For Debate

Peptide therapy for Type I diabetes: the immunological homunculus and the rationale for vaccination

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Abstract

The autoimmune process that produces Type I (insulin-dependent) diabetes mellitus seems to respond favourably to therapeutic vaccination with a peptide of the 60 kDa heat shock protein. This article is a personal review of the observations and the thinking that gave rise to the idea of therapeutic peptide vaccination. The rationale for vaccination therapy is compared to other approaches aimed at specific immunological treatment for autoimmune disease.

Keywords. Type I diabetes - autoimmune disease - vaccination - HSP60 - peptide p277 - immune modulation - hypothesis driven research - immunological homunculus

Abbreviations. *AA* Adjuvant arthritis *APL* altered peptide ligand *CFA* complete Freund's adjuvant *EAE* experimental autoimmune encephalomyelitis *GAD* glutamic acid decarboxylase *HSP60* 60 kDa mammalian heat shock protein *HSP65* 65 kDa mycobacterial heat shock protein *IFA* incomplete Freund's adjuvant

An autoimmune disease

Type I (insulin-dependent) diabetes mellitus is caused by an autoimmune process that leads to inappropriate inflammation directed at the pancreatic islets [1]. The inflammation selectively causes the functional inactivation and ultimately the death of the insulin-producing beta cells by mechanisms which are not yet clear. We have yet to understand fully many important factors in the causes and pathogenesis of the autoimmune process. Nevertheless, the fact that Type I diabetes results from an autoimmune disease tells us that beta-cell destruction can be stopped by arresting the inflammatory autoimmune process. The objective is clear but the problem is how best to achieve it. Pioneering trials using cyclosporine treatment of new-onset patients proved that the disease process could be halted by immunosuppression [2]. The autoimmune destruction of beta cells resumed, unfortunately, when the cyclosporine treatment ended [3]. Moreover, the potential toxicity of prolonged treatment with this generally immunosuppressive agent has prohibited its use in Type I diabetes.

An immunological agent for treating the diabetogenic autoimmune process should have several characteristics:

(i)
    specificity for the disease process alone, without causing general immunosuppression;
(ii)
    effectiveness without a need for high doses or chronic treatment;
(iii)
    little or no toxicity.

My colleagues and I seem to have made some progress towards fulfilling these criteria by therapeutic vaccination with a peptide, p277, from the sequence of the 60 kDa heat shock protein (HSP60) [4]. The results of this first clinical trial need to be confirmed and enlarged to ascertain the possible usefulness of the approach; the subject of therapeutic peptide vaccination is still sub judice. But the very transition from pre-clinical observations to clinical trials raises questions of general interest. Why treat Type I diabetes autoimmunity with vaccination, and why use a peptide of HSP60? 'Vaccination' implies activating the immune system, and so vaccination, at first glance, would seem to be the opposite of immunosuppression. How can activation of the immune system 'suppress' an autoimmune disease? Professional research is said to be motivated by testing a hypotheses; what is the hypothesis that drove an investigation of a heat shock protein peptide in Type I diabetes on to clinical trails? How did we arrive at the concept of vaccination therapy and how did we suspect that peptide p277 might turn out to be an effective vaccine. How does one dare go from mice to people?

Origins of peptide vaccination

The discovery of p277 and the rationale for its use in Type I diabetes is based on studies of two experimental autoimmune disease models unrelated to diabetes: experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis (AA). Avraham Ben-Nun, my graduate student at the time, and I together with our colleague Hartmut Wekerle found that T-cell clones specific for a self-antigen could cause EAE in healthy rats [5]. If clones of autoimmune T cells were indeed the causal agents of EAE, might it be possible, to attenuate such cells and use them as vaccines to specifically prevent the active induction of EAE? Could the immune system be "educated" to regulate its own autoimmune T cells similar to the way the immune system can be vaccinated to resist active infection?

(The idea of vaccination for autoimmune disease was quite foreign to the thinking current at the time among autoimmunologists; the vaccination concept reflected my initial training in infectious disease by Gene H. Stollerman [*6*]. All experience is formative [*7*]).

We found that it was possible to induce resistance to active EAE by injecting rats with activated autoimmune T cells specific to the target antigen myelin basic protein; the vaccine T cells were attenuated by irradiation [*8*] or other means [*9*] to prevent them from adoptively transferring EAE. We coined the term T-cell vaccination to describe this type of immune cell treatment [*8*]. The T-cell approach to EAE has served as a model for studying other autoimmune diseases.

