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Research Article

Therapy with the hsp60 peptide DiaPep277™ in C-peptide positive type 1 diabetes patients†

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KEYWORDS

type 1 diabetes • hsp60 • immunomodulation • intervention therapy • C-peptide • HbA_{1c} • insulin • randomized controlled trial

ABSTRACT

Background

Type 1 diabetes results from a T-cell mediated autoimmune destruction of insulin-producing pancreatic beta-cells. The 60-kDa heat-shock protein (hsp60) is one of the known target self-antigens. An immunogenic peptide from hsp60, p277, arrested beta-cell destruction and maintained insulin production in newly diabetic non-obese diabetic (NOD) mice. A randomized, double-blind, phase Ib/II study of peptide treatment was undertaken in recent onset type 1 diabetes patients with remaining insulin production.

Methods

Forty-eight recent onset type 1 diabetes patients were assigned subcutaneous injections of 0.2, 1.0 or 2.5 mg peptide DiaPep277

(*n* = 12 per dosage) at entry, and 1, 6 and 12 months, or four placebo injections (*n* = 12). The primary clinical endpoints were safety and efficacy (glucagon-stimulated C-peptide production at 6 and 12 months); secondary endpoints were HbA_{1c} levels and daily insulin dose adjusted for body weight at 2, 6, 12 and 18 months.

Results

C-peptide levels decreased over time in all groups except the 2.5 mg-treated. The decrease in C-peptide production was less in treated patients *versus* placebo, mostly in the 2.5 mg group. HbA_{1c} increased significantly in the 1.0 mg group and in the 2.5 mg group at 2 and 18 months, respectively. No differences were seen in daily insulin doses. One patient was withdrawn from the study possibly owing to a treatment-related adverse event.

Conclusions

Multiple DiaPep277 peptide administration seems safe and may have a beneficial effect on C-peptide levels over time, but this finding is not supported by lower HbA_{1c} levels or daily insulin requirement. Further investigation on a larger scale is warranted.

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ARTICLE TEXT

Introduction

Type 1 diabetes is an autoimmune disease characterized by the selective destruction of insulin-producing pancreatic beta-cells [1]. Until recently, management of type 1 diabetes was limited to insulin replacement by subcutaneous injection. Unfortunately, glycemic control can be poor using this method, and complications such as hypoglycaemia can occur. Moreover, long-term loss of glycemic control can lead to secondary complications such as neuropathy, nephropathy and retinopathy, causing severe health impairment and eventually death [2][3]. Another option for replacement of insulin production is formed by transplantation of beta-cells (whole pancreas transplantation and more recently also isolated islets of Langerhans) [4][5]. In transplantation, however, surgery can cause complications, patients are subjected to severe immunosuppressive therapy, and recurrent autoimmune destruction and graft rejection still are major problems [5][6]. Furthermore, it is limited to a small number of patients because of donor shortage.

The methods mentioned above do not attempt to attack the cause of the disease: the autoimmune process. Both in pre-clinical and clinical setting many efforts are being made to attenuate this process in type 1 diabetes. The pathophysiology of type 1 diabetes has been extensively studied at the pre-clinical stage. The discovery of T-cell reactivity to several autoantigens in the NOD mouse model (e.g. insulin, GAD65 and IA-2) has led the way to a number of immunotherapeutic options that could cause reversal of type 1 diabetes in NOD mice. Unfortunately, so far very few of those options have shown to be effective in humans [7-9]. Non-selective immunosuppressive therapies such as anti-thymocyte globuline, plasmapheresis, azathioprine or steroids [10-13] were not able to stop human beta-cell destruction either. Cyclosporin treatment, although effective, caused many side effects [14][15]. The considerable effect of non-depleting humanized anti-CD3 antibody therapy on halting beta-cell destruction [16][17] is the first solid proof of successful immune intervention in human type 1 diabetes and thus gives hope for the future.

One of the autoantigens to which the autoimmune T-cells in NOD mice reacted was heat-shock protein 60 (hsp60) [18]. Treatment of NOD mice with a modified form of highly reactive hsp60 peptide p277 (DiaPep277) could attenuate beta-cell destruction, possibly by shifting cytokine production from a pro- to an anti-inflammatory state [19-21]. Although autoreactivity to hsp60 in humans has been studied less extensively, these observations formed the basis for a number of phase I and II trials using DiaPep277 in human type 1 diabetes patients. The long-term results of one of these investigations are reported here.

