Modulation of diabetes in NOD mice by GAD65-specific monoclonal antibodies is epitope specific and accompanied by anti-idiotypic antibodies

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Summary

Type 1 diabetes is caused by the autoimmune destruction of pancreatic beta cells. Here we show that administration of a human monoclonal antibody (b96·11) specific to the 65-kDa isoform of glutamate decarboxylase (GAD65) to prediabetic non-obese diabetic (NOD) mice significantly delays the onset of autoimmune diabetes. We found this effect to be epitope-specific, as only b96·11 showed this therapeutic property, while a GAD65-specific human monoclonal control antibody (b78) derived from the same patient, but specific to a different determinant of GAD65, had no significant effect on the progression of disease. Administration of b96·11 or b78 to NOD mice was accompanied by the generation of anti-idiotypic antibodies. Importantly, the induced anti-idiotypic antibodies were specific for the immunizing antibody and blocked the binding of GAD65 by the respective antibody. These findings suggest a potential role for the internal image of the GAD65 determinant recognized by b96·11 in the anti-idiotypic antibody, supporting an immunomodulatory role for GAD65-specific autoantibodies, as originally postulated by Jerne.

Keywords: autoimmune diabetes; autoantibodies; anti-idiotypic antibodies; NOD mouse; GAD65

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of the pancreatic beta cells. The pathogenesis of T1D and pathways that might be used to intercept the disease progression have been extensively studied in the non-obese diabetic (NOD) mouse (for a review, see Shoda et al.[1]). These inbred animals show mononuclear infiltration of the islets (insulitis) and evidence of beta cell loss already at 8–9 weeks of age, resulting in diabetes by ~30 weeks of age. The development of diabetes in the NOD mouse is strongly influenced by the environment, and stimulation, for example activation of the immune system by pathogens, induces T1D resistance.[2–4] The destruction of the pancreatic beta cells in the NOD mouse is thought to be mediated by both CD4^{+} and CD8^{+} T cells (for a review, see Haskins[5]). However, B lymphocytes appear to contribute to auto-immune diabetes in the NOD mouse, possibly through their role as antigen-presenting cells (for a review, see Wong and Wen[6]), and NOD mice deficient in B lymphocytes show a decreased incidence of diabetes.[7] A number of preventative therapies have been reported in the NOD mouse (for reviews, see Shoda et al.,[1] Atkinson and Leiter,[8] and Bach[9]). However, to date only treatment with antilymphocyte serum (ALS), and antibodies specific to CD4 or to CD3 have been demonstrated to reverse the disease.[10–12]

The beta cell autoantigens glutamate decarboxylase (65-kDa isoform; GAD65) and insulin have been identified as major candidates in triggering beta cell-specific autoimmunity.[13] Tolerization against these autoantigens has been attempted as a method for the prevention of the disease. GAD65 administration prevents beta cell destruction and the resulting diabetes in the NOD mouse, emphasizing the importance of GAD65 as an autoantigen

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Abbreviations: GAD65, 65-kDa isoform of glutamate decarboxylase; GAD65Ab, autoantibodies to GAD65; IVIG, intravenous immunoglobulin; RBA, radioligand binding assay; T1D, type 1 diabetes.

in the disease progression of the NOD mouse,[14–17] even though GAD65 is present only in very limited amounts in murine beta cells.[18] GAD65 administration has been suggested to result in tolerization of the T-cell-mediated immune response.[15,19,20] However, these results are in conflict with those of other studies.[21–24]

While autoantibodies to GAD65 (GAD65Ab) can be detected in the majority of new-onset T1D patients, the presence of GAD65Ab in the NOD mouse remains controversial.[25–27] Moreover, as in human T1D, the role of GAD65Ab in pathogenesis is poorly defined. While it is established that these antibodies are valuable markers for the disease progression, they are mainly viewed as innocent bystanders, resulting from the destruction of the pancreatic beta cells. However, recent studies suggest that GAD65Ab are involved in GAD65 processing and presentation and thus may modulate the immune response.[28–30] The effect of GAD65Ab on the disease progression in NOD mice was first studied by administering GAD65-specific monoclonal antibody GAD-6. The observed delayed onset of T1D in response to this treatment was attributed to possible impaired recognition of GAD65 by antigen-specific T cells.[31]

