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Autoantigen-based immunotherapeutics have been shown to activate regulatory responses capable of inhibiting T cell-mediated autoimmune disease in animal models. However, their efficacy generally declines, as treatment occurs later in the disease process, and their mechanism of action is a matter of intense debate. Here, we report that the early administration of β cell autoantigens (βCAAs) to nonobese diabetic (NOD) mice broadly diverts the natural development of potentially pathogenic Th1-biased autoimmune responses toward the Th2 phenotype through Th2 spreading. With disease progression, there was a steady decline in the ability of βCAA treatment to promote Th2-type cellular and humoral autoimmunity. Late in the disease process, some βCAAs were still able to induce Th2 responses and Th2 spreading (although to a much lesser extent), while other autoantigens were not. This attenuation of inducible Th2 immunity with disease progression is likely to reflect a reduction in the availability of uncommitted autoantigen-reactive precursor T cells. These findings suggest that there are inherent differences in the frequency of βCAA-reactive T cells and that, in advanced stages of autoimmune disease, regulatory responses may be best elicited with target tissue Ags against which large uncommitted T cell pools are still available. Since individuals presenting the first signs of autoimmune disease are likely to already have an advanced disease process, these findings may be useful for the rational design of Ag-based immunotherapeutics. *The Journal of Immunology*, 1998, 161: 5399–5403.

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tudies of T cell-mediated autoimmune diseases in animal models suggest that proinflammatory Th1 responses mediate the disease process (1–5). While the initial autoreactive T cell response is limited in its specificity, with disease progression, it gradually expands to involve additional target tissue Ags (2, 5–9). Autoantigen administration in modes that induce antiinflammatory Th2 or other regulatory responses is associated with the inhibition of autoimmune disease progression in animal models of organ-specific autoimmune disease (4, 9–16) although the mechanism(s) underlying this protection is a matter of intense debate (17–19).

Unlike in animal models, it is not yet feasible to identify individuals who are in the earliest stages of an autoimmune disease process and begin prophylactic treatment. Indeed, individuals who are presenting the first clinical signs of an autoimmune disease, or who are determined to be at high risk of developing autoimmune disease based on autoantibody screening, are likely already to have an advanced autoimmune disease process. However, autoantigen-based immunotherapies are generally less effective when administered later in the disease process (20, 21). Furthermore, while early treatment of young prediabetic nonobese diabetic (NOD) mice with several different β cell autoantigens (βCAAs) effect-

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Materials and Methods

Mice and treatments

NOD mice (Taconic Farms, Germantown, NY) were bred under specific pathogen-free conditions. Newborn mice were treated on day one and three with 200 µg of control or βCAAs i.p. in 50% IFA (Life Technologies, Gaithersburg, MD). Six-week-old and newly hyperglycemic animals (~18 wk in age, with blood glucose >250 mg/dl) received 200 µg of control or

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3 Abbreviations used in this paper: NOD, nonobese diabetic; βCAA, β cell autoan-
tigen; IDDM, insulin-dependent diabetes mellitus; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; HSP, heat shock protein; β-gal, β galactosidase; SFC, spot-forming colonies; ELISPOT, enzyme-linked immunospot; HRP, horseradish peroxidase.
βCAAs i.p. in 50% IFA, and again 10 days later. Splenic T cells from mice treated neonatally and at 6 wk in age were analyzed by ELISPOT when the mice reached 12 wk in age. Splenic T cells from mice that were treated at the onset of hyperglycemia (that remained outwardly healthy and were not treated with insulin) were analyzed 4–5 wk after the initial treatment. Only female mice were used in these studies.

Antigens
Mouse glutamic acid decarboxylase (GAD) and control Escherichia coli β-galactosidase were purified as previously described (2). The immunodominant heat shock peptide 277 (HSP) has been described elsewhere (20). Insulin B chain, which contains insulin’s immunodominant determinant (22, 23), was purchased from Sigma (St. Louis, MO).