In clinically manifest type 1 diabetes patients, the autoimmune process has been present for a considerable time. Consequently, any type of immunotherapy may have less effect in these patients than in euglycemic (pre-diabetic) subjects that are positive for anti-beta cell immune markers. The results of the present trial may allow for decisions on testing earlier in the disease process.

The present trial included C-peptide-producing patients up to 3 years from diagnosis of type 1 diabetes, in whom the autoimmune process presumably could still be halted. The trial combined a phase Ib and II design as it aimed to evaluate the safety of multiple DiaPep277 injections in human type 1 diabetes patients and the efficacy of DiaPep277 treatment through sustained beta-cell function and better metabolic control. The effects of DiaPep277 on both the humoral and cellular immune system will be subject to a separate report.

Materials and methods

Study design

This phase Ib/II study on hsp60 peptide DiaPep277 treatment was designed by Peptor Ltd, Rehovot, Israel, and performed as a multicentre, prospective, double-blind, randomized controlled trial that was conducted between November 1998 and February 2001. The study was designed as both phase Ib (endpoints: safety in large patient cohort with several exposures to peptide) and phase II (endpoints: pharmacological response and efficacy according to stimulated C-peptide production, percent HbA_{1c} and daily insulin dosage). Patients from eight separate hospitals were included. Criteria for inclusion were: age between 18 and 45 years, not pregnant or desiring to become pregnant, type 1 diabetes diagnosed within the last 42 months, stable medical condition in the last 6 months, and written informed consent by the patient. Halfway, the protocol was amended, requesting remaining basal C-peptide level of > 0.1 *nmol/L* and positive screening for islet autoantibodies (ICA, GAD and/or IA-2-antibody). Inclusion of ten type 1 diabetes patients per group was considered sufficient for the purpose of this study, but no formal power analysis could be made because of lack of information in human disease to estimate this. A total of 48 patients were included, five having a basal C-peptide level < 0.1 *nmol/L* and six testing negative for islet autoantibodies.

Patient randomization and treatment

Patients were double-blind randomized into four groups of twelve patients, treated with either placebo or 0.2, 1.0 or 2.5 mg DiaPep277 per injection. The randomization list was generated by the computer program RANCODE (version 3.6), which allocated patients to a certain double-blind treatment. The code could only be broken by an independent overall medical advisor for safety reasons. Each patient received a total of four subcutaneous injections: at the start of the trial, and after 1, 6 and 12 months (Figure 1). Baseline characteristics are depicted in Table 1.

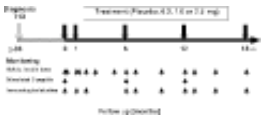


Figure 1. Scheme of DiaPep 277 administration, dosing and treatment arms [Normal View 3K | Magnified View 7K]

Table 1. Clinical and metabolic parameters of patients with type 1 diabetes mellitus for the different treatment arms at baseline. Data shown as median and interquartile range

	Placebo	0.2 mg DiaPep277™	1 mg DiaPep277™	2.5 mg DiaPep277™
Number (*n*)	12	12	12	12
Age (years)	29.8 (28-34)	32.8 (28-39)	32.1 (27-37)	28.9 (24-33)
Number (*n*) (female/male)	5/7	3/9	4/8	4/8
P277 antibodies (positive/negative)	3/9	3/9	2/10	2/10
BMI (kg/m^2)	25 (23-26)	22.5 (20-25)	22.5 (21-24)	21 (20-23)

Time from diagnosis (days)	298 (240-568)	541 (300-786)	385 (207-526)	662 (311-757)
HbA_{1c} (%)	6.6 (5.1-8.7)	5.95 (4.2-8.0)	6.15 (4.8-9.5)	5.4 (4.8-8.2)
Basal C-peptide (nmol/L)	0.30 (0.22-1.48)	0.18 (0.12-0.38)	0.23 (0.19-0.28)	0.25 (0.12-0.37)
Stimulated C-peptide (AUC) (*nmol**min/*L*)	9.17 (7.1-12.6)	4.76 (3.2-8.4)	6.62 (4.7-6.9)	6.18 (3.4-8.5)
Insulin dosage (U/kg/day)	0.43 (0.27-0.59)	0.36 (0.31-0.63)	0.50 (0.38-0.67)	0.40 (0.29-0.54)

