Immunological efficacy of heat shock protein 60 peptide DiaPep277™ therapy in clinical type 1 diabetes

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Summary

An immunogenic peptide (p277) from the 60-kDa heat shock protein (hsp60) arrested beta-cell destruction in non-obese diabetic mice. A randomized, double-blind, phase Ib/II clinical trial of DiaPep277 peptide treatment was performed in recent-onset type 1 diabetes patients with remaining insulin production. We studied the immunological efficacy of this peptide therapy and correlated this with clinical outcome. Forty-eight C-peptide-positive patients were assigned subcutaneous injections of 0·2, 1·0 or 2·5 mg p277 ($n = 12$ per dosage) at entry, and 1, 6 and 12 months, or four placebo injections ($n = 12$). T cell autoimmunity to hsp60, DiaPep277, glutamic acid decarboxylase and tetanus toxoid (recall response control) were assayed by proliferation and cytokine secretion assays (enzyme-linked immunospot) at regular intervals until 18 months after the first injection. All treated patients at each dosage of peptide demonstrated an altered immune response to DiaPep277, while the majority of placebo-treated patients remained non-responsive to treatment ($P = 0·00001$), indicating a 100% efficacy of immunization. Cytokine production in response to therapy was dominated by interleukin (IL)-10. IL-10 production before therapy and decreasing autoantigen-specific T cell proliferation were associated with beta-cell preservation. Third-party control immune responses were unaffected by therapy. No potentially adverse immunological side effects were noted. DiaPep277 is immunogenic in type 1 diabetic subjects and has immune modulating properties. Immunological monitoring distinguished therapy from placebo treatment and could determine immunological efficacy. Declining or temporary proliferative responses to peptide DiaPep277 treatment may serve as an immunological biomarker for clinical efficacy.

Keywords: autoreactive T cell, C-peptide, DiaPep277, hsp60, Immunotherapy, intervention, type 1 diabetes

Introduction

Type 1 diabetes mellitus (T1D) is characterized by the autoimmune destruction of insulin-producing pancreatic beta-cells [1]. CD4+ T cell responses to T1D-associated antigens play a central role in the pathophysiology of the disease [1–5]. Islet autoimmunity may result from insufficient negative selection of autoreactive T cells in the thymus [6] or insufficient immune regulation [7,8]. CD4+ T cells are able to enhance autoantibody formation and CD8+ T cell cytotoxicity, as well as up-regulate macrophage-, cytokine- and natural killer-mediated killing [1]. Several T1D-associated autoantigens to which T cells can react have been

identified, among which are (pre) pro-insulin, 38 kDa, islet-associated antigen 2 (IA-2), glutamic acid decarboxylase (GAD) 65, GAD67, islet cell antibodies 69, carboxypeptidase H, fetal antigen-1, Imogen38 and heat shock protein (hsp) 60 (reviewed in [9]).

Therapy for the disease has been limited to treatment of symptoms and replacement of beta-cell function either by injecting exogenous insulin or, more recently, by transplanting donor pancreas or beta-cells. Reversal of the underlying autoimmune process by intervention therapy has shown limited success in humans [10–13]. Recently, however, it was shown that treatment of C-peptide-positive new-onset T1D patients with anti-CD3 monoclonal antibody could preserve

beta-cell function, underscoring the relevance of T cell-targeted therapy [14,15].

Immunization with an immunogenic peptide of auto-antigen hsp60, p277, was able to stop beta-cell destruction in the non-obese diabetic (NOD) mouse model [16–18]. One hypothesis to explain this finding was that the peptide could activate regulatory mechanisms and skew T cell and cytokine responses from a pro- to an anti-inflammatory state [19]. A recent study showing up-regulation of $CD4^+/CD25^+$ regulatory T cells by hsp60 or the p277 peptide through Toll-like receptor 2 signalling provides a further insight into the mechanism of action of p277 treatment [20]. The potential of this peptide immunotherapy in humans was tested subsequently in a series of clinical trials [21–23]. These trials were performed with recent-onset T1D patients in whom the beta-cell destruction process presumably may still be halted. One of these trials suggested preservation of insulin (C-peptide) production after 1 year [24]. Long-term follow-up supports this finding [23], even though this was not accompanied by an effect on percentage glycosylated haemoglobin ($HbA1_c$) or insulin dosage. No beneficial effect of treatment could be demonstrated in a subsequent trial in Hungary [22].

The first phase II clinical trial using the biologically stable form of p277 [17] focused on clinical safety and efficacy, but also tested the effects of DiaPep277 on immune modulation by assaying the antibody and T cell cytokine responses to treatment. The results of the clinical end points reported previously indicated that DiaPep277 treatment is safe and may delay loss of C-peptide production in some recently diagnosed T1D patients [21]. The present report focuses on the immunological response to DiaPep277 peptide treatment.