Joseph Holoshitz, then a Fellow in the laboratory, raised a T-cell clone (A2b) from rats induced to develop AA by immunization with complete Freund's adjuvant (CFA) [*10*]. The A2b clone was arthritogenic in irradiated rats and served as a probe to study AA. Willem van Eden, then a post-doctoral Fellow, discovered that clone A2b recognised an epitope that was cross-reactive with cartilage proteoglycan and an antigen of M. tuberculosis present in CFA [*11*]. The Mycobacterial antigen recognized by A2b was later found to be the 65 kDa heat shock protein (HSP65), and we succeeded to identify a peptide from the HSP65 sequence bearing the target epitope [*12*]. Moreover, the HSP65 molecule itself or its peptide epitope could be used as a vaccine to inhibit AA [*12*, *13*]. Thus, we found that effective vaccination therapy for autoimmune disease might include target peptides as well as attenuated T cells. The AA study also provided us unknowingly with HSP65 antigen for later use in diabetes.

The immunological homunculus concept of autoimmunity

The immunological homunculus theory of autoimmunity summarizes the ideas we used to develop peptide vaccination in Type I diabetes. The homunculus is part of the argument.

The term immunological homunculus [*14*, *15*, *16*] refers both to a set of empirical observations and to set of theoretical conclusions derived from the following observations.

Empirical observations:

(i)
    Healthy immune systems contain a detectable background of autoimmune T cells and autoantibodies reactive with the auto-antigens targeted in the prevalent autoimmune diseases.
(ii)
    Healthy immune systems and the immune systems of animals recovered from experimental autoimmune diseases feature several mechanisms that can contain and regulate the autoimmune repertoire, such as anti-idiotypic [*17*] T cells and B cells; anti-ergotypic T cells [*18*]; CD25 regulatory T cells [*19*]; and autoimmune T cells with anti-inflammatory (Th2/3) phenotypes [*20*].

Explanatory hypotheses:

(i)
    These regulatory mechanisms function naturally to prevent autoimmune disease [*14*, *16*].
(ii)
    Autoimmune diseases arise from the failure of natural regulatory mechanisms to prevent or contain the autoimmune responses to injury or infection that arise naturally [*14*, *16*].
(iii)
    The natural treatment of autoimmune disease is to activate the regulatory mechanisms, not to suppress the autoimmune clones: specific vaccination, not immunosuppression [*21*]. The

vaccination will activate the immune system to control its own renegade autoimmune effector clones.

This last hypothesis is the rationale for vaccination therapy of autoimmune disease.

HSP60 and Type I diabetes

Our pre-clinical studies of Type I diabetes actually began with an investigation of the effect of immune response genes on the T-cell response to the insulin molecule [22]. Ruth Maron and Dana Elias, who was doing her PhD thesis jointly with Yoram Shechter and myself at the time, showed that antibodies to the insulin receptor could arise spontaneously in the course of autoimmunization to insulin [23]. Moreover, these anti-receptor antibodies could cause desensitisation of the insulin receptor and functional insulin resistance [24]. We detected such anti-receptor antibodies in humans with Type I diabetes [25] and in BB rats [26]. The question was whether NOD mice might also develop such antibodies in the course of their autoimmune insulitis.

To test this question, Dana Elias bled individual NOD mice at regular intervals from 1 month of age until the appearance several months later of hyperglycemia, a clinical sign of Type I diabetes [27]. Since not all NOD mice actually develop diabetes, it was possible to test whether there might be a difference in anti-receptor antibodies between mice that did or did not finally develop the disease.

The NOD study took place while we continued to investigate HSP65 as a target antigen in AA. It so happened that I attended a conference on autoimmune disease where my presentation of HSP65 (a 65 kDa antigen) in AA was preceded by a report of a then unknown 64 kDa antigen targeted by autoantibodies in Type I diabetic patients [28]. Might the unknown 64 kDa antigen in Type I diabetes and the 65 kDa antigen in AA be the same molecule? In fact, a person who had heard both presentations left an unsigned note on my chair at the meeting asking me that very question. Upon my return to the laboratory, it was a simple matter to include HSP65 as a "control" antigen in our study of the autoantibodies to insulin and to the insulin receptor in the NOD mouse sera. We found that the pre-diabetic NOD mice actually made large amounts of antibodies to HSP65 as they developed insulitis. The anti-HSP65 antibodies declined spontaneously after the onset of clinical diabetes in the mice [27]. In contrast, the NOD mice that did not develop diabetes did not produce increased amounts of anti-HSP65 antibodies. We then found that HSP65 vaccination could induce or prevent the development of diabetes depending on the adjuvant context: HSP65 administered in saline prevented diabetes and HSP65 in CFA induced accelerated diabetes [27].