In our previous research we detected disease-specific GAD65Ab epitopes in T1D.[32,33] Our goal was to investigate the role of GAD65Ab with distinct epitope specificities in the pathogenesis of T1D. We examined possible GAD65Ab-mediated immune modulation by administrating disease-specific monoclonal GAD65Ab to young NOD mice. These GAD65Ab showed distinct epitope specificities. One of the antibodies (b96·11) recognizes an epitope located in the middle region of the molecule, which is recognized by the majority of GAD65Ab-positive sera derived from T1D patients[32,33] and has been associated with progression towards T1D.[34] Another antibody (b78) recognizes an epitope located in the C-terminal region, which is not shared by patients with T1D, but appears to be common in patients with stiff person syndrome (SPS).[35]

Materials and methods

Antibodies

Both GAD65-specific human monoclonal antibodies used in this study were derived from a patient with autoimmune polyendocrine syndrome type 2.[36] B96·11 recognizes an epitope located at amino acid residues 308–365,[36–38] while b78 recognizes an epitope located at the C-terminus of GAD65 (512–540).[35,36] Both antibodies are of the immunoglobulin G1 (IgG1) subtype and their light chains are of the lambda subclass. The IgGs show 80% similarities in the heavy chain and 79% similarities in the light chain, with the majority of differences located in the complementarity determining regions (CDRs) (Fig. 1). The antibodies were purified from supernatants of the
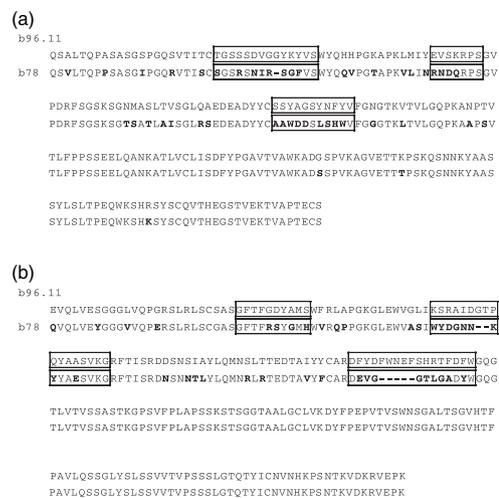

Figure 1. Alignment of light (a) and heavy (b) chains of monoclonal antibodies b96·11 and b78 (accession numbers 917308 and 917304, respectively). Complementarity determining regions (CDRs) used for homology comparison are boxed. Amino acid substitutions between the two antibodies are identified in bold font.

respective B-cell line using Protein G Sepharose (Invitrogen, Carlsbad, CA).

Polyclonal human IgG (The Binding Site, Birmingham, UK) does not contain GAD65 binding reactivity (data not shown). All antibodies were dialysed against phosphate-buffered saline (PBS) and sterile-filtered. The final concentration of the antibodies was 1 mg/ml in PBS.

Radioligand binding assay (RBA)

Recombinant human ^{35}S-GAD65 was produced in an *in vitro* coupled transcription/translation system with SP6 RNA polymerase and nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI) as described previously.[39] Briefly, animal sera (5 µl) or IgG was incubated with ^{35}S-GAD65. After an overnight incubation at 4°, antibody-bound ^{35}S-GAD65 was separated from unbound antigen with Protein A Sepharose (PAS) (Invitrogen) as a precipitating agent as previously described.[40] The immunoprecipitated radioactivity was counted on a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences, Boston, MA). In competition RBA we incubated GAD65-specific monoclonal antibody at its half-maximal binding concentration with serum from the injected animals. All samples were analysed in triplicate determinations.

Enzyme-linked immunosorbent assay (ELISA)

Detection of human antibodies. Mouse sera were analysed for the presence of human antibodies as follows: 96-well

MAXI-SORP plates (Nalge Nunc International, Rochester, NY) were coated with goat anti-human antibodies (Bethyl Laboratories, Montgomery, TX) (1 : 100) overnight at 4°. The plates were blocked with 1% bovine serum albumin (BSA) in PBS to reduce non-specific binding. Mouse serum was added to the wells and incubated for 2 hr at 37°. Human antibodies were detected by incubation with 50 µl/well peroxidase-labelled goat anti-human IgG (Bethyl Laboratories) (1 : 10 000) for 1 hr at 37°. The plates were washed and incubated with the peroxidase substrate o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St Louis, MO). The reaction was stopped with 1 M sulphuric acid solution and the plates were read using a microplate reader at 450 nm. A standard curve consisting of human IgG dilutions was included in each assay.

