\mu$ g/mL), and the numbers of T cells producing various cytokines are enumerated by counting spots in a cytokine capture assay. Interferon  $\gamma$ , a Th1 cytokine, and interleukins 4, 10, and 13, Th2 cytokines, were measured. T-cell responses were also measured to bacterial recall antigens *Mycobacterium tuberculosis* (purified protein derivative; PPD) and tetanus toxoid as described.<sup>7</sup>

### Statistical analysis

A sample size of 15 patients in each treatment group was judged sufficient in this exploratory study, but additional patients were recruited as insurance against dropouts. No formal power calculation could be done, because we had no previous results for guidance. Analysis of the results and comparisons between treated and control groups were done by a biostatistical consulting agency, AL-STAT, by use of a two-tailed *t* test for a two-sample study with unequal variance.

## Results

Of 47 patients screened, 35 were eligible. 18 were assigned DiaPep277 and 17 placebo (figure 1). By the end of the follow-up period, four patients had been lost to follow-up (one was excluded for drug use and three refused to undergo the glucagon stimulation assay). The DiaPep277 and placebo groups were similar in terms of age (29.3 [SD 11.9] vs 23.1 [6.9] years), body-mass index (22.1 [2.9] vs 21.9 [2.7] kg/m<sup>2</sup>), duration of disease (14.5 [9.9] vs 12.6 [6.6] weeks), baseline C-peptide concentration (0.44 [0.30] vs 0.53 [0.40] nmol/L), and insulin requirements on entry (0.35 [0.14] vs 0.37 [0.19] U/kg).

Patients on placebo showed a progressive loss of glucagon-stimulated C-peptide, indicating a progressive, cumulative loss of  $\beta$  cells with time (figure 2). Indeed, the rapid loss of C-peptide supports the diagnosis of type 1 diabetes. The group of patients assigned DiaPep277, by contrast, maintained their production of C-peptide after glucagon stimulation. The differences between the DiaPep277 and placebo groups were significant at 7 months and 10 months of follow-up (0.92 [SD 0.25] vs 0.35 [0.22] nmol/L, *p*=0.043, at 7 months; 0.93 [0.35] vs

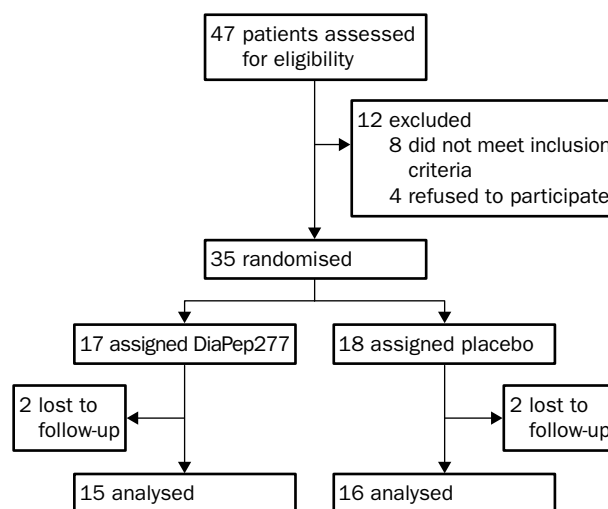


Figure 1: Trial profile

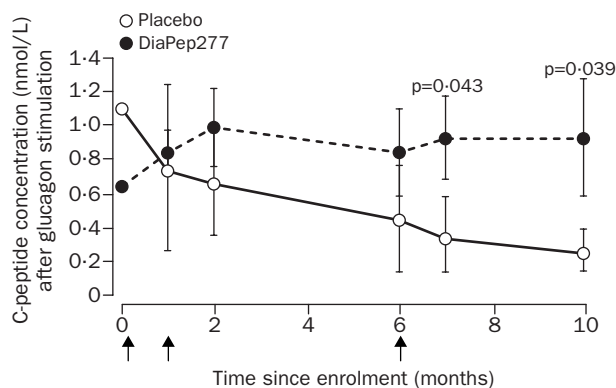


Figure 2: Mean and SD stimulated C-peptide concentrations. Arrows indicate times of treatment.

0.26 [0.11] nmol/L,  $p=0.039$  at 10 months). There was a positive correlation between C-peptide concentrations at entry and at 2 months in both groups (correlation coefficient 0.65 in the DiaPep277 group, 0.70 in the placebo group). At 10 months, however, the correlation coefficient of the DiaPep277 group was 0.82 and that of the placebo group only 0.02. Thus, the individuals with higher C-peptide concentrations at the time of initiation of DiaPep277 treatment showed better preservation of C-peptide concentration 10 months later.

