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Regulation of NOD mouse autoimmune diabetes by T cells that recognize a TCR CDR3 peptide

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Abstract

NOD mice spontaneously develop type I diabetes resulting from autoimmune destruction of their insulin-producing β cells. Among the self-antigens targeted by NOD autoimmune T cells is a peptide, p277, from the sequence of the 60 kDa heat shock protein (hsp60). Common to the anti-p277 T cell populations of NOD mice is an idiotope, C9, that spans the CDR3 region of the C9 TCR. We now report: (i) that the C9 idiotope peptide can be presented directly to anti-C9 anti-idiotypic T cells by C9 T cells, (ii) that spontaneous anti-C9 anti-idiotypic T cell activity falls as disease progresses, but immunization can activate the anti-idiotypic T cells to regulate the autoimmune process, (iii) that the anti-idiotypic T cells secrete IFN-γ, but appear to control the disease by down-regulating the IFN-γ produced by the pathogenic population of anti-p277 T cells, (iv) that intrathymic administration of the C9 idiotope peptide at 1 week of age can accelerate the disease, and (v) that administering the p277 target peptide can up-regulate the anti-idiotypic T cells and arrest the disease process. Thus, the development of NOD diabetes can be regulated by a balance between anti-idiotypic and anti-target peptide autoimmunity, and anti-idiotypic regulation can lead to changes in the cytokine secretion of the autoimmune T cells involved in the disease process.

Introduction

NOD mice spontaneously develop diabetes mellitus caused by autoimmune T cells that destroy the insulin-producing β cells of the pancreatic islets (1). The autoimmune inflammatory process can be detected beginning at ~4 weeks of age as peri-vascular and peri-islet insulitis that progresses to intra-islet insulitis culminating in clinical diabetes appearing at ~14–17 weeks of age in female NOD mice. Male NOD mice exhibit a slower progression and lower incidence of diabetes. A peptide, p277, from the sequence of the mammalian hsp60 molecule was identified as containing a target epitope for diabetogenic T cells (2). Peptide p277 was found to be involved in the pathogenesis of autoimmune diabetes in mice: NOD mice spontaneously develop T cell reactivity to peptide p277 during the insulitis that precedes the outbreak of clinical diabetes; anti-p277 T cells can adoptively transfer diabetes (2); induction of autoimmune diabetes in C57BL/KsJ mice by an ultra low dose of the toxin streptozotocin is associated with T cell reactivity to hsp60 and to peptide p277 (3); and standard strains of mice not prone to develop diabetes can be induced to develop transient hyperglycemia and insulitis by immunization to peptide p277 conjugated to an immunogenic carrier such as ovalbumin (OVA) (4).

Interestingly, the development of diabetes in NOD (5–7) or in C57BL/KsJ mice (8) could be arrested by the administration once or twice of unconjugated p277 peptide emulsified in incomplete Freund's adjuvant. Peptide treatment was marked by down-regulation of T cell proliferation to p277 and by up-regulation of anti-p277 antibodies of the IgG1 and IgG2b isotypes. These changes in autoimmune reactivity were accompanied by a shift of the autoimmune process from a pro-inflammatory T_h1-like response to an anti-inflammatory T_h2-like response (7).

However, administration of a target peptide is not the only way to induce specific control of autoimmune disease processes. Experimentally induced autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE)

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have been found to be susceptible to regulation by anti-idiotypic T cells inducible by T cell vaccination (9,10) and by vaccination with relevant TCR peptides (reviewed in 11) or TCR DNA (12). To explore the possibility of anti-idiotypic regulation of NOD diabetes, we studied a TCR idiotypic peptide, the C9 CDR3β idiotope, present in populations of T cells reactive to the p277 peptide (13). This paper reports that the spontaneous diabetes of NOD mice can be regulated by anti-idiotypic T cells, arising naturally or activated by vaccination with the TCR CDR3 peptide.

Methods

Mice

Inbred NOD/Lt mice were raised and maintained under specific pathogen-free conditions at the animal breeding center of this Institute from a breeding nucleus originally provided by Dr E. Leiter (Jackson Immunoresearch, Bar Harbor, ME). Our female NOD mice reach a cumulative incidence of 80% or greater by 8 months of age, while the male NOD mice stay diabetes-free up to 6 months of age and reach a cumulative incidence of ~40% by 1 year of age. Groups of six to 12 female mice were used in these studies, unless stated otherwise.

Blood glucose

Blood glucose was measured using a Glucose Analyzer 2 (Beckman Instruments, Brea CA) and hyperglycemia was defined by a blood glucose concentration of 13 mmol/l or greater.

Peptides and antigens

Peptides were synthesized by standard Fmoc using a automated ABIMED synthesizer AMS422 (Langenfeld, Germany) as described (5,6). The peptides were purified by reverse-phase HPLC and their compositions were confirmed by amino acid analysis. The C9 CDR3 peptide used was ASSLGGNQDTQY and the control N4 CDR3 peptide was ASSLWTNQDTQY. The sequence of p277 used in the experiments shown here was VLGGGVALLRVIPALDSLTPANED. This peptide is substituted at positions 6 and 11 with valine (V) in place of the cystein (C) in the native sequence. Substitution of the two C residues by V enhances greatly the stability of the peptide without affecting its immunological activity: the V-substituted peptide is completely cross-reactive with the native peptide by T cell and antibody assays, and both peptides have the same therapeutic effect on diabetes (in preparation). All the effects shown in this paper have been repeated with either variant of p277. The sequence of MT-p278 is EGDEATGANIVKVALEA. The sequence of GAD peptide 34 (residues 509–528; GADp34) is IPPSLRTLEDNEERMSRLSK (14). Bovine insulin was purchased from Sigma (St Louis, MO). Recombinant hsp60 was prepared as described (2). Concanavalin A (Con A) was purchased from Sigma Israel (Israel).

Recombinant TCR-flagellin constructs

The bacterial strains used in this study included *Salmonella typhimurium* strain LB5000 (restriction negative and modification proficient) and *Salmonella dublin* strain SL5928 (aromatic-dependent, non-motile vaccine strain). Plasmid pLS408 was used to clone the C9 TCR CDR3 idiotope. Transformation of plasmids to *S. dublin* was achieved by the standard $CaCl_2$ method via *S. typhimurium* strain LB5000 as mediator, as described previously (15).

Cloning of the C9 idiotope into pLS408

Two complementary oligonucleotides specifying the ASSLGGNQDTQY idiotope were synthesized and purified. After mixing the oligonucleotides at a 1:1 ratio (100 ng of each) and heating at 90°C for 5 min, the mixture was allowed to cool to room temperature for oligonucleotide annealing. The C9 idiotope was then ligated with an *Eco*RV-digested pLS408 and the mixture was used to transform *S. typhimurium* strain LB500. The selection of the transduced bacteria was based on a motility assay and the lysed bacteria or desaggregated flagella were immunoblotted with a rabbit antibody to flagella antigen *d*.