The hsp60 peptide (DiaPep277) was manufactured and supplied by Peptor Ltd. (Rehovot, Israel). The sequence of the peptide was VLGGGVALLRVIPALDSLTPANED, the human hsp60 residues 437-460 with two cysteine-valine substitutions at locations 442 and 447 for reasons of stability [18]. The peptide was administered subcutaneously in the upper arm in a 10% lipid preparation (10% Intralipid™, Pharmacia Upjohn) suitable for human injection. Placebo treatment consisted of 40 mg mannitol in the same 10% lipid preparation.

Patient follow-up

All patients regularly visited their physician for regular medical treatment including possible adjustment of insulin dosage (Figure 1). Patients' beta-cell function was followed up by measurement of basal and stimulated C-peptide. For the stimulated C-peptide measurement, 1 mg of glucagon was administered intravenously and the area under the curve (AUC) of C-peptide production was calculated based on blood samples taken at - 5, 0, 2, 6, 10 and 20 min. Long-term glycemic control (HbA_{1c}) and general lab parameters were followed up as well as possible occurrence of adverse events. Follow-up was completed 18 months after the start of treatment. Primary clinical endpoint of the study was glucagon-stimulated C-peptide at 6 and 12 months after start of treatment; secondary endpoints were HbA_{1c} levels and daily insulin dose adjusted for body weight at 2, 6, 12, and 18 months. Production of antibodies against DiaPep277 was followed up at 0, 1, 2, 6, 7, 10, 12, 13, 15, and 18 months, using a DiaPep277-specific ELISA developed by Peptor Ltd.

Five patients, randomly distributed between the groups, were withdrawn from the study because of non-compliance. Two patients were lost to follow-up: one patient because of an adverse event (increased proteinuria and haematuria possibly related to DiaPep277 treatment) and one patient because of withdrawal of consent. A total of 41 patients completed the study: n = 10 in the 0.2 mg group, n = 12 in the 1.0 mg group, n = 10 in the 2.5 mg group, and n = 9 in the placebo group.

Statistical analysis

Treatment groups were compared at baseline using one way, non-parametric ANOVA for unpaired samples (Kruskal Wallis) to assess randomization. To compare single treatment arms over time, Wilcoxon signed rank test for paired samples was used. Changes over time in clinical parameters were compared between groups by non-parametric Mann-Whitney *U* test. Clinical endpoints were correlated using parametric Pearson's correlation test. As our study was based on an *a priori* hypothesis in its design, namely, differences in the level of beta-cell function in treatment groups *versus* placebo, we did not correct for multiple testing.

Results

Baseline comparison

The four different treatment groups were compared regarding a number of baseline parameters to assess randomization (Table 1). Several differences between the groups could be noted, e.g. C-peptide tended to be higher in the placebo group than others, while the time between diagnosis and start of the treatment tended to be shorter. However, none of the differences proved to be statistically significant.

In the course of the study, several patients were either withdrawn or lost to follow-up. Only one patient was

withdrawn because of an adverse event possibly related to therapy. This did not influence the results. The patients that were withdrawn were randomly distributed between the groups, and randomization was not violated (data not shown).

Safety

During the trial, a total number of 11 patients (26.8%) suffered from adverse events that could possibly be attributed to the trial medication (Table 2). These patients were distributed equally over the treatment groups. For only one of them this was a reason to withdraw the patient from the study. This patient, in the 0.2 mg group, was diagnosed with progressive proteinuria and haematuria. Since a relation to the trial medication could not be ruled out, the patient was withdrawn from the study after 13 months. Another patient in the 0.2 mg group became pregnant during the study and had a miscarriage that could have been remotely related to the trial medication. This participant was one among the five patients withdrawn from further studies because of non-compliance. A patient in the 2.5 mg group suffered from allergic reactions as well as a number of hypoglycaemic episodes, one of them causing hospitalization. Both events were thought to be remotely related to the trial medication. However, the patient's physician did not think these events were a reason to withdraw this patient. The other patients' adverse events included injection site dermatitis, gastrointestinal problems, fever, abnormal liver function, and hypoglycaemia (Table 2). None of them was a reason to withdraw the patient from the study.