Subjects, materials and methods

Patients and treatment

The study design, detailed treatment protocol and features of the peptide used in this trial have been described in detail earlier [21]. Briefly, 48 recently diagnosed T1D patients were randomized double-blindly into four groups of 12 patients each, and were treated with either placebo, or 0·2, 1·0 or 2·5 mg DiaPep277 per injection. Each patient received a total of four subcutaneous injections: at the start of the trial, and after 1, 6 and 12 months. Patients' beta-cell function (basal C-peptide, stimulated C-peptide area under the curve), long-term glycaemic control ($HbA1_c$), insulin dosage and general laboratory parameters were followed, as well as possible occurrence of adverse events. Follow-up was completed 18 months after the the start of treatment. Immunological tests were performed at the start of the trial ($t = 0$) and at 1, 2, 6, 7, 10, 12, 13, 15 and 18 months.

Five patients had a basal C-peptide level less than 0·1 nmol/l and six tested negative for islet autoantibodies at the start of the study. Five patients, distributed randomly

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 (increase in pre-existing 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.

Lymphocyte proliferation test

Peripheral blood lymphocytes were isolated from heparinized blood of the patient and tested freshly as described previously [1]. Briefly, 150 000 peripheral blood leucocytes (PBLs) were cultured in round-bottomed 96-well plates (Costar, Cambridge, MA, USA) in Iscove's modified Dulbecco's medium with 2 mmol/l glutamine (Gibco, Paisley, Scotland, UK) and 10% pooled human serum in the presence of antigen, 10% T cell growth factor (Biotest, Dreieich, Germany) or medium alone in a total volume of 150 µl at 37°C, 5% CO_2. After 5 days of incubation, each culture was pulsed for 16 h with 0·5 µCi/well of [^{3}H]-thymidine. Proliferation was expressed as stimulation index (SI: median of triplicates in presence of stimulus divided by the median of triplicates with medium alone).

A new measure for T cell proliferative activity to peptide needed to be designed because of the novelty of this monitoring technology and the generally low precursor frequencies of peptide epitope-specific T cells in peripheral blood mononuclear cells (PBMCs) (usually less than 10 cells per million). Similar measures applying to peptide-specific T cell responses have been described previously [25,26]. Lymphocyte stimulation tests were performed using three concentrations of DiaPep277: 2, 10 and 50 ug/ml. Each concentration was tested in six wells, accounting for a total of 18 wells tested. A well was scored positive if its thymidine count was above medium ± 3 standard deviation (s.d.). Firstly, points were assigned for the number of positive wells: 0–1 well: 0 points, 2–6 wells: 1 point, 7–12 wells: 2 points, 13–18 wells: 3 points. Secondly, points were assigned for positivity upon testing of different concentrations of DiaPep277: 0 point was assigned if positivity was observed with one of three concentrations tested; 2 points with two of three; and 3 points when positivity was observed with all concentrations. If no positive wells were observed, no points were assigned.

Thirdly, the amplitude of the response was valued: negative wells (< medium ± 3 s.d.) were assigned 0 points for this third parameter. Positive wells (< medium ± 3 s.d.) were assigned 1 point if their median response was < 3, 2 points if it was 3 < SI < 6 and 3 points when SI > 6. By this analysis, each sample was assigned a total number of points between 0 and 9. All tests and analyses were performed blinded from therapy and clinical outcome.

Although this design leads to a conservative estimate of reactivity, it involves and weighs all features of proliferative

responses and, as such, provides an accurate and objective measure of reactivity.

Antigens

Several autoantigens were tested in the lymphocyte stimulation test. DiaPep277 was provided by Peptor Ltd (currently operating as DeveloGen Israel Ltd, Rehovot, Israel) [15] and tested at concentrations of 2, 10 and 50 µg/ml. Human hsp60 recombinant protein was provided by Peptor Ltd and tested in 2- and 10-µg/ml concentrations. The responses to insulin (25 µg/ml; Sigma Chemical Co., St Louis, MO, USA), glutamic acid carboxylase 65 (GAD65) (10 µg/ml; Diamyd, Stockholm, Sweden) [16,27] and IA-2 (10 µg/ml, provided by Dr M. Christie, London, UK) were also tested. As unrelated control, antigen tetanus toxoid (TT) [1·5 flocculation units (LF)/ml or 12·0 IU/ml, National Institute of Public Health and Environmental Protection, the Netherlands] was tested. Recombinant human interleukin (IL)-2 (35 U/ml, Cetus, Amsterdam, the Netherlands) was used as a separate positive control in every experiment.