Purification of flagellin

Expression of *fliC* genes from high copy number plasmids resulted in the synthesis of exceptionally long, and thus fragile, flagella. Flagellin was prepared by vigorous vortexing an overnight culture of SL5928, harboring a plasmid encoding the C9 idiotope, followed by centrifugation. The supernatant was transferred to a new tube and the content (flagellin) was analyzed by SDS–PAGE.

T cell lines

The hsp60-specific T cell clone C9 was derived from unimmunized NOD spleen cells as described previously (16). The NOD OVA-specific T cell line was a gift from Dr A. Cooke. Both lines were maintained in culture by repeated stimulation in the present of their respective antigen and irradiated (3000 rad) syngeneic splenocytes as antigen-presenting cells (APC). Short-term lines of T cells were developed to the C9 idiotope by immunizing NOD/Lt mice with the activated C9 clone, 50,000 cells per mouse, intradermally in the hind foot pads. The anti-idiotypic lines were obtained by culturing the draining lymph node cells with irradiated C9 clone cells as immunogen for three cycles of stimulation; each cycle consisted of 3 days of culture with antigen and irradiated (3000 rad) syngeneic splenocytes as APC followed by rest for 7 days in medium supplemented with 10% T cell growth factors without added antigen or APC as described (2,4). The anti-clonotypic activity of the line was validated by testing the proliferative responses to the C9 CDR3 peptide or to the control N4 clone CDR3 peptide.

T cell proliferation

Groups of five female NOD mice, 6 weeks old, were immunized in the hind foot pads with 100 μg of the C9 CDR3 peptide emulsified in oil (incomplete Freund's adjuvant; Difco, Detroit, MI) in PBS or with the C9 CDR3–flagellin construct or with flagellin. Ten days later, the draining popliteal lymph nodes were removed and the T cell proliferative responses were assayed *in vitro* to the T cell mitogen Con A (1.25 μg/ml) or to various peptides (10 μg/ml) using a standard assay (7). Dose–response curves were obtained using concentrations of peptides up to 25 μg/ml (not shown). The concentration of

10 μg/ml was chosen to illustrate the results because this concentration produced the optimum response. T cell responses were detected by the incorporation of [methyl-^{3}H]thymidine added to the wells in quadruplicate cultures for the last 18 h of a 72 h culture. The stimulation index (SI) was computed as the ratio of the mean c.p.m. of antigen-containing wells to control wells cultured without antigens. The SD from the mean c.p.m. were always $<$10%. Background c.p.m., in the absence of antigens, was 800–1500 c.p.m.

T cell proliferation without APC

The proliferative response of the anti-clonotypic T cell line was tested in the presence or absence of syngeneic irradiated splenocytes as APC. Following the last antigen-driven stimulation of the line, the activated T cells were separated from the APC on a Ficoll gradient, followed by 7 days culture in T cell growth factor medium (16). One day before the proliferation test, a sample of the T cell line cells was stained with monoclonal hamster anti-CD3 145-2C11 (ATCC, Rockville, MD) and with MKD6 anti-I-A mouse mAb. For the second antibody, FITC-labeled anti-hamster IgG was used to detect the CD3$^+$ T cells and phycoerythrin-labeled anti-mouse IgG + IgM was used to detect the I-A$^+$ APC. By FACS analysis, the T cell line contained 98% CD3$^+$ cells and no detectable I-A$^+$ cells.

To test T cell proliferation, the anti-clonotypic T cell line was seeded at 50,000 cells/well to which 50,000 irradiated (3000 rad), activated C9 clone T cells were added, in the presence or absence of irradiated (3000 rad) syngeneic splenocytes. To ensure that neither the C9 clone cells nor the anti-clonotypic T cell line were contaminated with functional APC, we tested in parallel the proliferative responses of an anti-OVA NOD T cell line to OVA, using irradiated splenocytes, C9 clone cells or the anti-idiotypic line as added APC.

Transfer of protection by the anti-idiotypic T cell line

The short-term anti-idiotypic T cell line was propagated and activated as described above. A control T cell line was raised by repeated activation against a pooled sample of Con A-activated NOD T cells. The lines were activated by culture with irradiated stimulator cells for 72 h, and the responding T cells were collected and separated on a Ficoll gradient as described (16). The T cells (10^7) were then injected i.p. into 6-week-old NOD female mice. Two weeks later and monthly thereafter, the mice were monitored for the development of diabetes, determined the by the measurement of blood glucose.

Thymic inocculation

Seven-day old NOD female mice were injected intrathymically to induce 'tolerance' to the following peptides: C9 CDR3, p277, MTp278 or GADp34. A dose of 0.1 mg peptide was injected in 50 μl of PBS, using a 1 ml syringe with a 30G needle.

ELISA assay

NOD mice were tested for antibodies binding to the C9 CDR3 peptide, to the p277 peptide or to hsp60 as described (7,8). Briefly, 10 μg/ml of the various antigens were applied to assay plates (Maxisorp; Nunc, Roskilde, Denmark) suitable for the binding of peptides and the plates were incubated with the test sera. The binding of antibodies to the adherent antigens was detected using alkaline phosphatase-conjugated anti-mouse IgG + IgM, or isotype-specific anti-mouse IgG1, IgG2a or IgG2b (Jackson Immunoresearch, West Grove, PA). A significant amount of antibody was defined as an OD 405 nm reading of $>$0.25, which is 3 SD above the mean ELISA reading obtained in the sera of 10 normal BALB/c mice.

Statistical significance

The InStat 2.01 program was used for statistical analysis. Student's *t*-test and the χ^2-test were carried out, where appropriate, to assay significant differences between experimental and control groups.

