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99th Dahlem Conference on Infection, Inflammation and Chronic Inflammatory Disorders: Immune therapies of type 1 diabetes: new opportunities based on the hygiene hypothesis

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Summary

Insulin-dependent (type 1) diabetes is a prototypic organ-specific autoimmune disease resulting from the selective destruction of insulin-secreting β cells within pancreatic islets of Langerhans by an immune-mediated inflammation involving autoreactive CD4+ and CD8+ T lymphocytes which infiltrate pancreatic islets. Current treatment is substitutive, i.e. chronic use of exogenous insulin which, in spite of significant advances, is still associated with major constraints (multiple daily injections, risks of hypoglycaemia) and lack of effectiveness over the long term in preventing severe degenerative complications. Finding a cure for autoimmune diabetes by establishing effective immune-based therapies is a real medical health challenge, as the disease incidence increases steadily in industrialized countries. As the disease affects mainly children and young adults, any candidate immune therapy must therefore be safe and avoid a sustained depression of immune responses with the attendant problems of recurrent infection and drug toxicity. Thus, inducing or restoring immune tolerance to target autoantigens, controlling the pathogenic response while preserving the host reactivity to exogenous/unrelated antigens, appears to be the ideal approach. Our objective is to review the major progress accomplished over the last 20 years towards that aim. In addition, we would like to present another interesting possibility to access new preventive strategies based on the 'hygiene hypothesis', which proposes a causal link between the increasing incidence of autoimmune diseases, including diabetes, and the decrease of the infectious burden. The underlying rationale is to identify microbial-derived compounds mediating the protective activity of infections which could be developed therapeutically.

Keywords: anti-CD3, autoimmunity, hygiene hypothesis, tolerance induction, type 1 diabetes

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Introduction

Identifying insulin-dependent or type 1 diabetes (T1D) as a polygenic autoimmune inflammatory disease is a relatively recent finding which occurred by the end of the 1970s. The academic diabetes community reacted rapidly to this important discovery, concentrating efforts to approach, first, the major issue of the early diagnosis of the immunological disease and secondly, to devise immune-based therapeutic strategies to delay and/or prevent disease progression. Compared to other autoimmune diseases, approaching the pathophysiology of T1D was problematic because of the difficulties in having direct access to the target organ in patients. However, on the positive side, spontaneous experimental models of the disease became available: the biobreeding (BB) rat and the non-obese diabetic (NOD) mouse which, despite their obvious limitations, helped significantly to dissect the various stages of disease progression [1]. It appeared clearly from these models that the abnormal metabolic control, as assessed by hyperglycaemia and glycosuria, the hallmarks of T1D clinical diagnosis, was preceded by a long phase defined as 'prediabetes' during which the β cell autoantigen-specific inflammatory response developed silently, yet progressively. Thus, in NOD mice progressive

infiltration of the islets of Langerhans by mononuclear cells, also termed insulitis, evolves in two distinct phases [1]. Insulitis appears by 3–4 weeks of age and up to 8–10 weeks is confined to the periphery of the islets (peri-insulitis) without any sign of active destruction of insulin-secreting β cells. As disease progresses, by 10–14 weeks of age the infiltrating cells invade the islets quite abruptly, i.e. aggressive insulitis, and rapid β cell destruction occurs causing overt hyperglycaemia.

The orchestrated mechanisms leading to β cell destruction all represent potential targets for therapeutic intervention. These mechanisms involve a central triad constituted by β cells, autoantigen-presenting cells and T lymphocytes. Autoantigen-presenting cells are heterogeneous and include dendritic cells (DCs), macrophages and B lymphocytes. The observation that B cell-deficient NOD mice are disease free indicates that disease development is B cell-dependent [2]. In addition to their antigen-presenting role, macrophages and DCs are also key inflammatory effector cells. T lymphocytes involved in T1D are functionally heterogeneous, comprising pathogenic T cells and specialized subsets of regulatory T cells. β cell destruction involves pathogenic T cells, as demonstrated by the capacity of 'diabetogenic' $CD4^+$ and $CD8^+$ lymphocytes from the spleen of diabetic NOD mice to transfer disease into syngeneic immune-compromised recipients [NOD neonates, irradiated adult NOD mice, NOD severe combined immunodeficiency (SCID) mice] [3]. In parallel, there is evidence to show that disease progression is controlled by T cell-mediated immune regulatory circuits involving distinct subsets of regulatory T cells [4,5]. It is also important to stress that β cells must not be viewed simply as 'passive' targets that are killed immediately by the immune-mediated insult. In a first step they 'suffer' from the inflammatory environment created by the insulitis that, in a partially reversible fashion, inhibits their capacity to secrete insulin but also provides all the premises for establishing 'cross-talk' between the β cell and the immune cells and cytokines from the environment [6]. It is only in a second step that the β cell is eventually destroyed through apoptosis.