Cytokine enzyme-linked immunospot analysis

A new measure to determine cytokine production profiles was developed, applied and subsequently validated allowed high-throughput, large scale analyses of both pro- and anti-inflammatory cytokine responses with a high degree of reproducibility and low intra- and interassay variation [28,29]. Fresh PBMCs were resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, 25 mM hepes, 100 IU/ml penicillin, 100 µg/ml streptomycin [tissue culture (TC) medium; all Life Technologies, Breda, the Netherlands] supplemented with 20% human antibody serum at a density of 8×10^6/ml and 250 µl of cell suspension dispensed into wells of a 48-well plate. Peptide or antigens in TC medium were added and plates incubated at 37°C, 5% CO_2, tilted by 5°. To additional wells, TC medium containing TC medium or phorbol myristate acetate (PMA)/ionomycin (5 ng/ml and 745 ng/ml final concentrations respectively) were used as negative and positive controls respectively. After 24 h, 0·5 ml prewarmed TC medium/10% antibody was added. After a further 24 h, non-adherent cells were resuspended gently using prewarmed TC medium/2% antibody and wells washed with a further 1 ml. After centrifugation at 200 *g*, cells were resuspended at a concentration of 1×10^6/300 µl and 100 µl dispensed in triplicate into wells of 96-well enzyme-linked immunosorbent assay plates (Nunc Maxisorp, Merck, Poole, UK) precoated with monoclonal anti-interferon (IFN)-γ, anti-IL-10, IL-5, IL-13 or anti-IL-4 capture antibody and preblocked with 1% bovine serum albumin in phosphate-buffered saline (PBS), as specified in the manufacturer's instructions (U-Cytech, Utrecht, the Netherlands). When sufficient cells were available, all five cytokines were analysed for each test stimulus, otherwise

they were analysed in the order of priority IFN-γ, IL-10, IL-13, IL-4 and IL-5. After capture at 37°C, 5% CO_2 for 7 h (or overnight in case of IL-4), cells were lysed in ice-cold water, plates washed in PBS/Tween 20 and incubated with biotinylated monoclonal anti-IFN-γ, anti-IL-10, anti-IL-5, anti-IL-13 or anti-IL-4 detector antibody overnight at 4°C. After washing, enzyme-labelled polyclonal goat anti-biotin anti-serum was added to each well for 1 h at 37°C and after further washing the reaction was developed using the activator solutions provided. Enzyme-linked immunospot (ELISPOT) plates were developed for between 5 and 10 min, with development halted after the appearance of spots in the positive control wells. Plates were dried and read in a Bio-Reader 3000 (BioSys, Karben, Germany). Typically, spots of 80–120 µm size were counted. All cytokine assays demonstrated highly reproducible detection of T cell responses to the recall antigen TT in all subjects, with intra-assay coefficients of variation between 9·3 and 12·3% on replicated analyses. Triplicate values were pooled to provide mean [standard error of the mean (s.e.m.)] values for spot numbers per well. Results are reported as mean (s.e.m.) spots per 100 000 cells, the approximate number of PBLs in the bulk starter culture from which each single ELISPOT well was derived. In the absence of any currently accepted definition of positivity, mean values in test wells were corrected for the number of spots in the absence of a stimulus at start of the monitoring ($t = 0$), to avoid the influence of T cell responses to hsp60 naturally present, mainly in the mitochondria of leucocytes prior to initiation or in the course of the peptide therapy. Indeed, in our previously reported preclinical studies we demonstrated increased baseline reactivity upon immunization with murine hsp60 that were attributed to cross-reactivity with endogenous mitochondrial hsp60 in antigen-presenting cells [30]. Consequently, the only true baseline value is that of PBMC not primed with hsp60 peptide, i.e. $t = 0$ in our trial.

Statistics

Differences between dichotomous variables were calculated by Fisher's exact test. Differences between continuous variables in two groups were calculated by non-parametric Mann–Whitney *U*-test and in three or more groups by Kruskal–Wallis test. Correlation coefficients were determined by linear regression analysis. Differences between correlation coefficients were analysed by *F*-test. All analyses were performed using GraphPad Prism software, version 4·0.

Results

Proliferative T cell responses to DiaPep277

First, a distinction was made between patients responding to the peptide prior to initiation of the trial ('spontaneous' responders, more than three points in the assessment of

Table 1. Overview of proliferative patterns to p277 between patients treated with placebo or with DiaPep277.

Prior to trial	Non-responders			Responders	
	A	B	C	D	E
Trial	No response	Increase	Temporary response	Decrease	Steady response
Placebo	5	1	0	0	6
All treated (0·2/1·0/2·5 mg)	5 (2/3/0)	6 (0/3/3)	14 (4/3/7)	7 (6/1/0)	4 (0/2/2)

The number of patients showing a change in their T cell response is significantly different between placebo- treated and peptide-treated patients (A + E *versus* B + C + D, $P = 0·0007$ by Fisher's exact test). The number of 'tolerant' *versus* 'non-tolerant' patients at the end of the study is also different (C + D *versus* B + E, $P = 0·001$).

proliferation) and patients not responding to the peptide prior to the trial. Scores per time-point per patient are available in Supplementary material, Appendix SI. In non-responding patients, three types of responses could be identified on the basis of their patterns of proliferative reactivity over time: patients remaining non-responsive, patients showing a pattern of progressive increase in T cell proliferation to the peptide and patients in whom a pattern of temporary responses was observed. In patients responding prior to the trial, two types of proliferative reactivity patterns could be identified: a decrease in proliferative response to the peptide after initiation of peptide therapy and a steady response in time (no change compared with time point zero).