Results

Spontaneous anti-CDR3β T cell reactivity

The discovery of the shared TCR C9 CDR3β idiotope in populations of anti-p277 T cells in NOD mice (13) led us to investigate whether the onset of insulitis in NOD females might be accompanied by spontaneous T cell reactivity to the C9 CDR3β peptide idiotope, as well as by T cell reactivity to the p277 target peptide. Figure 1(A) shows the dynamics of the proliferative reactivities of splenic T cells to the hsp60 molecule, to the p277 target peptide and to the C9 CDR3 peptide measured over time. The cumulative incidence of clinical diabetes is also shown. It can be seen that between 4 and 6 weeks of age, coincident with the onset of insulitis, the beginning of spontaneous T cell proliferative activity to hsp60 and its p277 peptide could be detected. This early anti-hsp60 reactivity was accompanied by a sharp rise in T cell proliferative reactivity to the C9 CDR3 peptide. However, this anti-idiotypic reactivity was short lived and spontaneously fell as the reactivity to hsp60 and peptide p277 rose and peaked, coincident with the development of overt diabetes. With the completion of β cell destruction, the T cell reactivities to hsp60 and to p277 also spontaneously fell. There was no spontaneous reactivity (not shown) to a control hsp60 peptide p278, to a control TCR CDR3 peptide N4 that differs from C9 by two amino acids (13) or to a control *Escherichia coli* lysate containing the plasmid pEX2. In contrast to the early fall in anti-C9 reactivity and early rise in reactivity to p277 seen in female NOD mice, male mice, which manifest a lower incidence and a milder progression of diabetes (1), showed a prolonged duration of their anti-C9 activity lasting to 15 weeks or longer (Fig. 1B). It thus appears that T cell reactivity to the C9 CDR3 peptide is activated spontaneously in the pre-diabetic phase of insulitis and that this anti-idiotypic reactivity falls as the reactivity to p277, the target of the C9 T cell clone, climbs.

The C9 idiotope is recognized on C9 T cells

Figure 2 shows the results of experiments done to test whether T cell reactivity to the C9 CDR3 peptide is equivalent to T cell reactivity to intact C9 T cells. Groups of 8-week-old female NOD mice were immunized with intact, irradiated C9 T clone cells or with an anti-OVA control T clone (Fig. 2, upper). Other groups of mice were immunized with the C9 CDR3 peptide genetically engineered into a flagellin molecule (Fig. 2, lower).

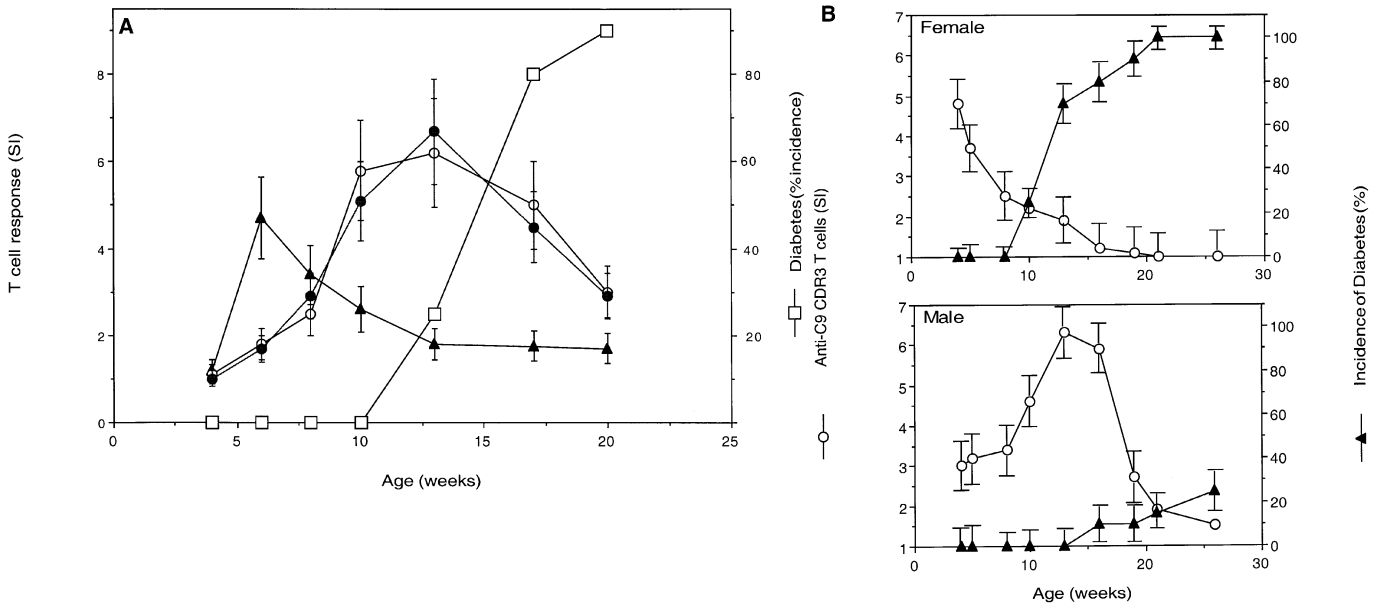

Fig. 1. Dynamics of T cell reactivities during the development of insulin-dependent diabetes mellitus (IDDM). (A) Female NOD mice of various ages were scored for the development of diabetes (□), and for spontaneous T cell proliferation to hsp60 (●), to the p277 peptide (○) and to the C9 CDR3 peptide (▲). (B) Groups of female (upper) and male (lower) mice were studied for the development of diabetes and for spontaneous T cell proliferative activity to the C9 CDR3 peptide.

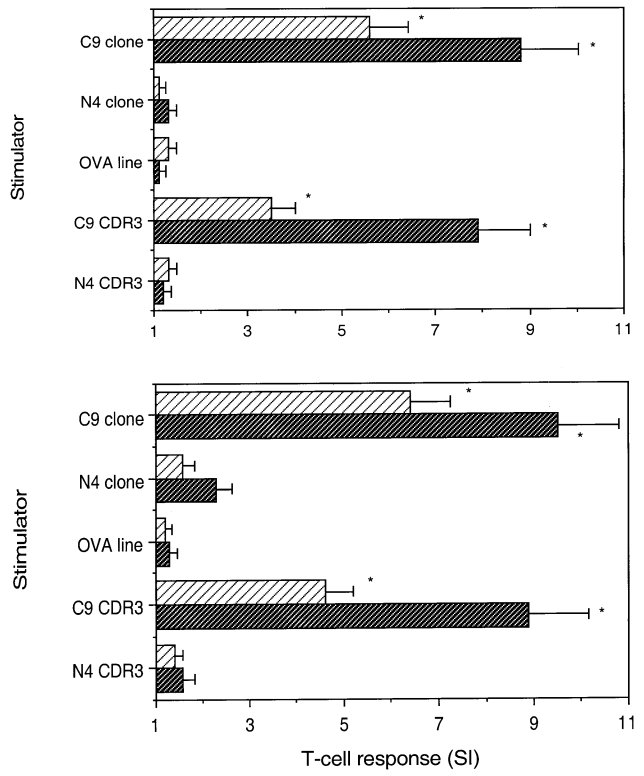

Fig. 2. The C9 idiotope is the C9 CDR3β peptide. Female NOD mice 10 weeks old, were vaccinated with T cell clones C9 (close-hatched)or anti-OVA (open-hatched) (upper panel) or with flagellin (open-hatched) or recombinant C9 CDR3β-flagellin (close-hatched) (lower panel). Ten days later, the spleens were removed and assayed for T cell proliferation activities to T cell clones C9, N4 or anti-OVA, or to the C9 CDR3β or N4 CDR3β peptides as stimulators $^{*}P < 0.01$.