ELISPOT
Splenic T cells were isolated from individual β-gal- and βCAA-treated mice, as well as from unmanipulated aged-matched NOD mice, and the frequency of Ag-specific T cells secreting IFN-γ, IL-4, and IL-5 was determined using a modified ELISA spot technique (5, 24). Briefly, 10^6 splenic mononuclear cells were added per well (in triplicate) of an ELISPOT plate (Millipore, Bedford, MA) that had been coated with cytokine capture Abs and incubated with peptide (20 μM) or whole protein (100 μg/ml) 24 h for IFN-γ, or 40 h for IL-4 and IL-5 detection. After washing, biotinylated detection Abs were added, and the plates were incubated at 4°C overnight. Bound secondary Abs were visualized using HRP-streptavidin (DAKO, Carpinteria, CA) and 3-amino-9-ethylcarbazole. Abs R4-6A2/XMG 1.2-biotin, 11B11/BVD6-24G2-biotin, and TRFK5/TRFK4-biotin (PharMingen, San Diego, CA) were used for capture and detection of IFN-γ, IL-4, and IL-5, respectively.

Autoantibody characterization
Sera were collected at the time of sacrifice, and the isotype of GAD autoantibodies was characterized using an ELISA assay as previously described (5). Briefly, GAD (BioSyn, Stockholm, Sweden) was bound to 96-well plates (Nunc, Roskilde, Denmark) at 10 μg/ml in 0.1 M NaHCO3 (pH 8.5) at 4°C overnight. The wells were rinsed with PBS and then blocked with 3% BSA in PBS for 1 h. Mouse sera were added (0.1 ml of a 1:500 dilution) and incubated 1 h at 37°C. Following washing, bound Ig was characterized using affinity-purified HRP-coupled goat anti-mouse IgG1 (Promega, Madison, WI) or whole protein insulin B chain (Sigma) or HRP-coupled goat anti-mouse IgG2a (Southern Biotechnology Associates Birmingham, AL) and ABTS. Sera from untreated BALB/c and AKR mice were used as negative controls.

Results
Responses to nontarget tissue Ags are unaffected by the disease process
It is unknown whether the gradual expansion of the proinflammatory autoimmune response during disease progression in NOD mice affects their ability to respond to foreign Ags. Since the cascade of spontaneous autoreactive T cell responses is limited to target tissue Ags (5), we surmised that the disease process should have little impact on T cell immunity to nontarget tissue Ags. To test this supposition, NOD mice were treated neonatally, at 6 wk in age, or at the onset of hyperglycemia, with a control foreign Ag (β-gal or a hen egg lysozyme peptide (HEL,11–25)) in IFA, an adjuvant that promotes vigorous Th2-biased responses (24). The mice developed polarized Th2 responses to the injected Ag; i.e., only IL-4- and IL-5- (and no IFN-γ) secreting T cells were detected. Notably, the magnitude of the Th2 response to the injected foreign Ag was similar, regardless of what stage of the disease process the NOD were immunized with the Ag (Fig. 1). Thus, the ability of NOD mice to mount Th2 responses to foreign Ags is not affected by the initiation or the progression of their spontaneous autoimmune disease process.

Attenuation of inducible Th2 responses to target tissue autoantigens with disease progression
Unmanipulated NOD mice did not display detectable spontaneous Th2-type splenic T cell responses to βCAAs at any age (see below). In contrast, NOD mice treated with a βCAA just after birth developed vigorous Th2 responses to the injected autoantigen that, however, varied in frequency depending on the administered βCAA (Fig. 1). When treatment occurred shortly after the onset of insulinitis at 6 wk in age, Th2 responses to the injected βCAA were about half as frequent as those elicited following neonatal treatment. When treatment was further delayed until the onset of hyperglycemia, the frequency of Th2 cells responding to the injected βCAA were only 32% and 10% of that which was induced following neonatal treatment with GAD and HSP, respectively. No detectable Th2 responses were elicited by insulin B chain treatment at this late stage of the disease process. Thus, βCAAs vary in their ability to prime Th2 responses, and there is a steady decline in their ability to induce Th2 responses with disease progression. While it is possible that the reduction in detectable primed Th2 responses is due to alterations in the migration and distribution of βCAA-reactive T cells with disease progression, we do not favor this explanation since the response to non-target tissue Ags was unaffected by the disease process.

Diminished Th2 spreading with disease progression
Early treatment with βCAAs (at birth and at 6 wk in age) not only induced Th2 immunity to the injected Ag, but also led to the development of Th2 responses to other unrelated βCAAs, creating an amplificatory cascade of this antiinflammatory limb (Fig. 2). Thus, treatment with GAD induced GAD-specific Th2 responses and led to the development of Th2 immunity to HSP and insulin. Similarly, early treatment with HSP led to the development of Th2 immunity to βCAAs. This effect was attenuated if treatment occurred later in disease progression, with the frequency of Th2 cells responding to other unrelated βCAAs (at birth and at 6 wk in age) not only induced Th2 immunity to the injected Ag, but also led to the development of Th2 responses to other unrelated βCAAs, creating an amplificatory cascade of this antiinflammatory limb (Fig. 2). Thus, treatment with GAD induced GAD-specific Th2 responses and led to the development of Th2 immunity to HSP and insulin. Similarly, early treatment with HSP led to the development of Th2 immunity to βCAAs. This effect was attenuated if treatment occurred later in disease progression, with the frequency of Th2 cells responding to other unrelated βCAAs being reduced.