While we were working with HSP65, however, it was reported that the 64 kDa antigen of Type I diabetes patients was glutamic acid decarboxylase (GAD) [29] and therefore the 64 kDa antigen, GAD was clearly not HSP65. But the discovery of GAD only made HSP65 all the more intriguing.

We approached the question of HSP65 in diabetes, as we had learned from our earlier studies of EAE and AA, by isolating anti-HSP65 T cells from NOD mice with autoimmune insulitis. Some of these T-cell clones could cause diabetes in recipient mice, so it was reasonable to suppose they saw a target antigen in the mice. Since mice express mammalian HSP60 and not Mycobacterial HSP65, we tested whether the T-cell clones might respond to human HSP60. Indeed, we found that HSP60 was the true target of autoimmunity in the mice [30]; mycobacterial HSP65 was only mildly cross-reactive with human HSP60 (human and mouse HSP60 are about 97% identical).

We further probed the HSP60 molecule with our T-cell clones and identified a 24-amino acid peptide containing a major T-cell epitope: amino acids 437-460 in the sequence of human HSP60. We named this peptide p277 according to its order number at the peptide synthesis unit. Subcutaneous injection of

100 μg of p277 in incomplete Freund's adjuvant (IFA, mineral oil without Mycobacteria) to NOD mice inhibited the development of spontaneous diabetes [30]. Now we had a peptide vaccine for NOD mice. The questions were how effective a vaccine was p277 and how did p277 actually arrest the autoimmune process.

Peptide p277 in NOD diabetes

Experiments using native p277 were hampered because the peptide was unstable; it tended both to aggregate and to break apart. Professor Mati Fridkin of the Department of Organic Chemistry suggested that the stability problems with p277 might be solved by substituting the two Cystein residues of the native sequence with other amino acids. We found that we could stabilize the peptide by replacing the Cysteins with Serines or Alanines, but these substituted peptides were not recognized by the anti-p277 T-cell clones, and we found that these substituted peptides failed to arrest the progression of diabetes. Fridkin then suggested that we use Valines in place of the Cysteins and that solved the problem. Valine-substituted p277 was completely cross-reactive with the native sequence both to T cell clones and monoclonal antibodies. Most importantly, the Valine-substituted p277 peptide was as effective in arresting NOD diabetes as was the native peptide [31]. Subsequent studies were carried out using the Valine-substituted p277 variant. Human HSP60 and p277 were cross-reactive with mouse HSP60 and its p277 peptide that differed by one amino acid [32].

Once the peptide was stabilized, we tested its effectiveness in treating NOD mice with advanced insulitis. A variety of antigenically non-specific treatments given to very young NOD mice, 4 to 6 weeks of age, had been shown to abort the later development of diabetes [33]. The challenge was whether late treatment with p277 could stop fully developed insulitis. We found that a single subcutaneous injection of p277 in IFA was effective when given just before the onset of clinical hyperglycemia (12 weeks of age), when half the mice were already diabetic (15 weeks of age), and even when two-thirds of the mice were diabetic (17 weeks of age) [31]. Peptide p277 was effective in arresting beta-cell destruction even late in the autoimmune disease process.

Peptide p277 in toxin-induced Type I diabetes

We also found p277 vaccination to be effective in a toxin-induced model of Type I diabetes. A sufficiently large dose of the toxin streptozotocin quickly kills beta cells, and so causes acute, toxic diabetes in most strains of mice. However, certain strains of mice respond to low doses of streptozotocin that cause sub-clinical damage by later developing a form of chronic autoimmune insulitis and diabetes [34]. We found that very low doses of streptozotocin induced Type I diabetes in C57BL/ksJ mice, and the process was accompanied by the development of autoimmunity to HSP60 and to its p277 epitope [35]. Moreover, p277 (100 μg in IFA) administered after the toxic insult could abort this toxin-induced Type I diabetes [36]. In contrast to the NOD mice that responded to a single dose of p277, the toxin-induced autoimmune disease required several doses of p277 in IFA. Thus, peptide p277, albeit at different dose schedules, was effective in stopping the progress of autoimmune diabetes induced by a toxin, as well as that developing spontaneously in NOD mice.