This recombinant flagellin protein was used as an immunogenic carrier for immunization to the CDR3 peptide to avoid the use of adjuvants, such as complete Freund's adjuvant, that can by themselves affect the development of diabetes (17). Control immunization was done with non-recombinant flagellin. Ten days later, T cell proliferative responses were assayed in each group to whole T cells C9, N4 or anti-OVA, or to the CDR3β peptide sequences of the C9 or N4 clones. It can be seen that immunization using either the intact C9 cells or the recombinant C9 CDR3β peptide was immunologically equivalent. The spontaneous anti-idiotypic T cell responses to the C9 clone and to the C9 CDR3β peptide were mutually augmented by either immunization. These responses were immunologically specific: there were no detectable reactivities to any of the control cells or peptides. Thus, the C9 CDR3β peptide seems to define the immunological identity of intact C9 T cells defined by anti-C9 T cells.

C9 T cells can present their idiotope

To learn whether intact C9 T cells could present their own idiotope to anti-C9 T cells, we developed a short-term line of anti-C9 T cells. Both the C9 and anti-C9 populations of T cells were purified of contaminating APC such that neither cell population was capable of supplying APC activity for a response to OVA of the anti-OVA T cell clone (Fig. 3). Nevertheless, the C9 clone could activate the anti-C9 T cell line and the addition of splenocyte APC did not enhance the C9–anti-C9 reactivity. Thus, it appears that C9 T cells can present their own idiotope to anti-C9 anti-idiotypic T cells. FACS analysis of the C9 clone showed it to be composed almost entirely of $CD4^{+}$ T cells; the anti-C9 anti-idiotypic T cell line was composed equally of $CD4^{+}$ and $CD8^{+}$ T cells (not shown).

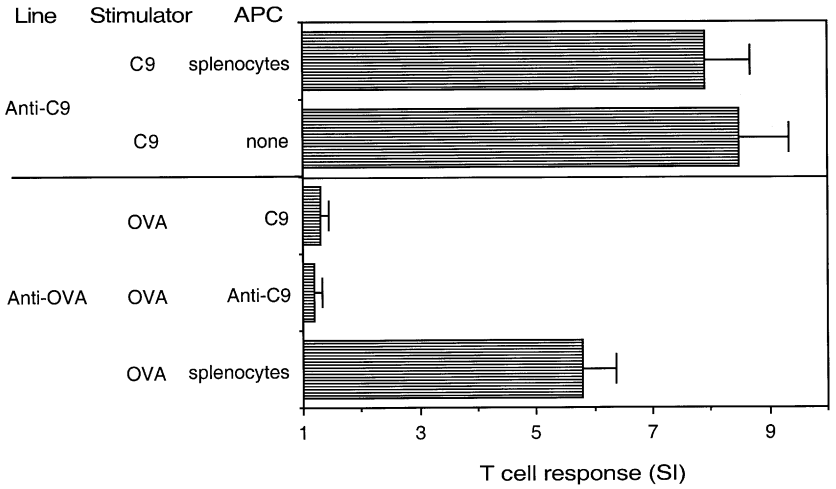

Fig. 3. Clone C9 presents its idiotope without added APC. The C9 clone and an anti-C9 line were purified of other APC by density-gradient separations. The purified C9 and anti-C9 T cells were irradiated (3000 rad) and used as APC to activate the proliferative response of purified anti-C9 or of anti-OVA T cells. The anti-OVA response was done in the presence of the OVA antigen. Splenocytes were added to some cultures to function as APC.

Anti-idiotypic reactivity inhibits the development of diabetes

To test the function of the anti-C9 CDR3β response, we vaccinated female NOD mice at the age of 6 weeks with the C9–CDR3β flagellin recombinant protein or with flagellin alone, and observed the treated and untreated control mice for the development of diabetes. Figure 4 shows that 100% of the mice in this experiment that were untreated or that had been treated with flagellin alone developed diabetes by 8 months of age. In contrast, the mice that had been vaccinated with the C9 CDR3β recombinant flagellin protein showed delayed onset of diabetes and only 40% became diabetic by 8 months of age ($P < 0.05$ at ages 3.5, 5, 7 and 8 months).

To confirm this finding, we transferred to NOD female mice anti-C9 T line cells or control T cells raised against syngeneic Con A T blasts. We found that the anti-C9 T cell line significantly protected the mice against the development of diabetes ($P < 0.01$); by 5 months of age only 20% of the mice had developed diabetes and this number rose to only 30% by 8 months, an age when 100% of the untreated mice and of the anti-Con A control T cell treated mice were diabetic (Fig. 5). Thus, both adoptive transfer and active immunization experiments indicated that an anti-C9 anti-idiotypic T cell response can inhibit the development of diabetes in NOD mice.

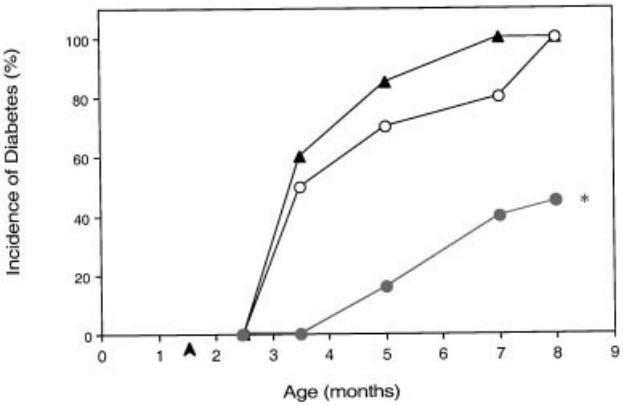

Fig. 4. Vaccination to CDR3β inhibits the development of IDDM. Six-week old NOD female mice were untreated (▲) or treated with flagellin (○) or with recombinant C9 CDR3β–flagellin (●). The mice were observed for the development of IDDM. Those vaccinated to the recombinant C9 CDR3β flagellin showed significantly reduced development of IDDM (*$P < 0.01$).