The hygiene hypothesis and autoimmune diabetes

During recent years the epidemiology of T1D has become alarming. The European continent is among the most affected parts of the world, with four of the five countries showing the highest figures, namely Finland, Sweden, Norway, the United Kingdom and Canada [7]. In addition, the disease is affecting younger children; two recent reports from a Finish and a European cohort fully support these preoccupying conclusions [8,9].

This trend is not only valid for autoimmune diabetes. In fact, over the past three decades, in industrialized countries the prevalence of allergic and autoimmune diseases has increased tremendously [10]. Over the same period of time there has been an obvious decrease in these countries of the incidence of many infections due to the improvement of hygiene standards and of medical care (use of antibiotics, vaccination campaigns and better socio-economic conditions). In northern European countries, in particular, rheumatic fever and hepatitis A are good examples to illustrate this tendency. Intestinal infections are another interesting example; their frequency has decreased significantly in developed countries, especially in young children, and it has been proved that there are major quantitative and qualitative differences in the intestinal flora in developed countries *versus* less-developed environments; i.e. colonization with Gram-negative bacteria occurs later. Major parasitic infections such as plasmodia or schistosoma are mostly non-existent in developed countries, and even infestation with minor parasites such as *Enterobius vermicularis* (pinworms) has decreased significantly over the last 10–20 years [11].

The working hypothesis proposing a causal link between the increasing incidence of allergic diseases and the decrease of infections was referred to as the 'hygiene hypothesis', coined by Strachan in 1989 [12], and has been extended to autoimmune diseases [10]. As formulated in its original inception, the hypothesis predicts that increased hygienic living conditions, the use of antibiotics and sterile food preparation will result in the continued segregation of the immune system from positive microbial exposure, thus favouring an increased susceptibility to immune-mediated disorders.

The best direct evidence in support of the hygiene hypothesis has been collected from experimental animal models. In susceptible strains of mice or rats, spontaneous autoimmune diseases develop faster and with a higher incidence in animals bred in a specific pathogen-free environment compared to those bred in conventional facilities. This is true in NOD mice and in BB rats and in rats with collagen or adjuvant-induced arthritis [10]. Disease is prevented in NOD mice by infecting the young mice with bacteria, viruses or parasites (i.e. mycobacteria, lymphocytic choriomeningitis virus, murine hepatitis virus, lactate dehydrogenase virus, schistosoma, filariae) [10]. Similarly, infection of lupus-prone New Zealand black (NZB) mice or NZB–New Zealand white (NZB–NZW) F_1 hybrid mice with lactate dehydrogenase virus or *Plasmodium berghei* prevents disease very effectively [10].

As a whole, based on epidemiological and experimental data there is now widespread recognition of the effect of infections on susceptibility to both allergic and autoimmune diseases. Such protective effect of infectious agents against immune-mediated diseases has clear public health and clinical implications: if one could characterize efficiently the microbial compounds that are responsible for the protective activity, these could be used therapeutically to prevent autoimmune and allergic diseases. There are, however, two major but not mutually exclusive problems: first, better characterization of the key microbial compounds and secondly,

fine dissection of the cellular and molecular mechanisms mediating the protection.

Lessons from immune intervention trials in recently diagnosed autoimmune diabetes: from immunosuppression to operational tolerance

The identification of T1D as an immune-mediated disease led rapidly to immune intervention approaches. As a high priority, the academic diabetes community considered conducting well-designed innovative randomized trials, mainly placebo-controlled, the rationale of which was the direct continuation of preclinical data derived from animal studies. The balance today is that major proofs of concept emerged from three major immune intervention approaches.