Table 2. List of patients with adverse events possibly related to trial medication. Some patients went through more than one adverse event

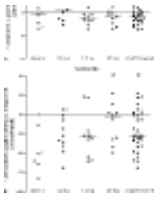
	Placebo	0.2 mg DiaPep277™	1 mg DiaPep277™	2.5 mg DiaPep277™	All patients
(*n*)	9	10	12	10	41
Injection site dermatitis	-	1	1	1	3
Fever	1	-	-	1	2
Nausea	-	1	1	-	2
Abnormal hepatic function	-	-	2	-	2
Hypoglycaemia	1	1	-	1	3
Myalgia	1	-	-	-	1
Anxiety	-	1	-	-	1
Any event	2 (22.2%)	4 (40%)	2 (16.7%)	3 (30%)	11 (26.8%)

Clinical endpoints

The primary clinical endpoint, glucagon-stimulated C-peptide production, measured by the area under the curve [22], was assessed by comparing absolute values in a single treatment arm over time (Figure 2), and by comparing those changes in the three treatment groups to the changes in the placebo group (Figure 3).

Figure 2. Glucagon-stimulated C-peptide production per treatment arm over time. Groups: A Placebo, B 0.2 mg, C 1.0 mg, and D 2.5 mg. Shown are median (bars) and individual data. Open circles depict patients negative for islet autoantibodies at the time of inclusion. * Indicates a *p*-value < 0.05, ** indicates a *p*-value < 0.01 *versus* timepoint 0
[Normal View 15K | Magnified View 41K]

Figure 3. Changes in glucagon-stimulated C-peptide production per treatment



arm over time at 6 months (A) and 12 months (B) after the start of the treatment. Shown are medians (lines) and individual data. Open circles depict patients negative for islet autoantibodies at the time of inclusion. * Indicates a *p*-value < 0.05 *versus* placebo
[Normal View 8K | Magnified View 19K]

C-peptide production significantly decreased over 12 months in all groups except the 2.5 mg DiaPep277 group (Wilcoxon matched pairs test; Figure 2(A-D)). The decrease in C-peptide production over 12 months proved to be significantly less in the 2.5 mg than in the placebo group (p = 0.03 by Mann-Whitney-U test; Figure 3(B)). Exclusion of islet antibody-negative patients from the analysis did not alter these findings (p = 0.008). Both the 0.2 mg and 1.0 mg group showed less decrease than the placebo group as well, but these differences were not statistically significant. All treated groups combined showed significantly less decrease after 12 months than the placebo group (p = 0.046, p = 0.022 excluding antibody-negative subjects; Figure 3(B)). Regarding the secondary clinical endpoints (Figure 4), the only change in absolute HbA_{1c} percentage (not adjusted for insulin dose) over time was in the 2.5 mg group (Figure 4(D)), showing a small but statistically significant increase at 18 months (p = 0.02). This change, however, did not differ from the placebo group (not shown). A trend towards a higher percentage of HbA_{1c} over time was noted in the 0.2 mg group (p = 0.08, start *vs* 18 months). The absolute daily insulin dosage did not decrease over time in any of the groups (Figure 4(E-H)). HbA_{1c} and insulin dosage were negatively correlated with stimulated C-peptide production (p = 0.006 and p = 0.02, respectively). They were positively correlated with each other (p < 0.0001).

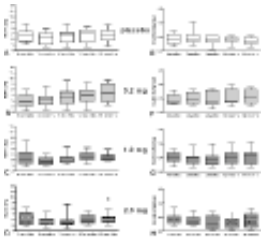


Figure 4. Secondary clinical endpoints per treatment arm over time (A-D HbA_{1c} [%], E-H insulin dosages [U/kg body weight/day]). Shown are median, interquartile range and total range. * Indicates a *p*-value < 0.05 *versus* timepoint 0
[Normal View 14K | Magnified View 37K]

Discussion

The promising pre-clinical results using DiaPep277 peptide immunotherapy in NOD mice led us to assess its clinical application [18]. The present trial was the first designed to evaluate safety as well as long-term clinical and immunological properties of DiaPep277 immunotherapy in different concentrations.