Eleven of the 12 patients in the placebo group displayed unaltered proliferative responses to the peptide compared with two of 12 (0·2 mg), five of 12 (1·0 mg) and two of 12 (2·5 mg) of the peptide-treated patients. An increase in reactivity (temporary or persistent) was seen more often in the groups receiving the higher dosages of peptide (1·0 and 2·5 mg; Table 1). Overall, 27 of 36 patients receiving peptide therapy showed proliferative reactivity to the peptide at some point before or during the trial. This was significantly different from the placebo-treated patients, who manifested no change in reactivity compared with baseline ($P = 0·0007$). Decreasing or temporary proliferation in response to DiaPep277 at the end of the study was also significantly different from the placebo group ($P = 0·001$). The number of patients responding to the peptide prior to the trial was relatively high (35%).

Cytokine production in response to DiaPep277

Cytokine production profiles were determined after *in vitro* stimulation with the peptide and enumeration of lymphocytes producing IFN-γ, IL-4, IL-5, IL-10 and IL-13. In order to allow comparison between therapy groups, we determined the major cytokine produced (i.e. the highest number of spots) in each patient prior to therapy, and following each injection. Cytokine measurements and interpretations were performed blinded from therapy assignment. All cytokine values were normalized for the baseline response to medium before therapy to correct for background responses to endogenous protein without overlooking natural responses

to hsp60, possibly as a result of peptide therapy. The number of cytokine-producing cells was generally low prior to the trial, and mainly of an anti-inflammatory or T helper 2 (Th-2) nature. Individual data per patient per time-point are available in Supplementary material, Appendix SII.

Table 2 shows an overview of cytokine responses, separated into responders and non-responders, similarly to the proliferative assay. Sixteen patients (33%) responded to the peptide by proliferation prior to therapy. In the placebo group, seven of nine patients not responding prior to the trial remained non-responders during the course of the follow-up. Two placebo-treated patients developed an IL-10 response. Two of three placebo-treated patients responding to the peptide prior to therapy showed an incidental change in response.

Eighteen of 23 (78%) peptide-treated patients not responding prior to the trial developed a cytokine immune response to the peptide in the course of the follow-up. All patients already responding to the peptide altered their response compared with the baseline response. The quality of the response was dominated by IL-10 production in patients who responded before the trial and those developing a response in the course of the treatment.

Immune monitoring by cytokines distinguished peptide therapy from placebo treatment significantly: 31 of 36 (86%) treated patients displayed an altered immune response following therapy *versus* four of 12 (33%) placebo-treated control subjects ($P = 0·001$).

Combined monitoring of proliferation and cytokine production

The combined immunological monitoring of proliferation and cytokine analysis provided an even better predictive measure of therapy and immunological efficacy of treatment. Prior to therapy, 24 of 48 patients (50%) were reactive (by proliferation and/or cytokine production) to the peptide. Of the patients in the treated group, 36 of 36 (100%) were identified correctly by immunological measures, while five of 12 (42%) of the placebo-treated subjects showed evidence of an altered immune response to the peptide ($P = 0·00001$). In this combined measure, sensitivity was 100% and specificity 58%, positive predictive value 88% and negative predictive value 100%. Total predictive value was 90%.

Table 2. Cytokine responses to DiaPep277 stratified for cytokine measurements only. Dominant cytokine production is shown prior to therapy and after each injection with peptide. Cytokine measurements and interpretations were performed blindly. Each line of boxes in a column represents a single patient, e.g. seven placebo-treated displayed no cytokine response. Time-points with cytokine production above background are indicated by colour [interferon-γ = red, interleukin (IL)-4 = yellow, IL-5 = orange, IL-10 = green, IL-13 = blue]. In case of absence of measurable cytokine production (irrespective of proliferation), boxes are white.

Prior to trial	Non-responders		Responders	
Trial	No response	Response	No change	Change

(Placebo, 0·2 mg, 1·0 mg, 2·5 mg rows — colour-coded patient boxes)

T cell and cytokine responses to hsp60, GAD65 and TT

To study the influence of DiaPep277 treatment on another autoimmune response shown to be associated with the pathogenesis of T1D, we analysed the proliferative responses in PBMC to hsp60 protein, the candidate target autoantigen GAD65 as well as the recall antigen TT (vaccine, third party control; Fig. 1).

Responses to any of the antigenic stimuli were not different between peptide-treated and placebo-treated groups and between the groups with different dosages of peptide therapy, with the exception of the T cell proliferative response to GAD65, which was decreased significantly in the 0·2 mg group at 15 months compared with the placebo group ($P = 0.006$; remaining significant after correction for multiple comparisons, Fig. 1c).