A first approach, begun in the mid-1980s, was that of generalized immunosuppression trials, the most extensive ones using cyclosporin [13,14]. Results demonstrated for the first time that a T cell-directed immune intervention could reverse established hyperglycaemia, challenging the prevailing dogma at that time that too many β cells have been destroyed at this stage of the disease to allow any chance for metabolic reconstitution. Both experimental and clinical data have accumulated since, indicating that at diabetes onset a good proportion of potentially functional β cells are still present, although they are impaired severely in their insulin-secreting capacity due to the effect of the immune-mediated inflammation. This explains the temporary improvement seen after beginning insulin treatment, and provides a rationale for the use of therapies that remove or inhibit aggressive islet-infiltrating cells. In spite of the significant rate of disease remission observed in cyclosporin-treated patients, disease relapse was observed invariably upon drug withdrawal, implying that indefinite administration would be necessary, which was unrealistic for safety reasons (i.e. nephrotoxicity and overimmunosuppression).

More recently, the use of a depleting CD20 monoclonal antibody (rituximab) was extended from other organ-specific autoimmune diseases such as multiple sclerosis [15] to T1D [16]. The reasoning was based on the evidence that B lymphocytes play a key role not only in autoantibody production but also in autoantigen presentation. In addition, encouraging data were reported in experimental models [17,18]. Results showed an improvement in stimulated C-peptide values shortly after the course of rituximab; values then declined progressively. The problem is to balance this efficacy with the massive B lymphocyte depletion induced by the treatment. Peripheral B cell counts ranged between approximately 10% and 69% of baseline levels at 6 and 12 months, respectively.

The second strategy, developed mainly over the past decade, consisted of more ambitious forms of immune therapy not aiming at immunosuppression but at inducing/restoring self-tolerance to well-defined β cell antigens. The rationale was based on the well-established notion that antigen delivery depends upon the molecular form of the antigen and its route of inoculation, and may lead either to effective immunization or to immune tolerance. This concept stemmed from pioneering experiments performed by D. W. Dresser in the early 1960s, showing that heterologous immunoglobulins that are immunogenic if administered in aggregated form induce specific unresponsiveness/immune tolerance, or 'immune paralysis', if injected intravenously (i.v.) in non-aggregated form [19]. Thus it made sense to use well-defined autoantigens as therapeutic tools to attempt inducing/restoring self-tolerance in T1D. As in many other autoimmune diseases, in T1D various candidate autoantigens have been incriminated as potential triggers and targets of the disease. These include the main β cell hormone proinsulin/insulin itself, glutamic acid decarboxylase (GAD), a β cell-specific protein phosphatase IA-2, a peptide (p277) of heat shock protein 60 (hsp60), the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a preferential target of pathogenic $CD8^+$ T cells, and the most recently characterized zinc transporter ZnT8. Targeting some of these antigens has proved successful in NOD mice, as disease was effectively prevented by administration of protein or specific peptide antigens such as pro-insulin, insulin, GAD, the p277 peptide of hsp60 using various routes [i.v., subcutaneous (s.c.), oral, intrathymic, intranasal] [20].

Although highly effective in the experimental setting, the transfer to the clinic of β cell autoantigen-induced strategies was beset by a number of difficulties. Antigens used in patients included insulin or altered insulin peptides, GAD65 and the hsp60 p277 peptide (DiaPep277). Most applications have been via administration of the antigen or peptide alone, and one approach has included the administration of antigen plus adjuvant. Insulin has been the main antigen used clinically. It was readily available for clinical use; experiments in animal models consistently showed effects in preventing diabetes; and several evidences suggested that insulin could be a primary autoantigen in T1D. Insulin has been used as an immunotherapy via s.c., i.v., oral and intranasal routes. Two trials performed after diabetes onset in approximately 100 patients have tested the use of oral insulin at a limited dose range without observing efficacy [21,22]. In addition to these, a Phase I/II and subsequently a larger Phase II efficacy clinical trial were performed in recently diagnosed patients using NBI-6024, an altered peptide ligand (APL) of the 9–23 insulin B chain peptide. The data were reported recently, describing no effect [23]. In 2001 a randomized, double-blind, Phase II study tested the therapeutic potential of DiaPep277 [24]. Initial results appeared encouraging, but were not confirmed in subsequent studies.