Anti-idiotypic vaccination down-regulates the T cell response to the p277 target peptide

To investigate the effect of anti-idiotypic regulation on the spontaneous T cell response to the p277 target peptide, we vaccinated 1-month-old NOD female mice with the C9 T clone or with C9 CDR3β recombinant flagellin. Control vaccination was done with an anti-OVA T cell clone or with flagellin alone. We then studied the spontaneous T cell responses of the mice at 3 months of age, when untreated NOD mice show their peak T cell proliferative reactivity to p277 and to hsp60 (see Fig. 1). Figure 6 shows that vaccination with flagellin alone or with the anti-OVA T cell line did not significantly affect these T cell proliferative responses. In contrast, these responses were significantly reduced in the mice that had been vaccinated with C9 cells or with the recombinant C9 CDR3β flagellin ($P < 0.01$). Anti-idiotypic activation, therefore, leads to inhibition of the spontaneous T cell proliferative reactivity to hsp60 and p277 that marks the progression of the diabetic process.

Effects of anti-idiotypic vaccination on T_h1- and T_h2-associated reactivities

It has been proposed that T_h1-type T cells are involved in the destruction of β cells and that arrest of the autoimmune process might be achieved by down-regulating T_h1-like activity and up-regulating T_h2-like activity (7,18). We therefore measured the effects on IFN-γ secretion of vaccination to the C9 idiotope. Figure 7 shows that 3-month-old untreated NOD

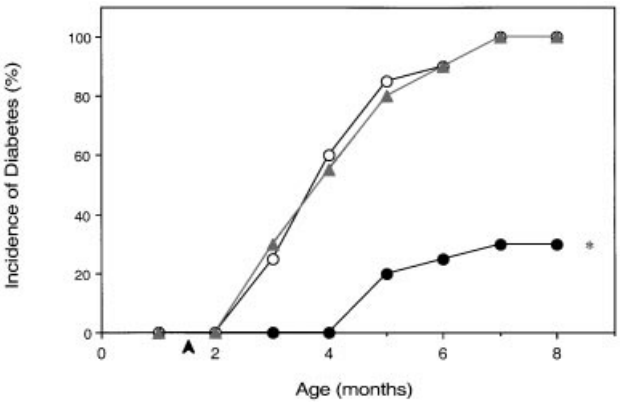

Fig. 5. Transfer of anti-C9 T cells inhibits the development of IDDM. Female NOD mice, 6 weeks old, were untreated (▲) or inoculated with 10^7 short-term line cells, anti-C9 (●) or anti-Con A blasts (○). The mice receiving the anti-C9 T cells manifested a significant inhibition of development of IDDM (*$P < 0.01$).

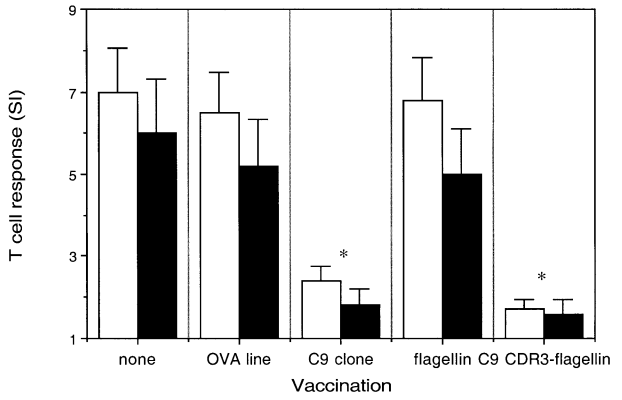

Fig. 6. Spontaneous T cell proliferative reactivities to hsp60 or to p277 are reduced by vaccination to C9 CDR3β. Ten-week old NOD female mice were vaccinated with 10^7 irradiated T cells of clone C9 or anti-OVA, or with flagellin or recombinant C9 CDR3β–flagellin. Four weeks later, the proliferative responses of their splenocytes were tested to hsp60 (open bars) and to p277 (closed bars). The responses of the mice that had been vaccinated to C9 or to C9 CDR3β flagellin were significantly reduced (*$P < 0.01$).

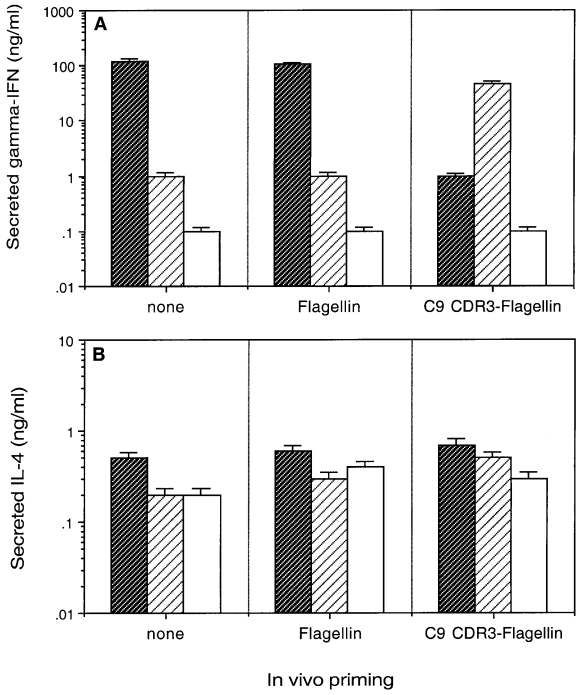

Fig. 7. (A) Vaccination to C9 CDR3β up-regulates the IFN-γ response to the C9 CDR3 peptide and down-regulates the IFN-γ response to the p277 peptide. Ten-week-old NOD mice were vaccinated with flagellin or with recombinant C9 CDR3β-flagellin and 4 weeks later their splenocytes were assayed for IFN-γ secretion induced by culture with peptides C9 CDR3β (vertical bars), N4 CDR3β (open bars) or p277 (cross-hatched bars). The differences in the responses to C9 CDR3β and to p277 after C9 CDR3β-flagellin vaccination were significant (*$P < 0.01$). (B) The IL-4 response was tested at the same conditions as (A) and was unaffected.

mice and NOD mice that had been vaccinated with flagellin secreted appreciable amounts of IFN-γ in response to stimulation *in vitro* with the p277 peptide. The high IFN-γ response (100 ng/ml) to the p277 peptide was associated with a low IFN-γ response (1 ng/ml) to the C9 CDR3β peptide (Fig. 7A). In contrast, the mice that had been vaccinated with C9 CDR3β recombinant flagellin showed a marked rise in their IFN-γ response to the C9 idiotope (50–100 ng/ml) accompanied by a marked decrease in their IFN-γ response to the p277 peptide (1 ng/ml). The secretion of IFN-γ was specific; a control peptide, N4 CDR3β, did not elicit a significant IFN-γ response. Thus, the induction of an IFN-γ response to the C9 CDR3β peptide was associated with a decrease in the IFN-γ response of the autoimmune T cells to the target hsp60 and p277 molecules. In contrast to the shift in production of IFN-γ, no marked effects on the production of IL-4 were noted (Fig. 7B).