Detection of anti-idiotypic antibodies. The method used was as above, but human recombinant Fab or human IgG at the indicated concentrations was used for the initial coating. Mouse serum were added to the wells and incubated for 2 hr at 37°. Bound murine antibodies were detected with 50 µl (1 : 10 000) of peroxidase-conjugated goat anti-mouse IgG (Bethyl Laboratories) per well.

A standard curve for the determination of antibody levels (dilutions of goat anti-human IgG; Bethyl Laboratories) was generated for each assay. Negative controls consisted of mouse serum obtained from mice injected with PBS only.

Mice

Female NOD mice were purchased at 3–4 weeks of age (Jackson Laboratories, Bar Harbor, ME). The mice were maintained in specific pathogen-free conditions in the animal facility at the University of Washington, Seattle. All animal experimentation was approved by the Animal Care and Use Committees of the University of Washington. The animals (groups of eight) were injected intraperitoneally (i.p.) weekly with 10, 50 or 100 µg antibody or PBS. The injections started at 5 weeks of age and continued until the animals reached 35 weeks of age or developed diabetes. All animals were monitored for the development of diabetes. Hyperglycaemia was determined by weekly weighing and blood glucose level tests. Blood glucose levels were measured with a Bayer Ascensia Elite meter and strips (Bayer HealthCare Diabetes Care, Tarrytown, NY) when the animal experienced a loss of 5–10% of body weight. Diabetes was defined by weight loss of 5–10% of body weight and blood glucose levels of >300 mg/dL for two consecutive weeks.

Upon confirmation of diabetes, the animal was sedated with ketamine/xylazine and killed by heart puncture. The pancreas was perfusion-fixed in 4% paraformaldehyde and embedded in paraffin wax. Sections of 5-µm were mounted on glass slides and stained with haematoxylin and eosin for histological analysis.

Insulitis scoring

A minimum of 41 islets/group were scored for insulitis. Scoring was performed under double-blinded conditions. The degree of insulitis was graded according to the following: normal islet, score 1; perivascular/periductal infiltration, score 2; peri-insulitis, score 3; mild insulitis (< 25% of the islet infiltrated), score 4; and severe insulitis (more than 25% of the islet infiltrated), score 5.

Statistical analysis

The control animals injected with PBS or polyclonal human IgG were combined into one group, because no difference in incidence rate, age at disease onset, or degree of insulitis was detectable. Similarly, groups injected with b78 IgG (50 and 100 µg) showed no difference in incidence, age at disease onset, or degree of insulitis and were therefore combined into one group.

Weight gain in the different animal groups was compared using the Mann–Whitney U-test.

Differences in the incidence rates in the different treatment groups were compared using the χ^2 test with 1 degree of freedom, Fisher's exact test (two-sided), and permutation test (two-sided). All analyses indicated a significant difference between the control group and animals injected with 100 µg of b96·11. We report the results of the most conservative analysis, namely the χ^2 test with 1 degree of freedom.

Results

Injections with b96·11 delay the onset and reduce the incidence rate of diabetes

Animals were injected with GAD65-specific monoclonal antibody, human polyclonal antibody, or PBS. The animals were monitored weekly for diabetes development. Animals injected with PBS or human polyclonal IgG did not differ in their incidence rate of diabetes and we combined the data obtained from these animals. The mice started to develop diabetes at 7 weeks of age. Nineteen of twenty-four animals (80%) developed diabetes by week 28. Injections with 50 and 100 µg of b78 IgG yielded cumulative incidence rates of 50 and 38%, respectively, with no significant difference between the two dosage groups or with respect to the control animals.

Injections with 50 and 100 µg of b96·11 resulted in a cumulative incidence rate of 25% (two of eight animals). The two animals in the lower dose group developed diabetes at week 15, and the first animal in the 100-µg group developed diabetes at week 18 and the second animal at
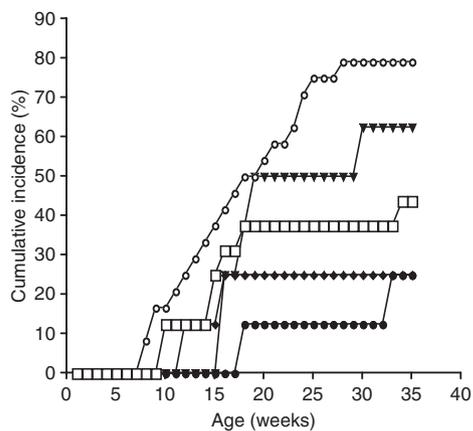
Figure 2. Cumulative incidence rate of diabetes development. Control animals were injected with phosphate-buffered saline (PBS) or human polyclonal antibody (white circles). Animals injected with 100 and 50 µg of b78 immunoglobulin G (IgG) showed no difference in disease progression and were grouped together (white squares). Animals injected with 10, 50 and 100 µg of b96·11 are represented as black inverted triangles, diamonds and circles, respectively. Animals were monitored weekly for diabetes development.