The concentrations of haemoglobin A1c seen over 10 months in both groups were around 7% throughout the study. This value indicates that patients in both groups received adequate treatment.<sup>12</sup> Thus, any differences between the groups could be attributed to the effects of treatment, not to any difference in metabolic control.

The DiaPep277 group required less exogenous insulin to maintain adequate control than did the placebo group (figure 3). The difference at 10 months was significant ( $p=0.042$ ).

T-cell responses to human hsp60, peptide p277, and bacterial antigens were assessed at 10 months (figure 4). Patients on placebo showed more interferon  $\gamma$  ( $p=0.041$ ) and less interleukin 13 ( $p=0.048$ ) in response to hsp60 than to p277. Thus, hsp60 seems to activate more of a Th1 response than does p277 in controls. Compared with the placebo group, patients assigned DiaPep277 produced less interferon  $\gamma$  ( $p=0.04$ ) and more interleukin 10

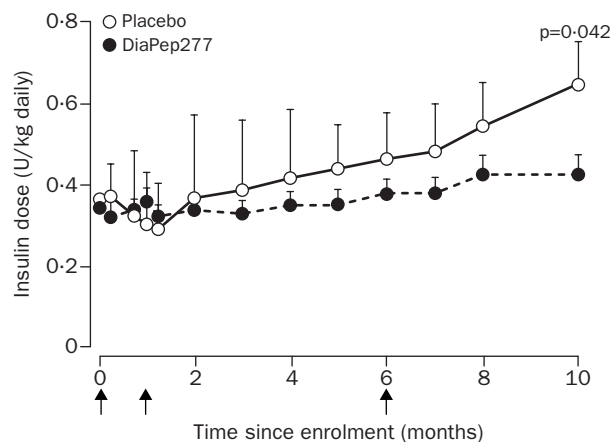


Figure 3: Mean and SD insulin requirements. Arrows indicate times of treatment.

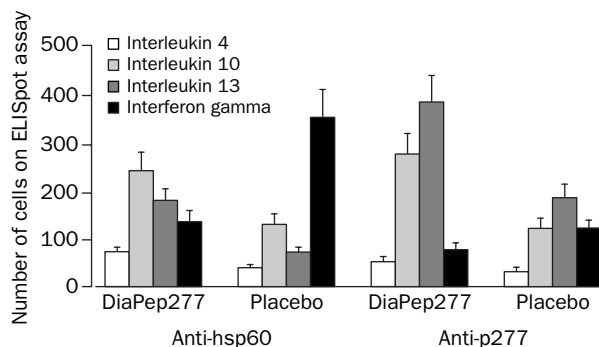


Figure 4: Stimulated cytokine production and SD 10 months after initiation of treatment

( $p=0.03$ ) and interleukin 13 ( $p=0.04$ ) in response to hsp60; the increase in interleukin 4 was not significant ( $p=0.14$ ). In response to p277, the DiaPep277 patients produced more interleukin 10 ( $p=0.01$ ) and interleukin 13 ( $p=0.02$ ) than patients on placebo; the differences in interleukin 4 and interferon  $\gamma$  were not significant ( $p=0.13$  and  $p=0.12$ , respectively).

The induction of antibodies to p277 by DiaPep277 treatment could not be measured because many of the patients tested positive for such antibodies before they received treatment (data not shown). There were no great differences in the T-cell proliferation and cytokine responses between the groups to PPD or tetanus toxoid (data not shown). There was a positive correlation between the amount of interleukin 13 produced in response to hsp60 and the mean concentration of C-peptide at 10 months in the DiaPep277-group patients (correlation coefficient 0.75).

No adverse effects of treatment were noted, except for slight redness at the injection site in four patients which resolved within 24–48 h without treatment.

## Discussion

The results of this clinical study are generally similar to our earlier experience with peptide p277 administered in incomplete Freund's adjuvant to newly diabetic NOD mice.<sup>5</sup> The patients in the placebo group showed a fall in their ability to produce C-peptide and an increasing need for exogenous insulin over the period of 10 months after treatment. Both these changes can be attributed to the progressive loss of the few  $\beta$  cells that were still functional at the time the patients entered the study. Treatment with exogenous insulin instituted after the diagnosis of type 1 diabetes was effective in controlling hyperglycaemia, but it did not prevent the continuing destruction of  $\beta$  cells by the autoimmunity present in the placebo-treated patients.