Analysis of IgG antibody isotypes is a convenient way to detect the functional activation of cytokines *in vivo* in response to specific antigens (19). We therefore studied the IgG antibodies induced by vaccination with the C9 CDR3β peptide. Figure 8(A) shows that C9 CDR3β recombinant flagellin induced antibodies to the C9 CDR3β peptide that were primarily of the IgG2a isotype, which is regulated by IFN-γ (19). Hence, the IFN-γ response to the C9 CDR3β peptide, which we detected *in vitro* (Fig. 7), could be confirmed systemically by an IgG2a antibody response *in vivo*.

We also looked for antibodies to peptide p277 arising in the mice immunized to the C9 idiotope. Anti-p277 antibodies are not detectable in untreated NOD mice (7), but such antibodies appeared spontaneously in the sera of the mice immunized with the C9 CDR3β recombinant flagellin (Fig. 8B). These anti-p277 antibodies, in contrast to the anti-C9 CDR3 antibodies, were of the IgG1 and mostly of the IgG2b isotypes (Fig. 8B). IgG1 is induced by the T_h2 cytokine IL-4 (19,20) and IgG2b antibodies are induced by the 'suppresser' cytokine transforming growth factor (TGF)-β (21). Few or none of the anti-p277 antibodies were of the IgG2a isotype that requires IFN-γ. Thus, activation of the anti-C9 anti-idiotypic response automatically produced a change in the anti-p277

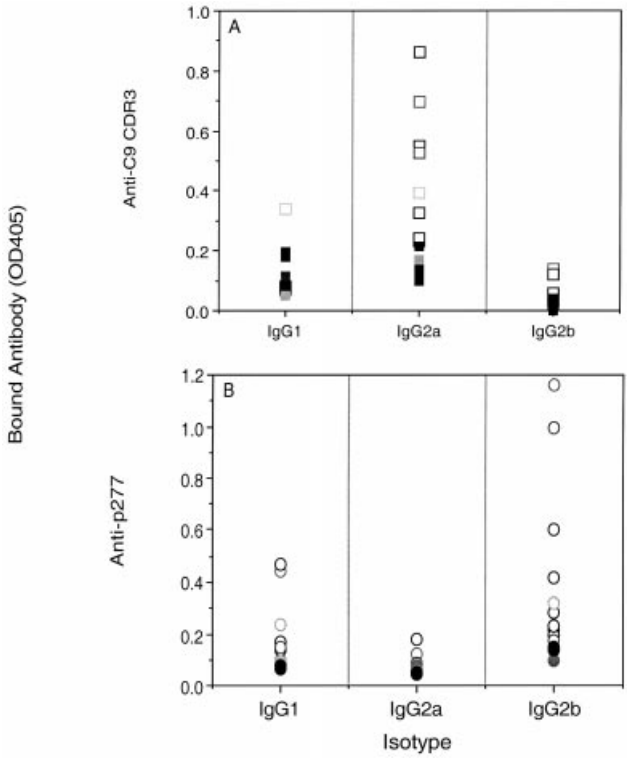

Fig. 8. Vaccination to C9 CDR3β induces anti-C9 CDR3β IgG2a antibodies and anti-p277 IgG2b and IgG1 antibodies. Ten-week-old female NOD mice were vaccinated with flagellin (filled shapes) or with recombinant C9 CDR3β–flagellin (open shapes). Four weeks later, the sera of individual mice were assayed by ELISA for antibody isotypes to C9 CDR3β peptide (upper panel) or to p277 peptide (lower panel). The response to C9 CDR3β was significantly of the IgG2a isotype ($P < 0.01$), while the response to p277 was significantly of the IgGI ($P < 0.05$) and IgG2b isotypes ($P < 0.01$).

response phenotype from T cell proliferation (Fig. 6) and IFN-γ secretion (Fig. 7) to T_h2-like antibodies (Fig. 8).

The effect of anti-idiotypic activation on the spontaneous appearance of anti-p277 antibodies was associated with down-regulation of other antibodies. Figure 9 shows that vaccination with C9 CDR3β was associated with a fall in the antibodies to the whole hsp60 molecule and to insulin that mark the spontaneous development of diabetes in NOD mice (16). Thus, activation of anti-C9 CDR3β immunity augmented anti-p277 antibody production (Fig. 8), while it decreased the production of antibodies to other antigens associated with the diabetic process (Fig. 9).

Peptide p277 treatment up-regulates anti-idiotypic T cell reactivity

We have previously reported that administration of peptide p277 itself can modulate T cell proliferative activity and cytokine secretion in response to p277 (7) and can arrest the development of diabetes (2,5–7). We therefore investigated whether treatment with the p277 peptide might automatically influence anti-idiotypic T cell reactivity to the C9 CDR3β idiotope. Figure 10 shows that treatment with the p277 peptide both down-regulated the proliferative response to p277 (Fig. 10A) itself and up-regulated the T cell proliferative response to the C9 CDR3β peptide (Fig. 10B). Modulation of the T cell responses to the p277 and C9 CDR3β peptides induced by p277 treatment was specific. Treatment with a control immunogenic peptide MT p278 (7) did not down-regulate the spontaneous T cell reactivity to p277 or up-regulate the reactivity to C9 CDR3. Therefore, activating a specific modification in the response to either of the two peptides, p277 or C9 CDR3β, can automatically modify the associated response to the other peptide.

Intrathymic administration of the C9 idiotope peptide accelerates diabetes

If anti-C9 CDR3 T cell reactivity functions physiologically to down-regulate the autoimmune process leading to diabetes, then measures that interfere with the development of anti-C9 CDR3β reactivity should accelerate the development of disease. Intrathymic administration of antigens early in life has been reported to inhibit the development of T cell immunity to the administered antigens, including antigens targeted in NOD diabetes (22,23). We reasoned that intrathymic administration of either the p277 peptide or the C9 CDR3β peptide should have contrary effects on the development of diabetes, if indeed reactivity to the p277 peptide were diabetogenic and reactivity to the C9 CDR3β peptide were anti-diabetogenic. Female NOD mice at 7 days of age were injected into the thymus with 0.1 mg of either of these peptides or with the GAD p34 peptide, to which NOD mice respond spontaneously (14). Figure 11 shows that thymic administration of the p277 peptide inhibited diabetes ($P < 0.035$) and, in contrast, thymic administration of the C9 CDR3β peptide led to accelerated diabetes ($P < 0.035$). Thymic injection of the GADp34 peptide had no influence on the development of diabetes. Thus, thymic T cell education to specific peptides seems to be important in determining the disease phenotype, both positively and negatively.