week 33 (Fig. 2). Only injections with 100 µg of b96·11 IgG resulted in a significant reduction in incidence rate and a delay in onset, as compared with the control animals ($P = 0.006$). Injections with b96·11 at the lower dosage showed only a trend towards prevention, suggesting that the effect was dose dependent. Injections with 10 µg of b96·11 induced no reduction in incidence or delay in onset. The injections with 100 µg of b96·11 IgG were repeated once more with similar results (data not shown).

GAD65Ab b96·11 reduces the severity of insulitis

The above effect was also reflected in the severity of insulitis. Insulitis was less severe in animals treated with 100 and 50 µg of b96·11 IgG as compared with the

Table 1. Degree of insulitis in non-obese diabetic (NOD) mice

	Score				
	1	2	3	4	5
b96·11, 100 µg	64	28	9	0	0
b96·11, 50 µg	54	29	15	2	0
b96·11, 10 µg	39	39	13	10	0
b78	17	34	32	15	2
Controls	22	19	15	24	19

Islets obtained from animals injected with b96·11 immunoglobulin G (IgG) (100, 50 and 10 µg) and b78 IgG, and from controls were scored as follows: normal islets, score 1; perivascular/periductal infiltration, score 2; peri-insulitis, score 3; mild insulitis (< 25% of the islet infiltrated), score 4; and severe insulitis (more than 25% of the islet infiltrated), score 5. The mean score for each group shows the percentage of islets in this group.

control group and animals injected with b78 IgG (Table 1 and Fig. 3). Insulitis severity showed a dose–response to injected b96·11 IgG.

Weight

The animals were weighed weekly to monitor their growth and potential weight loss as an early sign of diabetes. The last two weight measurements before mice were killed were removed, because the animals were killed after 2 weeks of consecutive hyperglycaemia and weight loss. The longitudinal weight measurements between groups were not significantly different (data not shown).

Presence of human IgG in mouse sera

Blood samples derived from the animals 2 weeks after the final injection were tested for the presence of human IgG by ELISA. We found a significant presence of human IgG in all animals, except in mice injected with PBS only
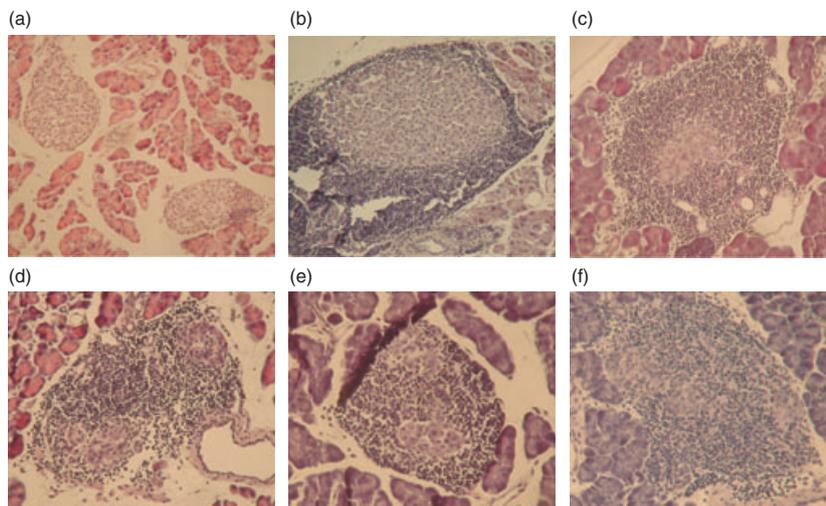

Figure 3. The histopathology of pancreatic islets at onset of diabetes or at 35 weeks of age. Representative images of pancreatic islets from animals injected with 100 µg (a), 50 µg (b) and 10 µg (c) of b96·11 immunoglobulin G (IgG), 100 µg of b78 IgG (d), 100 µg of human polyclonal IgG (e), and phosphate-buffered saline (PBS) (f). Pancreatic tissues were sectioned and stained with haematoxylin and eosin.