Because DiaPep277 is based on hsp60, the correlation between changes in immune response to hsp60 protein with TT and GAD was studied further for placebo-treated and peptide-treated patients separately (Fig. 2). All responses after therapy were included to obtain a complete overview of immunological specificity of immunization. Because of the wide range of proliferative responses that was observed, the data required correction for baseline response (divided by proliferation at $t = 0$). Subsequently, the data were normalized by taking the natural logarithm to be able to perform linear correlation analyses on proliferative responses of a non-linear nature. Immune responses to hsp60 in placebo-treated patients did not change differentially from those to tetanus or GAD65. The correlation between tetanus, GAD65 and hsp60 responses was significant for both placebo and treated patients ($P < 0.0001$ for both groups in all three comparisons). However, between placebo-treated and peptide-treated patients a trend towards difference was observed in the slope of the correlation for TT/hsp60 ($P = 0.06$, Fig. 2a) and a significant difference for GAD65/hsp60 ($P = 0.01$, Fig. 2b). This slope was not different when correlating TT with GAD65 (Fig. 2c). This suggests that the changes in proliferative response induced by peptide therapy were specific for hsp60, while a
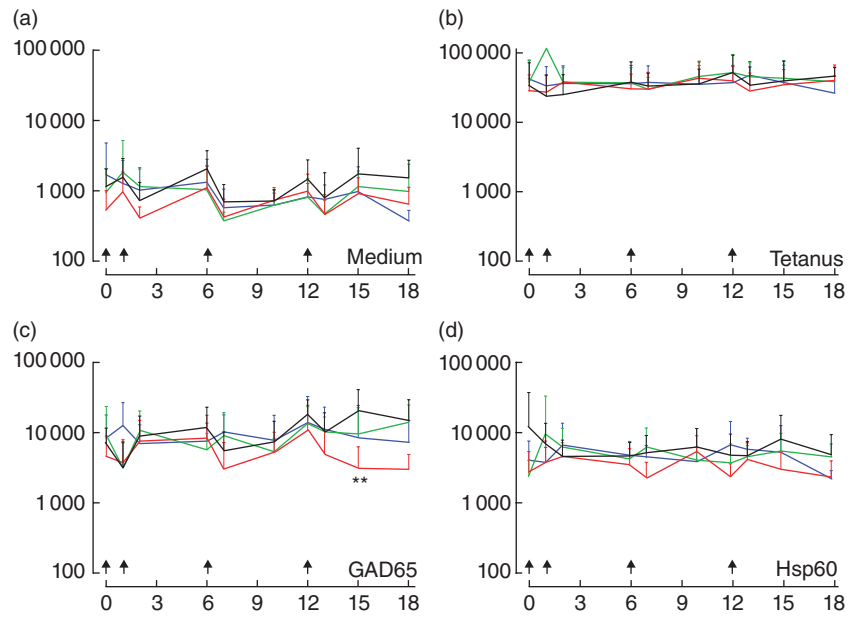
Fig. 1. Proliferative responses in peripheral blood mononuclear cells to medium (baseline response) (a), tetanus toxoid (b), glutamic acid decarboxylase 65 (c) and heat shock protein 60 (d). Arrows indicate injection time-points. Colours refer to treatment groups (black = placebo; red = 0·2 mg; green = 1·0 mg; blue = 2·5 mg). Indicated are raw counts per minute. **P-value < 0·01.

recall antigen and an unrelated islet autoantigen were less affected.

In both placebo-treated and peptide-treated patients a minority of patients reacted to hsp60 or GAD by cytokine production; this did not change significantly after treatment (see Supplementary material, Appendix SIII). Cytokine production in response to TT was seen both in placebo-treated and drug-treated patients (25% Th-1, 42% Th-2 and 19% Th-1, 9% Th-2 respectively). This did not change significantly after treatment.

Correlation between immune responses and clinical efficacy of DiaPep277 immunization

To assess the effect of peptide immunization on clinical efficacy, the change in C-peptide production over time (0–12 months) was studied by stratifying for various immunological parameters.

Patients showing proliferative responses to DiaPep277 prior to the trial (see Table 1) lost C-peptide production over time at a similar rate to patients who did not manifest such
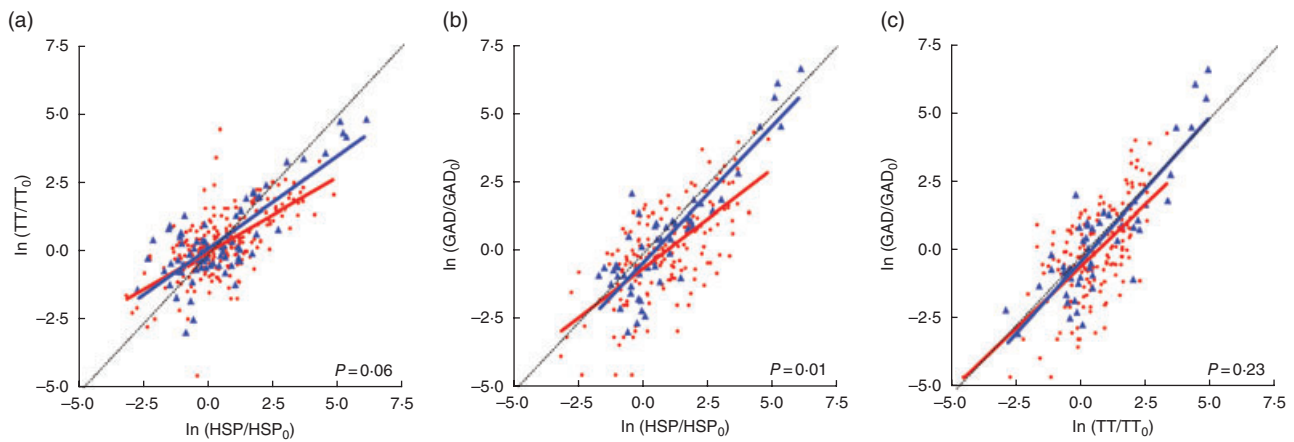