Discussion

The results reported here indicate that NOD diabetes can be regulated by anti-idiotypic T cell reactivity to the C9 TCR idiotope peptide (Figs 4 and 5), an idiotope defined by a TCR CDR3β chain sequence shared by individual NOD mice (13). This anti-idiotypic reactivity has several features.

(i) The dynamics of the anti-idiotypic reactivity correlate with the dynamics of the spontaneous disease process. The first appearance of spontaneous anti-C9 CDR3β T cell reactivity is associated with the onset of insulitis, and the spontaneous decay of the anti-C9 CDR3β reactivity is associated with rising T cell reactivity to hsp60 and its p277 peptide epitope (Fig. 1A). Moreover, the naturally delayed onset of disease in male mice is marked by greater persistence of their anti-C9 CDR3β reactivity (Fig. 1B).

(ii) The TCR CDR3β sequence defines the C9 idiotope and the C9 CDR3β peptide can serve as the idiotypic address of the intact C9 T cell (Fig. 2). The C9 T cell can present its own CDR3β peptide without the aid of other APC (Fig. 3). The determination of MHC class I and class II restrictions awaits the purification of lines of CD4 and CD8 anti-C9 T cells.

(iii) Although the details of idiotypic T cell interactions have yet to be characterized, the present observations suggest

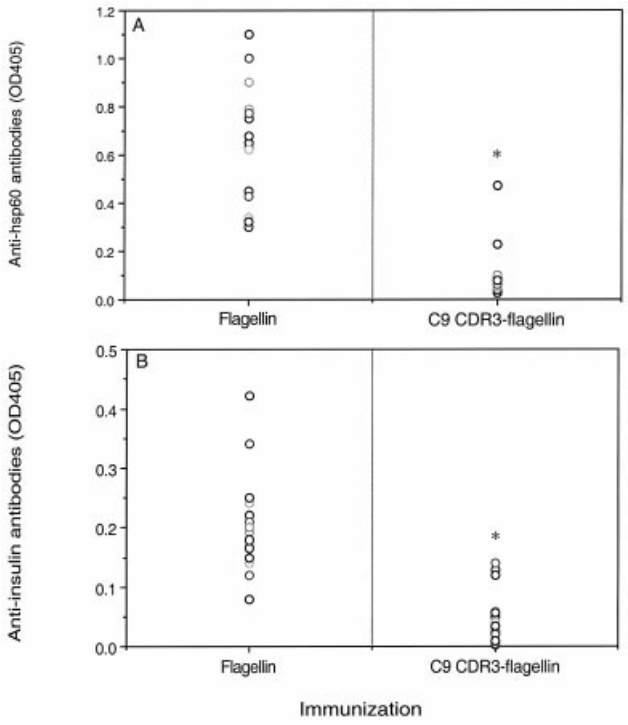

Fig. 9. Vaccination to C9 CDR3β down-regulates the spontaneous antibody responses to intact hsp60 (A) and to insulin (B). Ten-week-old female NOD mice were vaccinated to flagellin or to recombinant C9 CDR3β–flagellin. Four weeks later, their serum IgG antibodies were assayed by ELISA to intact hsp60 or to insulin. The antibody titers of the C9 CDR3β-vaccinated mice were significantly decreased (*$P < 0.01$).

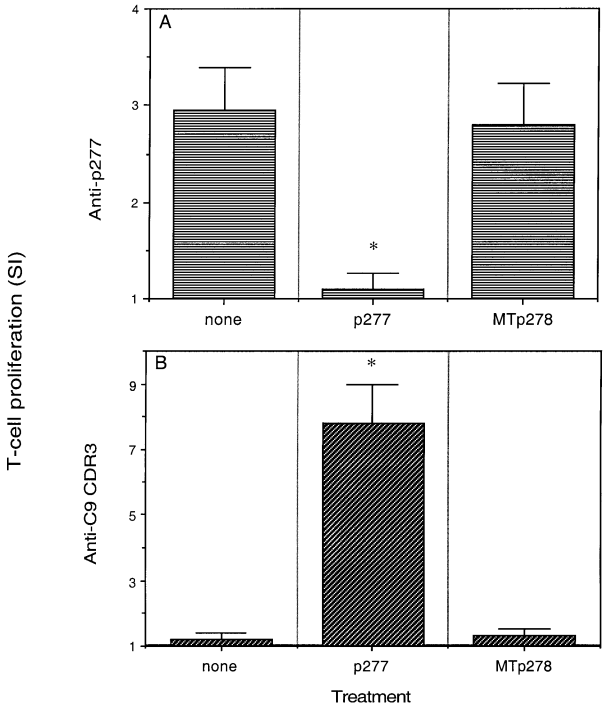

Fig. 10. Treatment with peptide p277 up-regulates the T cell proliferative response to C9 CDR3β peptide and down-regulates the response to p277 itself. Ten-week-old female NOD mice were vaccinated either with peptide p277 or with control peptide MTp278. Four weeks later, their T cell proliferative responses were assayed in response to peptides p277 (A) or C9 CDR3β (B). The differences in the responses of the p277-treated mice were significant (*$P < 0.01$).

that the resulting cytokine profiles of the interacting T cell populations differ. Activated anti-C9 CDR3β anti-idiotypic T cells proliferate (Figs 1–3) and secrete the T_h1 cytokine IFN-γ *in vitro* (Fig. 7A). The induction of IgG2a isotype antibodies is additional evidence for the T_h1-like character of the anti-idiotypic response to the C9 CDR3β peptide *in vivo* (Fig. 8A).

The finding that anti-idiotypic regulatory T cells may be 'pro-inflammatory' might explain the surprising observations that the transgenic expression of tumor necrosis factor (TNF)-α in the islets can prevent diabetes (26) and that the administration of TNF-α *in vivo* may protect NOD mice from developing spontaneous diabetes (27). Indeed, TNF-α appears to mediate the protective effects of complete Freund's adjuvant in BB rats (28).