Because of logistic issues and slow recruitment of qualifying patients in Belgium, a second trial was initiated in Israel, of which the interim results became available in the course of the completion of the first trial [23]. Our preceding study differs in several ways with the reported follow-up. It consisted of more patients who were recruited over a longer period; disease duration was longer, and residual beta-cell mass consequently smaller; and it included a patient group that was treated with a higher dosage of 2.5 mg. The study designs also differed with regard to the timing of glucagon stimulation, precluding integral comparison of the corresponding results between the two studies. Because of the largely overlapping time windows of these two trials, changes to adjust trial design were impossible.

Before drawing any conclusions about the clinical data, several issues require attention. First, similar to the reported second trial, randomization of the groups was not ideal. Although baseline characteristics did not differ significantly, baseline C-peptide production tended to be higher in the placebo group compared to the other groups. The shorter duration of clinically manifest disease in the placebo group may account for the difference in C-peptide production that was found, appreciating that most type 1 diabetes patients show C-peptide production at diagnosis, that steadily decreases with time. This inconvenience could partly be taken care of during analysis by comparing both absolute values and changes in C-peptide production, percentage HbA_{1c} and daily insulin dose.

Second, the trial suffered from a considerable loss to follow-up because of withdrawal of patients. In all cases, this occurred because of appropriate reasons. Withdrawal was distributed equally between the groups, and in only one case possibly related to trial medication. Nevertheless, the percentage of patients completing the study (85%) was rather

low.

Third, owing to ascertainment and recruitment difficulties causing a delay in initiation of therapy, the period between diagnosis and start of the treatment became longer than anticipated. Furthermore, some patients did not meet all amended inclusion criteria at the moment treatment started, in particular, with regard to C-peptide production and autoantibody positivity. However, basal C-peptide < 0.1 *nmol/L* was not considered as a reason for retrospective exclusion from the study, since all patients retained minimal C-peptide production. The production of islet autoantibodies *per se* is not critically required for the diagnosis of type 1 diabetes. Therefore, patients not meeting this criterion were included for analysis, but analyses were repeated excluding seronegative subjects. Indeed, exclusion did not affect the outcome.

The side effects of DiaPep277 administration seem limited. The number of adverse events was equally distributed between placebo and treated groups. Although one patient was withdrawn from the study because of an adverse event, in none of the cases a relation between DiaPep277 treatment and the adverse event was proven. Additionally, DiaPep277 treatment did not alter unrelated antigen-specific T-cell responses or DiaPep277 antibody titers (data not shown).

Despite the potential caveats discussed earlier, the trial shows differences associated with therapy. The 2.5 mg group showed no significant loss of initial glucagon-stimulated C-peptide production, whereas all other groups experienced a significant decrease in C-peptide production at 12 months. This finding was corroborated by the observation that the difference in decrease over time was significant between the 2.5 mg and the placebo group. All DiaPep277-treated patients combined showed diminished decrease compared to the placebo group. These findings are in accordance with the short-term results in the second trial by Raz *et al.*, although efficacy was confined to different dosages [23]. The apparent C-peptide preservation in peptide-treated patients can also be interpreted as an additional measure of safety, since it implies that peptide treatment does not accelerate the disease process.

These results, however, could not be confirmed by secondary clinical endpoints. Although both parameters were correlated with C-peptide production, daily insulin dosage did not change in any of the groups at any timepoint, and HbA_{1c} even increased in the 2.5 mg group at 18 months. Nonetheless, the number of patients with well-controlled diabetes appeared higher in the two groups treated with the highest dosage of DiaPep277, which is clearly important for the clinical well-being and long-term prognosis of the patients. HbA_{1c} values, however, are partly dependent on compliance of the patient to self-administer insulin, as well as the vigilance of the treating physician. Therefore glucagon-stimulated C-peptide production remains the most reliable measure for the severity of the autoimmune beta-cell destruction, and as such for the potential beneficial effect of DiaPep277 treatment [22].

In conclusion, the results of this trial may support clinical efficacy of DiaPep277 immunotherapy for type 1 diabetes. Treatment with a total of four injections appeared safe and especially in high dosages seemed to delay the decrease in C-peptide production compared to placebo-treated patients, although this was not accompanied by reduced long-term glucose levels or insulin needs. The use of DiaPep277 in type 1 diabetes is one of the limited number of immune intervention strategies that have shown potential in a clinical setting [16][17][24][25], and might be of importance in the fight against human autoimmune beta-cell destruction in the future. The present results warrant further investigation on a larger scale.

Acknowledgements

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