Fig. 2. Changes in immune response to heat shock protein (hsp) 60 protein, tetanus toxoid and glutamic acid decarboxylase (GAD) after treatment. Proliferative responses to antigen were calculated as fractions from the pretreatment value per patient and subsequently normalized by taking the natural logarithm (ln). Blue triangles indicate measurements of placebo-treated patients ($n = 79$), red dots of peptide-treated patients ($n = 253$). In all analyses, correlation between the measurements was highly significant ($P < 0·0001$ for both groups in graphs a–c). The P-value inside the graph assesses whether the differences in the slopes of the correlations are significant. The change in response after treatment was different between treated and placebo when comparing hsp with tetanus toxoid ($P = 0·06$) and with GAD ($P = 0·01$), indicating hsp-specific immune deviation by the peptide treatment.
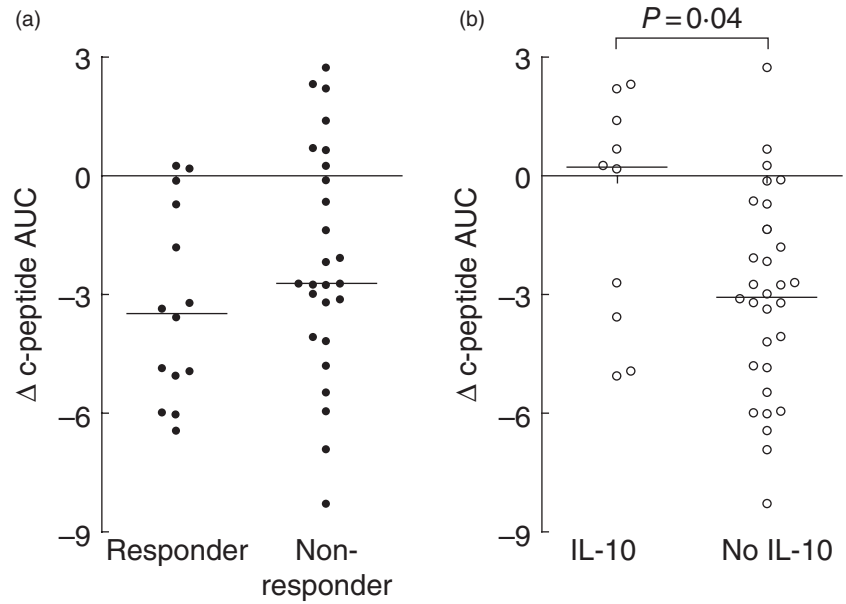
Fig. 3. Correlation between DiaPep277 immune status before peptide therapy and changes in C-peptide production afterwards. (a) Patients showing proliferation in response to peptide DiaPep277 prior to therapy ($n = 14$) showed similar loss of C-peptide production as non-responsive patients ($n = 26$). (b) Interleukin (IL)-10 response to DiaPep277 prior to therapy ($n = 10$) is associated with preservation of C-peptide compared with no IL-10 response ($n = 30$). Horizontal lines depict medians for each group.

baseline response (Fig. 3a). In contrast, IL-10 production in response to DiaPep277 prior to therapy was associated significantly with preservation of C-peptide (Fig. 3b). This correlation was even more significant in the subgroup of patients treated with p277 ($P = 0.03$).

Patients who became 'tolerant' to DiaPep277 during the study in terms of proliferation (defined by loss of immune reactivity or temporary proliferative response, Table 1) lost significantly less C-peptide than those who did not become 'tolerant' (Fig. 4, $P = 0.02$). Patients not responding to the peptide at any time ($n = 10$) were excluded from this analysis as the nature for their 'non-responsiveness' could not be determined. Cytokine production after therapy was not indicative: anti-inflammatory IL-10 production after the last injection did not correlate with changes in C-peptide produc-

tion (Fig. 4). Patients that produced IL-10 prior to treatment with declining or temporary proliferative responses to the peptide during treatment ($n = 4$) experienced better preservation of C-peptide than patients that did not express either of these two measures ($P = 0.04$, Mann–Whitney *U*-test).