The patients assigned p277, by contrast, maintained their ability to produce C-peptide and needed significantly less insulin to control their blood glucose concentrations. Our finding that patients with higher C-peptide concentrations at the initiation of treatment had a more pronounced effect of DiaPep277 treatment on the C-peptide concentrations at 10 months suggests that the treatment is more effective when there are more  $\beta$  cells that can be saved, though the number of patients in that analysis was small.

This trial was designed to test the effect of the peptide treatment in preventing the continued loss of C-peptide during the period shortly after the diagnosis of type 1 diabetes; it remains to be seen whether, and with what intervals, additional treatments might be needed to maintain long-term endogenous insulin production.

At present, there is no direct way of assessing  $\beta$ -cell destruction other than functionally. However, both the maintenance of C-peptide production and the lesser need for exogenous insulin can be explained most simply by the arrest of autoimmune  $\beta$ -cell destruction. DiaPep277 treatment was associated with modulation of the autoimmune response to hsp60 from a proinflammatory Th1 type to an anti-inflammatory Th2 type. The positive correlation between the concentration of C-peptide at 10 months and the numbers of T cells secreting interleukin 13 in response to hsp60 further supports this idea.

Investigation of the use of p277 in the NOD mouse model showed that modulation of the hsp60 autoimmunity detected peripherally was marked by spreading down-regulation of T-cell autoimmunity to various other self antigens such as insulin and glutamic acid decarboxylase.<sup>7</sup> This modulation was accompanied by the cessation of production of inflammatory cytokines by T cells in the islets, irrespective of their specific target self antigen.<sup>8</sup> The down-regulation of autoimmunity to several self antigens induced by immune modulation through use of one of them can be explained by bystander regulation.<sup>14</sup> Immunity to bacterial antigens was not affected by DiaPep277 in this trial nor in the NOD mice treated with peptide p277.<sup>7</sup> Thus the immune modulation induced by DiaPep277 seems to be specific for the autoimmunity associated with type 1 diabetes. Likewise, immunisation to foreign antigens does not seem to modify the diabetic process; childhood vaccinations and infections are not known to be risk factors for type 1 diabetes.<sup>15,16</sup>

Attempts to treat diabetes with non-specific immunomodulators such as steroids, plasmapheresis, antithymocyte globulin, and azathioprine had inconclusive results.<sup>17–20</sup> Cyclosporin, however, conserved endogenous insulin secretion, and its use proved that immune intervention can change the natural course of the disease.<sup>21–24</sup> But cyclosporin is of limited usefulness because it has potentially hazardous side-effects. The results obtained with DiaPep277 contrast with the failure of oral insulin to maintain residual  $\beta$ -cell function in a trial in recent-onset type 1 diabetes.<sup>25</sup>

The DiaPep277 molecule features two aminoacid substitutions in the native sequence of peptide p277, but the modified peptide does not function as an altered peptide ligand; it seems to have the immunological properties of the native p277 sequence.<sup>6</sup> The valine-cysteine substitutions were adopted only to stabilise the p277 peptide chemically; the native sequence and the modified sequence were equivalent in their recognition by both T cells and monoclonal antibodies, and the two versions of p277 were equally effective in the treatment of NOD mice.<sup>6</sup> Our use of DiaPep277 in small and infrequent doses shows that specific immunomodulation can be achieved by limited intervention, even late in the autoimmune process.<sup>5</sup> Other autoimmune diseases might be specifically modulated by the right amount of a suitable target peptide.<sup>26</sup>

The positive correlation between the concentrations of C-peptide at 10 months and at the initiation of DiaPep277 treatment indicates that early treatment is important in preserving  $\beta$ -cell function. The persistence of C-peptide, even if the treated patients continue to require exogenous insulin, might be expected to protect to some degree from the long-term complications of diabetes;<sup>27</sup> any amount of endogenous insulin production would add to glycaemic control. Obviously, the ideal population for treatment with DiaPep277 would be those

with subclinical autoimmunity whose  $\beta$ -cell destruction has not yet manifest clinically as type 1 diabetes; the permanent arrest of subclinical  $\beta$ -cell destruction would constitute cure. More information on the safety and effectiveness of DiaPep277 in recent-onset diabetes should pave the way to the pre-emptive treatment of those at risk.

#### Contributors

Itamar Raz and Muriel Metzger did the clinical work: patient screening, treatment administration, and follow-up. Dana Elias, Ann Avron, and Merana Tamir did immunological assays, and monitored patients. Irun R Cohen, Dana Elias, and Itamar Raz planned and designed the study.

#### Conflict-of-interest statement

Irun R Cohen is a member of Peptor's scientific advisory board.

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