(data not shown). The human IgG titre in animals injected with 100 µg of IgG was twice as high (median of 200 µg/ml) as that observed in animals injected with 50 µg (median of 100 µg/ml) and 5 times higher than that in animals injected with 10 µg of IgG (median of 40 µg/ml).

However, we could not detect reactivity to GAD65 in the mouse sera, with the exception of two sera, both obtained from animals injected with 10 µg of b96·11 (data not shown).

Presence of anti-idiotypic antibodies

Because the mice were injected with human IgG, we anticipated a human-specific immune response in the animals. ELISA analysis of the mouse sera revealed the presence of anti-idiotypic antibodies specific to human Fab in all animals injected with human IgG. The levels of the anti-idiotypic antibodies correlated with the dosage of injected IgG (data not shown). The median concentration of anti-idiotypic antibodies in animals injected with 50 or 100 µg of IgG was significantly higher compared with that in animals injected with 10 µg of IgG ($P = 0.0002$). No difference in the anti-idiotypic antibody levels was found when animals injected with IgG of different specificities were compared.

Anti-idiotypic antibodies prevent binding of GAD65-specific monoclonal antibodies to GAD65

To investigate the binding specificities of the anti-idiotypic antibodies, we tested whether they interfered with the binding of b96·11 and b78 to GAD65. In competition RBA we incubated the respective GAD65-specific monoclonal antibody at its half-maximal binding concentration with serum from the injected animals (Fig. 4a). We observed that sera from b96·11-injected mice inhibited the binding of b96·11 IgG and Fab to GAD65, but not the binding of b78 IgG or Fab. Similarly, sera from b78-injected animals specifically inhibited the binding of b78 IgG and Fab to GAD65, but not that of b96·11 IgG or Fab (Fig. 4b). Sera obtained from animals injected with human polyclonal antibody, or PBS, had no effect on the binding capacity of b96·11 or b78.

The level of anti-idiotypic antibodies specific to the antigen-binding site of the GAD65Ab depended on the dosage of injected IgG (data not shown). Animals injected with 100 µg of b96·11 had significantly higher levels of anti-idiotypic antibodies interfering with GAD65 binding than animals in the 10-µg group ($P = 0.04$).

Discussion

T1D-specific monoclonal GAD65Ab b96·11 significantly reduced diabetes development in NOD mice. This effect
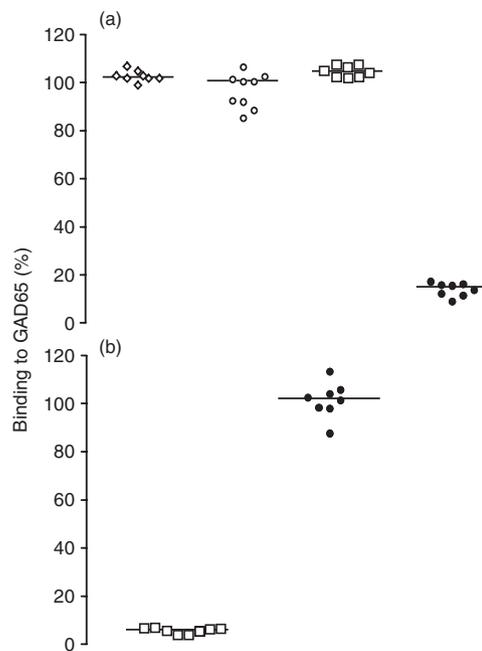

Figure 4. Effect of mouse sera on reactivity of autoantibodies to the 65-kDa isoform of glutamate decarboxylase (GAD65Ab) with GAD65. b96·11 (a) and b78 (b) were incubated at their half-maximal binding concentration with sera of animals injected with 100 µg of b96·11 (black circles), b78 (white squares), human polyclonal immunoglobulin G (IgG) (white circles), or phosphate-buffered saline (PBS) (white diamonds). The binding capacity was determined in a competitive radioligand binding assay (RBA) and is presented as per cent binding (non-competed binding equals 100%).

was antigen, epitope and dose specific, as neither administration of polyclonal human IgG nor that of GAD65-specific monoclonal antibody b78 had an effect on the development of diabetes, and only the highest dosage of b96·11 resulted in significant reduction.