In the 1·0 mg therapy subgroup, which represents the peptide dosage used in most clinical trials of DiaPep277, pre-IL-10 production was also correlated with preserved C-peptide production (median delta C-peptide 2180 *versus* −3172 in non-IL-10 patients, $P = 0.02$). Over time, four patients showed decreasing proliferation, five were 'steady responders' and three remained undetermined. 'Decreasing responders' showed a trend towards less loss of beta-cell function than did steady responders (median: −2955 *versus* −5725 ng/ml, respectively, $P = 0.2$). Patients with undeter-
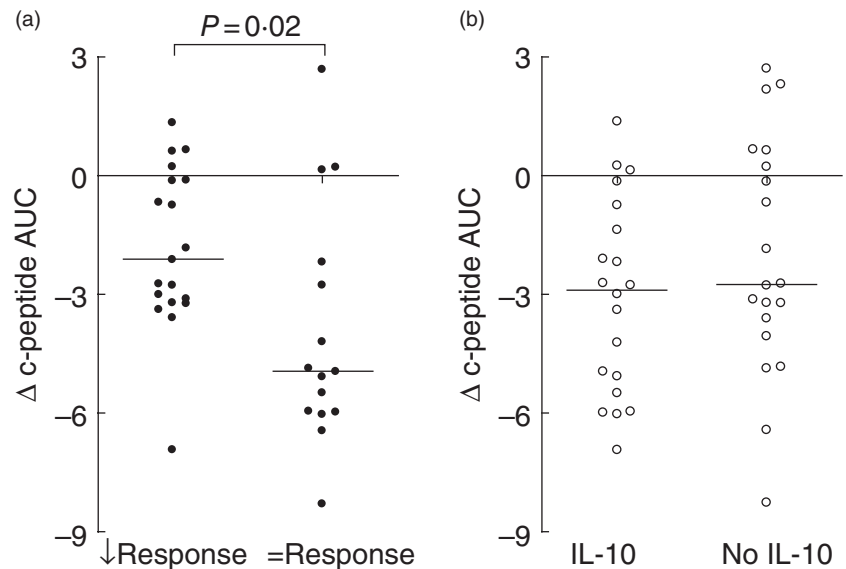

Fig. 4. Correlation between DiaPep277 immune status over time and changes in C-peptide production. (a) Patients showing modulated proliferation in the course of the study ($n = 19$) show preserved beta-cell function compared with patients who do not ($n = 15$). Six patients did not elicit proliferation at any time-point. (b) Interleukin (IL)-10 production after the last injection ($n = 21$) is not associated with C-peptide preservation when compared with no IL-10 production ($n = 19$). Horizontal lines depict medians for each group.

mined proliferation status also did better than steady responders (2180 ng/ml increase in C-peptide; $P = 0.06$).

Discussion

There is a pressing need for biomarkers associated with T1D disease progression. Perhaps even more important is the definition of immune correlates of efficacy in disease intervention studies, where distinction can be made between markers of immunological and clinical efficacy and safety. Because intervention in T1D requires immunotherapy targeting autoreactive T cells, it is conceivable that the immunological efficacy and safety of immune intervention can be monitored by studying changes in T cell autoreactivity to islets, provided that methods are available that allow sensitive, specific and reproducible measurement of immune responses relevant to disease.

The results of this study demonstrate that treatment with hsp60-based peptide DiaPep277 is immunologically safe and manifests immunological efficacy. Furthermore, the results suggest that IL-10 production in reponse to DiaPep277 before therapy and the decrease or loss of a proliferative response to DiaPep277 provide an immune correlate for clinical efficacy; these biomarkers, possibly reflecting 'tolerance' to DiaPep277, appear to be associated with improved clinical outcome. Importantly, immunological monitoring was performed without knowledge of the clinical follow-up.

When interpreting the results of this study, two observations have to be taken into account. First, the immunological responses were highly diverse among the subjects in kinetics, quality and quantity. For this reason, we had to develop novel methods of evaluation to allow interpretation of the results. Secondly, loss to follow-up and missing data in both the clinical study [21] and the immunology measurements reported here impose the need for caution. The innovative approach to immune monitoring applied here proved successful none the less in distinguishing subjects treated with the DiaPep277 peptide from those treated with placebo. This is confirmed further by the fact that both the proliferation assay and the cytokine analysis provided complementary and mutually supportive results regarding the quality and quantity of the immune response to peptides, autoantigens and control stimuli.

Both hsp60 and DiaPep277 were recognized frequently in our patient group, regardless of peptide therapy. Recognition of hsp60 protein and DiaPep277 peptide changed after peptide therapy in all peptide-treated patients, but not in the majority of placebo-treated patients. Regarding proliferative response patterns, the presence of steady increases in patients receiving a higher amount of peptide could reflect a dose escalation associated with stronger immune reactivity. A temporary response may reflect the induction of regulatory responses dampening the initiated proliferative response, or a direct induction of operational tolerance by stimulation of naive regulatory T cells. Alternatively, the loss of proliferative reactivity could reflect activation-induced cell death, anergy or exhaustion because of repeated stimulation of chronically activated autoreactive peptide-specific T cells. The lack of changes in proliferative response in the placebo-treated group implies that the reactivities defined in the course of the trial are related to the peptide therapy specifically.