In contrast to the T_h1-like character of the anti-idiotypic regulatory T cells, the effects of regulation on the autoimmune response to the target antigens appear to be expressed as a change in cytokine profile from a T_h1-like response to a T_h2-like response: T cell proliferation and IFN-γ secretion decreased (Figs 6 and 7), while anti-p277 antibodies of the IgG1 and IgG2b isotypes appeared (Fig. 8). The induction of IgG1 antibodies suggests that IL-4 may be involved (19,20) and the induction of IgG2b antibodies indicates that TGF-β may be activated in response to anti-idiotypic regulation (21). Indeed, the 'suppresser' activity of TGF-β (29,30) could explain the arrest of the disease process. Treatment of NOD diabetes with the p277 peptide itself was found previously to

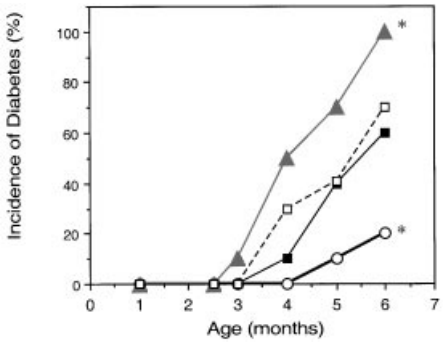

Fig. 11. Thymic injection of peptides p277 or C9 CDR3β affects the development of IDDM. One-week old female NOD mice were untreated (□), or injected into the thymus with peptides p277 (○), C9 CDR3β (▲) or GAD p34 (■) and monitored for the cumulative incidence of IDDM. The groups receiving p277 or C9 CDR3β were significantly ($P < 0.035$) lower (p277) or higher (C9 CDR3β) than the untreated or GADp34-treated mice.

result in a shift in the cytokines produced by splenic anti-p277 T cells from a T_h1-type response (IL-2 and IFN-γ) to a T_h2-type response (IL-4 and IL-10) (7). However, we were not able to detect a rise in secretion of IL-4 after anti-C9 CDR3β vaccination (Fig. 7) or of IL-10. Perhaps the secretion of anti-inflammatory cytokines was too limited in time, amount or site to allow detection using our present methods. Nevertheless,

the difference in isotypes (Fig. 8) between the antibodies to the C9 CDR3β peptide (IgG2a) and to the p277 peptide (IgG1 and IgG2b), although indirect, is strong evidence for a significant difference in cytokine 'help' *in vivo* for the two peptides in the same mice. Be that as it may, the fact that immunization with one peptide, the C9 CDR3β idiotope, can automatically elicit antibodies to another, non-cross reactive peptide, p277, argues for the existence of a regulatory network (9,10).

In contrast to up-regulation of anti-p277 antibodies, the spontaneous autoantibodies to intact hsp60 and to insulin that accompany the diabetogenic process were down-regulated by anti-C9 CDR3β vaccination (Fig. 9). The spreading of regulation from one antigen to additional antigens may be explained by 'by-stander suppression' (29,30); but this too needs to be established directly. In any case, the present results indicate that anti-idiotypic regulation of an autoimmune process does not require that the autoimmune T cell population be killed or completely suppressed by the regulators. Rather, the autoimmune T cell population may be induced by anti-idiotypic regulator T cells to express a different cytokine profile of activity (31). Indeed, vaccination with TCR DNA in the EAE model also seems to affect the disease by a shift in the cytokine profile to the target antigen from a T_h1 to a T_h2 type (12). In the EAE model, it was demonstrated that co-culture of TCR-specific and BP-specific T cells resulted in the functional inhibition of the encephalitogenic BP-specific T cells through a soluble, secreted lymphokine (32).

(iv) Finally, the anti-idiotope network seems to be bi-directional: treatment with peptide p277 in a way that arrests the diabetic process (2,5,6) automatically induces up-regulation of T cell proliferation to the C9 CDR3β peptide (Fig. 10). The notion that the diabetogenic process might involve a balance between competing T cell reactivities to TCR idiotope and target epitope peptides is compatible with the observation that early thymic administration of each peptide produced opposite effects: thymic administration of the p277 peptide inhibited disease, while thymic administration of the C9 CDR3b peptide accelerated disease (Fig. 11). Acceleration of disease by thymic administration of the C9 CDR3β peptide suggests that thymic education may be involved naturally in generating anti-idiotypic regulatory cells.

At present, we do not know how anti-idiotypic networks get organized, but the results reported here and elsewhere suggest the following possibility. The normal expression of hsp60 in the thymus (33) can lead to the positive selection of anti-p277 T cells in NOD mice; the p277 peptide bears a binding motif for the I-A^{g7} molecule of the NOD mouse (34). The positive selection of anti-p277 T cells could amplify T cells bearing the C9 CDR3β idiotope; indeed, the C9 CDR3β idiotope is detectable in the NOD thymus at an early age (13). It is conceivable that the C9 CDR3 idiotope could positively select anti-C9 anti-idiotypic T cells. Thus, the NOD mouse may be born with both C9 idiotypic T cells, which in the T_h1 mode damage β cells (2), and anti-C9 anti-idiotypic T cells, which can down-regulate the C9 T cells (shown here). The results of the intrathymic injection experiment (Fig. 11) can be explained by manipulation of these T cells at the site of their early development.

It is conceivable that the C9 idiotype–anti-idiotype network remains latently sub-clinical until some insult (viral infection, another autoimmune process?) triggers the onset of insulitis and thus the activation of the C9 anti-p277 T cells. This activation first triggers the anti-C9 anti-idiotypic regulators, but these regulators fail to control the autoimmune process and decay (Fig. 1). Activation of the anti-C9 anti-idiotypic regulators can be induced by vaccination (Figs 2, 4 and 5) and regulation can be restored (Figs 6–10). Alternatively, treatment with p277 itself (5–7) can allow the recovery of the anti-idiotypic regulators (Fig. 10). At present, there is no explanation for the ability of anti-idiotypic regulation to affect the cytokine profile (Figs 7–9), but similar observations have been made in TCR-DNA vaccination in EAE (12).

The demonstration of a shared TCR element in anti-idiotypic regulation of NOD diabetes confirms and extends observations made over the years regarding T cell vaccination (reviewed in 35), TCR peptide vaccination (reviewed in 11,36) and TCR DNA vaccination (12) in EAE and other models. The application of T cell vaccination (37) and TCR peptides (38) to human multiple sclerosis also has uncovered a negative correlation between anti-idiotypic T cells and target-specific autoimmune effector cells. Anti-idiotypic T cell populations have been reported to contain cytotoxic T cells and T cells of both the CD4 and CD8 phenotypes (9,37), and it is likely that autoimmune regulation may involve several mechanisms and even include non-idiotypic specifies (39).

This model needs detailed analysis, but in general the present results support the idea that spontaneous networks may underlay autoimmune phenomena (31), and suggest that both anti-idiotypic and target antigen regulation may rest on common pathways that regulate the cytokine profile, and thereby determine the biological consequences of autoimmune reactions to the dominant set of autoantigens, the immunological homunculus (40). Perhaps the very existence of the immunological homunculus is what makes complex regulation by simple peptides possible.

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Abbreviations

APC	antigen-presenting cell
Con A	concanavalin A
EAE	experimental autoimmune encephalomyelitis
hsp60	60 kDa heat shock protein
IDDM	insulin-dependent diabetes mellitus
OVA	ovalbumin
SI	stimulation index
TGF	transforming growth factor
TNF	tumor necrosis factor

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