FIGURE 1. Attenuation of inducible Th2 immunity to βCAAs with disease progression. Splenic T cells from NOD mice that were treated at birth, at 6 wk in age, or at the onset of hyperglycemia (~18 wk in age) with control non-target tissue Ags (β-gal or HEL,11–25) or βCAAs (GAD, HSP, or insulin B chain) were isolated, and the frequency of T cells secreting IL-4 in response to the injected Ag was determined by ELISPOT (5, 24). The data are represented as the mean number of IL-4-secreting spot-forming colonies (SFC) per 10^6 splenic T cells. The background level was ~5 SFC. The individual variation within each group was less than 15%. Experimental and control mice were tested simultaneously (in triplicate) in two separate experiments (n = 5 for each group). A similar pattern was observed for IL-5-secreting, Ag-reactive T cells (data not shown). Spontaneous IL-4 and IL-5 responses by splenic T cells from unmanipulated and IFA- (alone) treated, age-matched NOD and BALB/c mice to non-target tissue Ags and βCAAs were at background levels (data not shown).
Ag-specific Th2 responses tended to promote more extensive why GAD treatment could induce Th2 spreading to HSP, but not committed insulin-reactive T cells at this late stage and explaining was less than 15%. Experimental and control mice were tested simultaneously (in triplicate) in two separate experiments (n = 5 for each group). A similar pattern was observed for IL-5-secreting, Ag-reactive T cells. Control non-target tissue Ags primed vigorous Th2 responses to the injected Ag, which failed to spread to βCAAs (data not shown).

FIGURE 2. Th2 spreading declines with disease progression. Splenic T cells from mice treated at different stages of the disease process with control Ags or βCAAs were tested for Ag-induced IL-4 and IL-5 T cell responses by ELISPOT. The data are represented as the mean number of IL-4-secreting spot-forming colonies (SFC) per 10^6 splenic T cells. The background level was ~5 SFC. The individual variation within each group was less than 15%. Experimental and control mice were tested simultaneously (in triplicate) in two separate experiments (n = 5 for each group). The data are represented as the mean number of IFN-γ-secreting spot-forming colonies (SFC) per 10^6 splenic T cells. The background level was ~5 SFC. The individual variation within each group was less than 15%. Experimental and control mice were tested simultaneously (in triplicate) in two separate experiments (n = 5 for each group). The magnitude of IFN-γ responses to βCAAs in NOD mice that had been treated with β-gal at different ages was indistinguishable from that of IFA-treated or age-matched, unmanipulated NOD mice (data not shown).

immunity to GAD and insulin, and treatment with insulin led to the development of Th2 responses to GAD and HSP. The Th2 immunity induced by βCAA treatment did not spread to non-target tissue self Ags, and primed Th2 responses to non-target tissue Ags did not spread to βCAAs (data not shown). Thus, the spreading of Th2 immunity was restricted to target tissue Ags.

At each stage of the disease process, βCAAs that primed greater Ag-specific Th2 responses tended to promote more extensive spreading of Th2 immunity to other βCAAs. As the ability of βCAA treatment to prime Th2 responses declined with disease progression, there was a corresponding reduction in the spreading of Th2 immunity. While all βCAAs induced Th2 spreading following treatment at birth and (to a lesser extent) at 6 wk in age, when treatment occurred at the onset of hyperglycemia, only GAD treatment induced the spreading of Th2 immunity (to HSP, but not to insulin, Fig. 2). Treatment with HSP induced only low Th2 responses to itself, with no detectable Th2 spreading to other βCAAs. Treatment with insulin B chain failed to induce Th2 immunity to insulin or to other βCAAs, suggesting a paucity of uncommitted insulin-reactive T cells at this late stage and explaining why GAD treatment could induce Th2 spreading to HSP, but not to insulin. Thus, the ability of βCAAs to prime Th2 immunity and Th2 spreading depends both on the Ag and the stage of the disease process.