Peptide treatment led to a peptide-specific anti-inflammatory cytokine profile dominated by production of the regulatory cytokine IL-10 in a majority of patients. No significant changes in cytokine response to any of the other antigens were detected in the course of the immunological follow-up. The lack of induction of Th-1 cytokines implies that treatment with this peptide does not aggravate potentially harmful islet autoreactivity, a common concern with candidate immunotherapies based on target autoantigens. Furthermore, no immunological evidence of allergic correlates (i.e. IL-5 production or eosinophilia) was observed.

Proliferative immune responses to hsp60 in placebo-treated patients did not change differently from those to tetanus or GAD, whereas changes in immune responses to hsp60 in peptide-treated patients were frequently specific for hsp60. This again suggests that these changes in proliferative response were a result of peptide therapy. The selectivity of the immune modulation can also be considered as an indication of safety; it would be undesirable to change the immune response nonspecifically. Selectivity for hsp60 was not observed with regard to cytokine profiles. This may seem discordant with proliferation, but IL-2 was not included in the cytokine analyses while proliferation is largely a measure of the production of IL-2.

We appreciate that influence of DiaPep277 reactivity on the clinical kinetics of the disease should be interpreted with caution. However, it does provide interesting information corresponding with the efficacy found in the NOD mouse model [17]. Untreated patients responding spontaneously to peptide p277 by proliferation showed a trend towards increased loss of C-peptide production. It is conceivable that those patients may be less responsive to DiaPep277 treatment because they developed T1D despite a natural response to p277. Patients showing an IL-10 immune response prior to therapy showed preservation of C-peptide production, especially in individuals treated subsequently with DiaPep277. This result implies that the status of the immune response prior to therapy may be predictive for treatment outcome, and might be considered a relevant parameter in recruitment in future clinical trials. Indeed, it also provides immunological rationale for DiaPep277 therapy. No relationship between immune responses to DiaPep277 and age (as suggested from data by Schloot *et al.* [22]) was observed (unpublished observation). It is important to note that IL-10 production prior to therapy existed in 25% of peptide-treated patients and in only 8% of placebo-treated patients. Therefore, it remains to be determined whether the a priori immune status or the peptide treatment itself was primarily

responsible for the clinical effect. Decreased proliferation to DiaPep277 also correlated with a better outcome. However, a relation between IL-10 production and clinical outcome was absent upon therapy, implying that this cytokine is not a surrogate for development of regulation or tolerance by DiaPep277 immunization. It is conceivable that IL-10 could work in concert with transforming growth factor-α or indoleamine-2, 3-deoxygenase to induce operational immune tolerance.

Despite the limitations of the present study imposed by imperfections in this trial, our data indicate that loss of an immune response to DiaPep277 can be induced by immunization and is associated with preservation of C-peptide in humans. Such a mechanism has been observed in mouse models [16,18]; indeed, this mechanism may account for induction of tolerance associated with *in vivo* efficacy [21,23].

We have reported previously on biomarkers that, in combination with genetic stratification, predicted clinical remission ('honeymoon') in T1D patients following their initiation of insulin therapy [5], indicating the possibility to define biomarkers of disease progression and remission. Our present study demonstrates, for the first time, that immunological correlates of immunological and possibly even clinical efficacy can be defined and measured in a clinical intervention trial in T1D. While there is room for improvement, such efforts may help in designing future monitoring algorithms. T cell analyses also proved valuable to test and define mechanisms of action of candidate immunesuppressive therapies *in vitro* [31,32].

In conclusion, this study clearly confirms the applicability of DiaPep277 peptide-treatment in T1D patients. Treatment is immunologically effective, specific and safe. The possible association of DiaPep277 immune status with beta-cell function corresponds with earlier findings, and accords with the rationale of this trial. We contend that immunological studies add to our understanding of the mechanism of peptide immunization, and possibly therapeutic efficacy. The limitations of the present study call for further investigation regarding clinical efficacy of DiaPep277 immunization in T1D.

Acknowledgements

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Supplementary material

The following supplementary material is available as part of the online article (full text) from: http://www.blackwell-synergy.com

Appendix SI. Individual scores of proliferation per patient. Patients are grouped per treatment.

Appendix SII. Cytokine profiles per patient after *in vitro* stimulation with the peptide by cumulative qualification and enumeration and enumeration of lymphocytes per million PBMCs producing IFNg (red), IL-4 (yellow), IL-5 (orange), IL-10 (green) and IL-13 (blue) per patient, shown for placebo (A), 0.2 mg (B), 1.0 mg (C) and 2.5 mg (D). A. Placebo, B. 0.2 mg per injection, C. 1.0 mg per injection, D. 2.5 mg per injection.

Appendix SIII. Cytokine profiles in response to hsp60, GAD and Tetanus toxoid before the start of the trial and after each injection. Shown are percentages of patients reacting to either antigen by Th1, Th1, Th2 or Treg cytokine production in placebo- ($n = 12$) and p277-treated ($n = 36$) patients.

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