Mechanism of action

We studied the effect of p277 treatment on the immunology of Type I diabetes in two anatomical sites in the body: the spleen and the islets. The splenic T cells of NOD mice treated with p277 in IFA showed four features of interest [37]:

(i)

down-regulation of the Th1 cytokines IFNγ and IL-2, and up-regulation of the Th2 cytokines

IL-4 and IL-10 in the T-cell response to HSP60 and peptide p277;

(ii)

down-regulation of the T-cell proliferative responses to HSP60, p277, and GAD peptides;

(iii)

down-regulation of Th1-like IgG isotype antibodies to HSP60, GAD, and insulin; and

(iv)

no modification of a spontaneous Th1 cytokine response to a Mycobacterial HSP65 peptide.

Thus, the splenic T cells manifested a selective Th2 shift in the phenotype of their autoimmune response to the diabetes-associated antigens GAD, insulin and HSP60, while they maintained a Th1 response to a foreign bacterial antigen. The shift to Th2 autoimmunity regressed spontaneously after a month or two, the Th1 responses did not reappear, and the mice returned to a "pre-diabetic" immune state.

The autoimmunity responsible for Type I diabetes, however, takes place mainly in the islets, not in the spleen. Vitaly Ablamunits adapted a quantitative Elispot assay to measure various cytokines produced by T cells infiltrating the islets. The islet T-cell cytokine response was activated by mitogenic anti-CD3 stimulation, so the assay was not biased to any particular antigen. The islet-infiltrating T cells of the p277-treated mice showed marked down-regulation of INFγ production [38]. The

numbers of infiltrating leukocytes per islet generally decreased as did the degree of insulitis. Indeed, the T cells recovered from the islets of the p277-treated mice were less able to transfer diabetes into NOD recipient mice [39]; these T cells were less pathogenic than the islet-infiltrating T cells of mice treated with control peptide. Thus, peptide vaccination with p277 acted as a specific immunomodulator of the diabetogenic autoimmune process. Ohad Birk and Daniel Altmann developed transgenic mice hyper-expressing mouse HSP60, and these mice were resistant to diabetes [40]; even endogenous HSP60 could serve as an immunomodulator.

Peptide p277 in human Type I diabetes

To investigate whether p277 might be a suitable treatment for humans with Type I diabetes, my then student Boris Reizis characterized the peptide binding motifs of the MHC class II diabetes-susceptibility genes of the NOD mouse (IA^{g7}) [41] and the human (DQ8) [42], and found that both molecules expressed similar binding motifs and biochemical stability [42, 43]. Dr. Rivka Abulafia and Professor Itamar Raz of Hadassah University Hospital collaborated with us to show that Type I diabetes patients do make significant T-cell responses to HSP60 and to p277 [44]. These findings paved the way for clinical trials of p277 vaccination therapy in humans. It was only necessary to find an adjuvant suitable for human use to replace the IFA used as adjuvant in the mouse studies.

Professor Meir Shinitzky of the Department of Bio-physics collaborated with us in finding a metabolizable vegetable oil vehicle for the p277 vaccine. This approved vehicle is now used clinically; peptide p277 in the vegetable oil with a mannitol filler is called DiaPep277 [4]. DiaPep277 is being developed for clinical use by Peptor, Ltd. A phase 1 trail showed no adverse side effects in volunteers with longstanding Type I diabetes and with no detectable C-peptide. Six phase-2 trials were then initiated in early onset Type I diabetes, from which the first publication has emerged [4].

Comparative immuno-therapeutics

A comparison with other clinical immune therapies highlights the distinctive features of our use of peptide therapy. The effectiveness of p277 in arresting the destruction of residual beta cells in NOD mice with overt diabetes indicated that we could aim the clinical trials to persons who were already clinically diabetic. This decision freed us from the ethical dilemma of treating healthy "pre-diabetic subjects", not all of whom would be certain to develop clinical diabetes. By treating overt diabetics patients, we also avoided the problems prevention trials have faced in recruiting clinically healthy subjects.

A clinical trial of oral insulin in new-onset diabetic patients has failed [45], although oral insulin was shown to be effective in the NOD mouse [46]. A major difficulty in immuno-therapy by "oral tolerance" is that people differ in their absorption of antigens through the gut as well as in their genes, so that optimum doses and dose schedules probably differ between people. In contrast to oral administration, the injection of p277 through the subcutaneous route insured that each subject received the intended amount of active peptide.