Early, but not late, Ag treatment curtails the recruitment of autoreactive Th1 cells

Splenic T cells from unmanipulated NOD mice displayed pure Th1-type spontaneous immune responses to βCAAs at all stages of the disease process, and the induction of the Th2 immunity to nontarget tissue Ags (β-gal or HEL) at any age did not affect the development of these spontaneous autoimmune responses (Fig. 3, and data not shown). However, early treatment with a βCAA (at birth or at 6 wk in age) inhibited the development of Th1-type reactivity to the injected Ag (e.g., following treatment with GAD or HSP, Th1 responses to the injected Ag were about half of those in age-matched β-gal-treated mice). Furthermore, early βCAA treatment also inhibited the development of Th1-type reactivity to other un.injected βCAAs (Fig. 3). Notably, early treatment with GAD (which promoted the most extensive Th2 spreading; Fig. 2) most effectively inhibited the development of Th1-type reactivity against other βCAAs. HSP treatment (which induced less Th2 immunity) did not reduce Th1 reactivity to other βCAAs as effectively as GAD but was more effective than insulin B chain treatment (which induced the least Th2 immunity). In contrast, when βCAA treatment was delayed until near to the onset of hyperglycemia, it had little, or no, impact on Th1-type autoimmunity to the injected Ag or to unrelated βCAAs (Fig. 3). Thus, early, but not late, βCAA treatment can broadly curtail the recruitment of βCAA-specific T cells into the autoreactive Th1 limb, presumably through inducing Th2 bystander suppression of Th1 development and/or guiding the development of uncommitted βCAA-reactive T cells toward the Th2 phenotype.

A gradual reduction in the level of primed anti-βCAA humoral responses parallels the attenuation of inducible Th2 immunity

We next investigated to what extent Ag-based immunotherapy affected the development of humoral autoimmune responses. All NOD mice that were treated with a control Ag (β-gal) had low levels of Abs to GAD, regardless of the age at which they were treated. These Abs were predominantly of the IgG2 isotype and were at similar levels to those in unmanipulated NOD mice (data not shown). In contrast, mice treated neonatally with GAD displayed high levels of GAD Abs. These Abs were predominately of the IgG1 isotype (Fig. 4), which is indicative of Th2 help (25). As treatment was administered at later stages of the disease process, the level of primed IgG1 Abs to GAD declined, paralleling the attenuation of inducible Th2 cellular immunity with disease progression. Notably, mice that had been treated neonatally or at 6 wk
from their different patterns of expression and the extent to which responses can be experimentally primed to these Ags. IgG1 Abs to GAD using an ELISA assay (12). The background OD was between 4 – 6 samples. Experimental and control sera were tested simultaneously in two separate assays. The variance in absorbance values between triplicate samples from 4 – 6 mice. Experimental and control sera were tested simultaneously in two separate assays. The variance in absorbance values between triplicate samples from the two sets of experiments was <8%. Humoral responses to GAD in control NOD mice treated with β-gal were similar to those of unmanipulated NOD mice. Abs to GAD and insulin in sera from untreated BALB/c and AKR mice were at background levels (data not shown).

in age with insulin B chain also displayed increased levels of IgG1 Abs to GAD, consistent with the intermolecular spreading of Th2 immunity to GAD. Late in the disease process, insulin B chain treatment did not promote detectable Abs to GAD, as would be expected by the inability of this treatment to induce Th2 immunity at this stage (Figs. 1, 2). Thus, Ag-based immunotherapy can promote humoral autoimmune responses to uninjected target tissue autoantigens, but this effect declines with disease progression.

Discussion

The clonal deletion of self-reactive T cells during their thymic development is the major mechanism by which self tolerance is established (26). However, both central and peripheral tolerance induction are not fully effective since potentially self-reactive T cells, particularly those that are specific for Ags that are expressed only in a limited fashion in the periphery, are allowed to persist (27–32). Thus, the pattern of self Ag expression should determine the frequency of potentially reactive precursor T cells. Indeed, transgenic animal models of autoimmune disease have shown that neoantigens that are expressed at low levels in peripheral tissues often have little impact on T cell education and elicit strong immune responses after immunization (29, 32–35). Consistent with these notions, our data demonstrate that there are inherent differences in the frequency of spontaneously primed autoimmune responses to different βCAAs, as well as the extent to which T cell responses can be experimentally primed to these Ags.