Injections with human antibodies induced the formation of anti-idiotypic antibodies in the animals, some of which recognized the antigen-binding site of the monoclonal antibodies and consequently interfered with the binding to GAD65. We speculate that these anti-idiotypic antibodies present internal images of GAD65 bound by b96·11 and thus may mimic GAD65. Alternatively, the internalized GAD65/b96·11 complexes could induce a modulation of GAD65 processing, thus modifying antigen presentation.

The two GAD65-specific monoclonal antibodies were generated from the same individual but recognize different epitopes. B96·11 shares epitope specificities with GAD65Ab present in sera obtained from T1D patients,[32,33] and in a longitudinal study of healthy GAD65Ab-positive schoolchildren we showed that the specificity to the b96·11-defined epitope increases with time in high-risk children only.[34] GAD65Ab with epitopes similar to that recognized by b78 are not common in

T1D patients, but may be observed in sera from stiff person syndrome patients, where this antibody specificity correlates with the ability of the sera to inhibit GAD65 enzymatic activity.[35]

In mice, human monoclonal antibodies and their antigen complexes are probably taken up via pinocytosis, rather than via Fc-receptor binding. Earlier reports showed that human IgG injected i.p. into mice was eliminated from the animals with a half life of 2–3 days.[41] It is therefore surprising that we detected human IgG in circulation after 2 weeks. Moreover, the level of human IgG in circulation exceeds that of the injected bolus, suggesting that the IgG accumulates in the animals. The mechanism by which the antibodies are retained in the animals and the location of the antibody other than in blood need to be further investigated. Our initial efforts to localize the antibody in the animals were restricted to the pancreas. We did not find significant amounts of human antibody in the islet or elsewhere in the pancreas.

The injection with human IgG triggered the development of significant amounts of anti-idiotypic antibodies in the animals, some of which inhibited the binding of GAD65 by the respective monoclonal antibody or its Fab. Our data suggest that the anti-idiotypic antibodies recognize the GAD65-binding domain of their respective monoclonal antibodies, thus serving as an internal-image antibody. The anti-idiotypic antibodies may mimic GAD65 administration as the internal image binds to the idiotypic antibodies. We are currently developing monoclonal anti-idiotypic antibodies specific to b96·11 to test our hypothesis that these anti-idiotypic antibodies interfere with the pathogenesis of T1D.

The injection of b96·11 monoclonal antibodies may also affect the processing and presentation of GAD65, as was suggested in earlier studies. Antibody-induced modulation of antigen processing may affect the presentation of the antigen to auto-reactive T cells. Experiments to detect changes in GAD65-specific T-cell responses in the NOD mice are necessary to test this hypothesis.

The role of anti-idiotypic antibodies in the regulation of the immune system was suggested first in the network hypothesis by Jerne,[42] who proposed that idiotypes and anti-idiotypes create a homeostasis of the adaptive immune response. The presence of anti-idiotypic antibodies has been described for a number of autoantibodies[43] and autoimmune diseases (for a review, see Rossi *et al.*[44]). Anti-idiotypic antibodies may bind the antigen-binding site of the antibody and thus represent the internal image of the antigen. Supporting this hypothesis is the finding that the anti-idiotypic antibody serum levels correlate inversely with those of autoantibodies in several autoimmune diseases.[45–47] The presence of anti-idiotypic antibodies in healthy individuals that successfully block the binding of pathogenic autoantibodies has been demon-strated, suggesting that these anti-idiotypic antibodies regulate potential pathogenic autoantibodies.[48,49] Moreover, the successful treatment of autoimmune disorders with intravenous immunoglobulin (IVIG) has partially been explained by the presence of anti-idiotypic antibodies (for reviews, see Kazatchkine and Kaveri,[50] and Sapir and Shoenfeld[51]). IVIG treatment in T1D patients was administered with different results. In new-onset T1D patients the treatment produced an improvement with regard to C-peptide levels and two out of eight patients ceased to require insulin treatment.[52] In established T1D patients, IVIG did not show a change in remission frequency or beta cell function.[53] Recent studies suggest that the successful treatment of SPS patients with IVIG induces a reduction in GAD65Ab titres.[54] Moreover, insulin-mimicking anti-idiotypic antibodies have been described in the development of T1D in another animal model of the disease, namely the BioBreeding rat (BB rat).[55]

Our study may suggest that the humoral immune response in T1D is more complex than usually perceived.

Acknowledgements

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