Altered peptide ligands (APL) are a form of immuno-therapy based on the idea that autoimmune diseases are caused by the accidental emergence of autoimmune clones [47]. A main stream of thought is that the best way to stop the disease is to get rid of the autoimmune clones by killing them, by blocking their recognition of self-antigen, or by shutting them off. APL can be said to "trick" the autoimmune T-cell clones into shutting themselves off. It has been observed that peptide epitopes that bind to T-cell receptors with less affinity than the native peptide will "inactivate" or "energize" the peptide-specific T cells [47]. To hit every autoimmune T cell, present and future, with the APL inactivator requires the chronic administration of relatively large amounts of APL peptide.

The homunculus concept, in contrast to the classic clonal selection view of autoimmunity [15, 16], proposes that the autoimmune clones are built into the system naturally; the disease arises not from their appearance but from their faulty regulation (i.e. vaccinate, therefore, to reactivate regulation). The direct targets of vaccination are the regulatory cells, not the autoimmune clones. Vaccination therapy is driven by a hypothesis that differs from the hypothesis for APL therapy. In contrast to the APL approach, peptide vaccination is based on short exposures to limited amounts of peptide in a suitable adjuvant.

The immunological homunculus concept proposes that natural regulation recognizes immunologically unaltered self-epitopes. So it is important to note that Valine-substituted p277 does not function as an APL; valine-modified p277 is indistinct from the native p277 peptide at the level of the T-cell antigen receptor [31]. Valine-substituted p277 is merely stabilized chemically. The natural regulation we hope to activate by vaccination arises physiologically, I believe, in response to unaltered self-epitopes, and not to immunologically altered self-epitopes - APL. APL could be seen by T-cell clones as different from natural self-antigens. Indeed, trials of APL therapy in multiple sclerosis had to be stopped because of allergic reactions [48] and aggravated disease [49], all leading to reconsideration of the approach [50].

Of course, it is conceivable that suitable APL, administered not chronically but in a suitable adjuvant, could turn out to activate natural regulatory mechanisms [50]. Obviously then, the APL would become a vaccine. Adjusting the hypotheses, however, is not rare in biology and medicine; many useful therapeutic agents, such as aspirin, have shifted the rationale behind their use as we have learned more about how they work. In such a case, the hypothesis is not the driver of the research; the hypothesis is driven by the research. Although the homunculus theory drove our vaccination strategy, there was no hypothesis behind the research that linked HSP60 and p277 to the treatment of Type I diabetes. The

research was driven, as I have described here, by curiosity.

New questions

The effects of HSP60 and peptide p277 in Type I diabetes raise practical and theoretical questions. Curiosity brought us to p277 therapy, but fundamentally we still do not understand how the phenotype of autoimmunity to an HSP60 epitope is connected to beta-cell health or disease. Why should a chaperon molecule that is expressible in every cell in the body be an antigen in Type I diabetes, an autoimmune disease that is supposed to be organ-specific? How is it possible for p277 vaccination to cross-modulate autoimmunity to GAD, insulin and other antigens in the collective of autoimmunity associated with Type I diabetes? How is it that bacterial immunity is left intact? How does the set of homuncular self-antigens avoid the negative selection that is proposed to purge the immune system of autoimmune clones? How does homuncular regulation get organized selectively? Peptide p277 treatment leads to a relatively specific cytokine shift in the autoimmune process; but how does the p277 peptide activate this effect at the molecular level? The recent findings that the HSP60 molecule triggers the innate immune receptor toll-like receptor-4 [51] and that stimulation of the innate immune system by bacterial CpG can prevent NOD diabetes [52] suggest that the innate immune system might be involved in the specific immuno-modulatory effects of p277.

At the applied level, we have yet to determine the optimal dose and dose schedule of p277 peptide treatment. Which subjects are most likely to respond to treatment? How do we best monitor a patient's response? Are booster vaccinations needed to maintain the therapeutic effect? Adverse effects of treatment have not yet been seen but our experience is still limited to early observations in only a few hundreds of human subjects presently enrolled in ongoing trials. Trials in LADA patients are underway and prevention trials are being considered. The outcome will indicate whether the peptide vaccine approach might be applicable to other peptides in other autoimmune diseases. There is much to be tested and learned. The effectiveness of vaccination in autoimmune diseases drives new hypotheses about self-recognition and the natural regulation of autoimmunity [53].

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