Antigen treatment alone has also been tested in prediabetes. The Diabetes Prevention Trial (DPT)-1 study studied the ability of i.v. plus s.c. insulin or oral insulin therapy to prevent or delay diabetes onset in insulin autoantibody-positive individuals with relatively late pre-

clinical diabetes [25]. No delay of diabetes was observed in the i.v. plus s.c. trial. The same was true for the oral insulin trial although, in a hypothesis-generating analysis of a subgroup presenting high levels of anti-insulin autoantibodies (> or = 80 nU/ml), some suggestion of benefit was reported. A new trial is ongoing to test the hypothesis. Intranasal insulin has also been used as an immunotherapy to prevent T1D in islet autoantibody-positive children and adults: recently a large study in Finland reported no effect in delaying diabetes onset using daily intranasal administration of insulin at a single dose [26]. Another trial using the same strategy is ongoing in Australia. Finally, an ongoing trial (Pre-POINT) is testing oral and intranasal insulin vaccination as a primary therapy in islet autoantibody-negative children, and more recently the effect of antigen plus adjuvant (GAD-alum) in established T1D [27]. Although the primary end-point was not met (no significant effect on change in fasting C-peptide level after 15 months), fasting and stimulated C-peptide levels declined from baseline significantly less over time in the GAD-alum group than in the placebo group.

A third approach is based on experimental results obtained in the 1990s, showing that short-term CD3 antibody treatment (5 consecutive days) in recently diagnosed diabetic NOD mice induces permanent remission of the disease by restoring self-tolerance [28,29]; therapeutic trials were launched. The European multi-national multi-centre Phase II placebo-controlled clinical trial used the humanized Fc-mutated, aglycosylated ChAglyCD3 antibody [30]. A total of 80 patients presenting with new-onset T1D receiving insulin treatment for not more than 4 weeks were randomized to receive a short 6-day treatment with 8 mg of ChAglyCD3 (40 patients) or placebo (40 patients). In this trial only adult patients were included. As already reported, the antibody preserved β cell function very efficiently, maintaining significantly higher levels of endogenous insulin secretion compared to placebo-treated patients at 6, 12 and even 18 months after treatment. This effect translated into a very significant decrease in the patients' insulin needs during the same study period. The study has been extended and the data from the 4-year follow-up showed a remarkably sustained effect [30].

At variance with conventional therapies, CD3 antibodies provided the proof of concept that it was possible to obtain a long-term therapeutic effect following a single short-course administration of a therapeutic agent that induced immune regulation.

Can one derive new treatments for prevention of autoimmune diabetes from the hygiene hypothesis?

The clinical experience just reviewed outlines the difficulties of treating patients with established T1D. The preventive effect of infections on the progression of β cell aggression, which represents the basis of the hygiene hypothesis, applies to the early phases of the natural history of the disease [31]. It is thus logical to postulate that intervention aimed at 'reprogramming' the β cell-specific autoimmune response, as did infections in the past, might represent a simple and robust way to prevent T1D, inasmuch as the treatment proposed is totally safe (because by definition it will concern very young and still 'healthy' subjects). The search for such treatments is strictly dependent upon a better understanding of the immune mechanisms underlying the hygiene hypothesis.

How can infections protect from allergy and autoimmune diseases?

Subsets of helper $CD4^+$ T lymphocytes could be identified on the basis of the array of cytokines they produced. T helper type 1 (Th1) $CD4^+$ T cells produce preferentially interleukin (IL)-2 and interferon (IFN)-γ that essentially support T cell growth, macrophage activation and cell-mediated immunity. Th2 cells produce IL-4, IL-6, IL-10 and IL-13, which contribute to antibody production. More recently described Th17 cells are a major source of IL-17 and IL-21. The development of most autoimmune diseases involves cell co-operation processes with Th1 and Th17 $CD4^+$ cells, whereas the development of allergic diseases requires IL-4 and IL-5 produced by Th2 cells.

Based on initial reports pointing to the reciprocal down-regulation of Th1 and Th2 cells, some authors have suggested that in developed countries the lack of microbial burden in early childhood, which normally favours strong Th1-biased immunity, redirects the immune response towards a Th2 phenotype and therefore predisposes the host to allergic disorders. The problem with such an explanation was, however, that Th1 responses in the case of autoimmunity are not protective but pathogenic. These observations would fit with the concept of a common mechanism underlying infection-mediated protection against autoimmunity and allergy. Specialized subsets of T lymphocytes defined generally as regulatory T cells will be suitable candidates, as there is compelling data to show that they are highly effective in controlling both Th1- and Th2-mediated responses.

A second mechanism with relevance to the influence of infection on allergy and autoimmunity is antigenic competition, in which the immune response to an antigen is decreased by a concomitant immune response against an unrelated antigen. The competition is maximal when the unrelated antigen is administered a few days after the administration of the first antigen. Antigenic competition can affect antibody production [including immunoglobulin (Ig)E] and cell-mediated immune responses, as well as autoimmune and allergic responses. The precise molecular basis of antigenic competition remains unknown, despite numerous investigations.