The observed higher frequency of spontaneous and inducible T cell responses to GAD compared with HSP and insulin may stem from their different patterns of expression and the extent to which tolerance has been induced to these Ags; GAD is not expressed in the thymus and only at low levels in a few peripheral tissues, while HSP and (pro-) insulin are expressed in the thymus (36) and are ubiquitous in the periphery. Other factors contributing to the differences in immune responses to βCAAs may include 1) differences in positive selection of potentially reactive T cells; 2) Ir gene-dependent preferences in Ag presentation; and 3) the greater number of determinants within the larger whole GAD protein. However, there are clear differences between the immunogenicity of the HSP and insulin B chain peptides, and both these peptides are less immunogenic than GAD peptide 35 (5).

In addition, we show that the extent of an autoimmune disease process can have a profound impact on the degree to which regulatory responses can be primed to target tissue autoantigens. The early administration of βCAAs (neonatally, or just after the onset of hyperglycemia) induces vigorous Th2 responses to the injected βCAA and broadly diverts the natural development of Th1-biased autoimmune responses to other βCAAs toward the Th2 phenotype through Th2 spreading. While immune responses to foreign Ags are unaffected by the disease process in NOD mice, there is a progressive decline in the ability of each autoantigen to promote Th2 immunity with disease progression. Late in the disease process, some βCAAs were still able to induce Th2 responses and Th2 spreading (although to a lesser extent), while other autoantigens could not. Accordingly, the ability of Ag treatment to modulate βCAA-specific Th1/Th2 balances greatly declines with disease progression. The attenuation of inducible Th2 immunity to βCAAs (but not to non-target tissue Ags) with disease progression is likely to reflect an exhaustion of naïve βCAA-reactive T cells as they are recruited into the spontaneous cascade of autoreactive Th1 responses. This reduction in primable Th2 responses suggests that the rate at which naïve βCAA-reactive T cells are spontaneously recruited and committed to the Th1-biased autoimmune response exceeds the rate at which they are replenished by the thymus or by regeneration in the periphery.

We previously demonstrated that Th2 spreading following neonatal autoantigen treatment can lead to the broad amplification of humoral immunity to un.injected target tissue autoantigens (5, 9). Here, we show that this amplification of humoral autoimmune responses declines as the autoantigen treatment is administered at later stages of the disease process, paralleling the attenuation of inducible Th2 responses with disease progression. Thus, with the diminution of primed Th2 help, the development of IgG1 responses to both injected and un injected βCAAs trailed off. Such diversification of humoral autoimmune responses after Ag-based immunotherapy could potentially lead to unforeseen pathologies. A sobering study recently observed that the induction of Th2 responses against an oligodendrocyte cell surface protein may exacerbate experimental autoimmune encephalomyelitis through an Ab-mediated mechanism (37). However, there is little additional evidence of autoantibodies exacerbating T cell-mediated autoimmune diseases, and the IgG1 autoantibodies induced in this study by Ag treatment are thought to be very inefficient in fixing complement and mediating Ab-dependent cell cytotoxicity. Indeed, the plethora of anti-β cell autoantibodies associated with IDDM appear to be nonpathogenic, and high autoantibody levels (whether naturally occurring or induced by Ag-based immunotherapy) are actually associated with a lack of disease progression in NOD mice and man (12, 16, 38–40).

While early treatment with all of the βCAAs used in this study has been shown to efficiently prevent disease in NOD mice, these βCAAs vary in their ability to prolong the survival of transplanted syngeneic β cells in diabetic NOD mice (12). Notably, the ability...
of different autoantigens to induce Th2 immunity late in the disease process correlates with the extent to which their administration protects the transplanted islets. Furthermore, Sarvetnick and colleagues have recently shown that the protective effects of an IL-4 transgene that is expressed in the β cells of NOD mice is dependent on the availability of a large population of naive T cells (41). These observations suggest that the spreading of Th2 immunity among naive target tissue Ag-reactive T cells may be an important mechanism underlying the efficacy of Ag-based immunotherapeutics. If another cell type other than Th2 cells actually mediates this protection, it is likely that this population will follow dynamics similar to those which we have observed for Th2 cells.

Prophylactic treatment during the earliest stages of human autoimmune diseases such as IDDM and multiple sclerosis is not yet feasible, making it crucial to develop therapeutics that are effective late in the disease process. Our findings suggest that treatment with target tissue Ags against which large, uncommitted T cell pools exist, may elicit more extensive regulatory responses. Accordingly, rare target tissue Ags, cryptic βCAA determinants, or altered peptide ligands thereof may provide more effective Ags for immunotherapy late in an autoimmune disease process.

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