Differential Effects of Anti-B7-1 and Anti-B7-2 Monoclonal Antibody Treatment on the Development of Diabetes in the Nonobese Diabetic Mouse

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Summary

Insulin-dependent diabetes mellitus (IDDM) is thought to be an immunologically mediated disease resulting in the complete destruction of the insulin-producing islets of Langerhans. It has become increasingly clear that autoreactive T cells play a major role in the development and progression of this disease. In this study, we examined the role of the CD28/B7 costimulation pathway in the development and progression of autoimmune diabetes in the nonobese diabetic (NOD) mouse model. Female NOD mice treated at the onset of insulitis (2–4 wk of age) with CTLA4Ig immunoglobulin (Ig) (a soluble CD28 antagonist) or a monoclonal antibody (mAb) specific for B7-2 (a CD28 ligand) did not develop diabetes. However, neither of these treatments altered the disease process when administered late, at >10 wk of age. Histological examination of islets from the various treatment groups showed that while CTLA4Ig and anti-B7-2 mAb treatment blocked the development of diabetes, these reagents had little effect on the development or severity of insulitis. Together these results suggest that blockade of costimulatory signals by CTLA4Ig or anti-B7-2 acts early in disease development, after insulitis but before the onset of frank diabetes. NOD mice were also treated with mAbs to another CD28 ligand, B7-1. In contrast to the previous results, the anti-B7-1 treatment significantly accelerated the development of disease in female mice and, most interestingly, induced diabetes in normally resistant male mice. A combination of anti-B7-1 and anti-B7-2 mAbs also resulted in an accelerated onset of diabetes, similar to that observed with anti-B7-1 mAb treatment alone, suggesting that anti-B7-1 mAb's effect was dominant. Furthermore, treatment with anti-B7-1 mAbs resulted in a more rapid and severe infiltrate. Finally, T cells isolated from the pancreases of these anti-B7-1–treated animals exhibited a more activated phenotype than T cells isolated from any of the other treatment groups. These studies demonstrate that costimulatory signals play an important role in the autoimmune process, and that different members of the B7 family have distinct regulatory functions during the development of autoimmune diabetes.

Diabetes is an autoimmune disease that results in the destruction of the insulin-producing islet cells (1). Despite the development of new tools for the identification of individuals who are at risk for developing insulin-dependent diabetes mellitus (IDDM),[1] there are limited therapeutic options to offer these future patients. Thus, current attempts to develop useful immunosuppressive therapies depends on a more complete understanding of the pathogenesis of disease.

Illness in the nonobese diabetic (NOD) mice shares many common features with human IDDM, and this mouse strain has provided an important model for dissecting the pathogenesis of autoimmune diabetes (2, 3). NOD mice spontaneously develop insulitis early in life (between 2 and 4 wk of age). However, it is not until 10–20 wk later that this insulitis progresses to diabetes in ~80% of the female mice and in only 20% of the male mice. As in human IDDM, there is extensive evidence supporting a role for T cells and

[1] *Abbreviations used in this paper:* CtIg, control Ig; GAD, glutamic acid decarboxylase; H&E, hematoxylin and eosin; IDDM, insulin-dependent diabetes mellitus; MFI, mean fluorescence intensity; NOD, nonobese diabetic.

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MHC-restricted self-antigen recognition in the development of disease. First, T cells are among the earliest infiltrating cells in diseased islets (4, 5). Treatment of NOD mice with antibodies directed at T cell surface molecules such as CD4, CD8, and the TCR/CD3 complex prevent the development of disease and, in the case of anti-CD3 mAbs, blocked the progression of diabetes in a diabetic animal (6–8). Furthermore, diabetes can be precipitated by adoptively transferring an islet-specific clone into young NOD mice (9, 10).