Another mechanism by which bacteria, parasites and viruses could protect against immune disorders is via stimulation of Toll-like receptors (TLRs) that bind pathogen-

associated molecular patterns (PAMPs). TLRs represent the early molecular sensors of invading microorganisms and link innate with adaptive immune responses [32]. To date, 10 members of the TLR family have been identified in humans and 13 in mice, and a series of genetic studies have unveiled their respective ligands. Mammalian TLRs can be expressed either on the cell surface (i.e. TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6) or intracellularly (TLR-3, TLR-7, TLR-8 and TLR-9). The recognition of microbial ligands by TLRs results in the induction of inflammatory cytokines, type I IFNs and chemokines. Moreover, signalling from TLRs induces the up-regulation of co-stimulatory molecules on specialized antigen-presenting cells such as DCs, thus increasing their antigen-presenting capacity. This process, referred to as DC maturation, in turn primes naive T lymphocytes towards specialized functionally distinct T lymphocyte subsets, such as Th1, Th2, Th17 and regulatory T lymphocytes.

Although TLRs were considered initially as the crucial stimulatory receptors capable of activating early defence mechanisms against invading pathogens, emerging data suggest that their role is far more complex and articulated. Thus, some TLR agonists are effective at prevention of T1D in NOD mice [33–37]. It is worth stressing at this point that there is also published evidence showing that stimulation of some TLRs may also trigger autoimmunity (well in keeping with the autoimmunity-promoting ability of some infections) [38–44]. Thus, both the nature of TLRs and the specific mechanisms involved in the immunoregulatory pathways they mediate must be dissected carefully before their clinical use as disease prevention tools can be envisioned.

Therapeutic perspectives

Based on these epidemiological and experimental data, and opting for a systematic approach, we decided to test whether bacterial extracts which were on the market for the treatment of respiratory infections could reproduce the well-described protective effect of infections on the development of diabetes in NOD mice [45]. The product used initially was OM-85 (Broncho-Vaxom; OM Pharma, Meyrin/Geneva, Switzerland), a bacterial extract prepared from eight bacterial species frequently responsible for respiratory tract infections. OM-85 is of particular pertinence because it has been used extensively and safely in children suffering from repeated upper respiratory tract infections.

In NOD mice OM-85 effectively prevented T1D onset when administered intraperitoneally (i.p.) and orally at dosages compatible with clinical use. The effect is optimal (complete prevention) when the treatment is started early (at 3 or 6 weeks of age), but some protection is still achieved when the treatment is started at 10 weeks of age. We examined the role of Th2 cytokines, namely IL-4 and IL-10, in the protective effect of OM-85. Using genetically deficient mice and cytokine-neutralizing monoclonal antibodies, we have demonstrated that the therapeutic effect does not involve the Th2 cytokine IL-4 but is tightly dependent upon transforming growth factor (TGF)-β. Natural killer (NK) T cells also participate in the therapeutic effect, as CD1d$^{-/-}$ NOD mice are partially resistant to the protective effect of OM-85 [45].

Importantly, key mechanistic results were that OM-85 induced the production of IL-12 by DCs and of IL-10 essentially by B lymphocytes. It is important to stress at this point that there appears to be a tight dependency between the TGF-β-producing ability of OM-85 and the protective effect on the disease, because when a neutralizing anti-TGF-β antibody was administered immediately after OM-85, the protective effect of the drug was lost [45].

The second important finding was that, in spite of the fact that OM-85 is a mixture of several bacterial products, its protective effect on diabetes development appears to be mediated by components targeting TLR-4 [45]. Supporting this conclusion further are the recent data we obtained using *in vivo* instead of the intact bacterial extract: well-defined TLR-4 ligands OM-174-DP and OM-197-MP-AC that are currently under clinical development as adjuvants [46–50]. These are mimics of the lipid A portion of lipopolysaccharide (LPS), possessing many of the biological activities of LPS but devoid of its toxic effects [46,48,50]. OM-174-DP and OM-197-MP-AC protected NOD mice significantly from the development of diabetes, similarly to OM-85. As with OM-85 the therapeutic activity correlated with an effect on B lymphocytes, leading to their proliferation and IL-10 secretion.

The immunopharmacology of TLR ligands is just at its beginning, but the results appear encouraging enough to invest in this novel immune intervention avenue.

Disclosure

None of the authors has conflicts of interest to declare, or any relevant financial interest, in any company or institution that might benefit from this publication.

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