One hypothesis for the development of autoimmune disease is that the disruption of the normal mechanisms of peripheral tolerance may occur. For instance, recent studies have shown that the development of insulitis and diabetes in the NOD mouse correlates with the acquisition of T cell reactivity to glutamic acid decarboxylase (GAD) and a series of other islet antigens (11, 12). Indeed, NOD mice rendered tolerant to GAD by intravenous or intrathymic injection showed a lower incidence of IDDM compared with control animals. Unfortunately, the therapeutic potential for tolerizing islet-specific T cells before their encounter with antigen is limited. Therefore, efforts have been devoted to altering the functional activity of autoreactive T cells after antigen recognition in an attempt to promote a tolerant rather than an activated state. Recent work has demonstrated that in addition to TCR engagement by antigen, a second signal, known as costimulation, is also required for T cell activation (13, 14). Blockade of this costimulatory signal results in the induction of a state of antigen-specific nonresponsiveness known as anergy (13). Thus, one potential approach to inducing autoreactive T cells into a tolerant state might be to block these costimulatory events. Studies performed in transgenic mice support this possibility. The ecotopic expression of MHC class I or II molecules on nonprofessional APCs, such as the islets of Langerhans, is not enough to activate potentially autoreactive T cells (15, 16). However, the coexpression of the appropriate costimulatory molecules, such as B7-1, on the islets activates these cells and results in the autoimmune destruction of the islets (17–19). Therefore, the potential to regulate T cell costimulation provides a potent new approach to altering the functional activity of autoreactive T cells.

While the costimulatory signals involved in IDDM are currently unknown, there are many cell surface molecules that may deliver the necessary costimulatory signal. Evidence suggests that the major T cell costimulatory pathway involves the CD28-B7 family of costimulatory molecules. CD28 is expressed on the majority of naive and memory T cells (20, 21). Activation of T cells with anti-CD28 mAb blocks the induction of anergy and synergizes with anti-CD3 stimulation to increase both T cell proliferation and lymphokine production in vitro (22–25). Furthermore, F(ab) fragments of anti-CD28 inhibit the activation of T cells and, in some instances, renders them anergic (22).

There are two known natural ligands for CD28. B7-1 was the first ligand to be identified and is expressed on "professional" APC, such as dendritic cells, macrophages, and activated B cells (26–30). In vitro studies using B7-1 transfectants demonstrated that B7-1 costimulated both antigen- and mitogen-driven T cell proliferation and IL-2 production by interacting with CD28 (31, 32). Recently, we and others have identified a second CD28 ligand, B7-2, which is also expressed on "professional" APC (33–36). Cloning of the B7-2 molecules has revealed sequence similarity to B7-1, and it has been shown to bind to the CD28 molecule (37–39). Antibodies to B7-2 are more effective than anti-B7-1 mAb at blocking T cell responses to natural APC, such as in an allogeneic mixed lymphocyte reaction (40, 41). Furthermore, prolongation of allogeneic islet graft survival occurred under the cover of anti-B7-2 but not anti-B7-1 mAb treatment, suggesting that the B7-2 costimulatory molecule plays a more dominant role than B7-1 in this immune response (Zeng, J., Lenschow, D. J., and Bluestone, J. A., manuscript submitted for publication).

CTLA-4 is a cell surface molecule, with sequence homology to CD28, expressed on activated $CD4^+$ and $CD8^+$ T cells (42, 43). Although CTLA-4 may not function as a costimulatory molecule, a soluble fusion protein comprising the extracellular domain of CTLA-4 and the Fc portion of human IgG1 constant region, CTLA4Ig, binds both B7-1 and B7-2 and has been used to inhibit a variety of CD28-dependent immune responses, including in vivo antibody responses and allogeneic and xenogeneic graft rejection (44–46). In fact, in a xenogeneic islet transplant model, blockade of the CD28/B7 costimulatory pathway with CTLA4Ig led to the induction of donor-specific tolerance (46).

In this study, we examined the role of the CD28/B7 signaling pathway in the initiation and propagation of autoimmune diabetes in the NOD mouse. While blockade of this costimulatory pathway with either CTLA4Ig or anti-B7-2 mAb prevented disease development, treatment with anti-B7-1 or a combination of anti-B7-1 and anti-B7-2 mAbs resulted in a more rapid onset of disease in both female and male mice. These results indicate that the CD28/B7 costimulatory pathway is involved in the control of this autoimmune response and may provide a powerful target to alter the function of autoreactive T cells and human disease progression.

Materials and Methods

Mice. NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, Inc. (Germantown, NY) and were bred in a specific pathogen-free animal facility at the University of Chicago. C57BL/6 (B6) mice were purchased from The Jackson Laboratory and were maintained in a pathogen-free animal facility at the University of Chicago.

Antibodies. In initial studies, human CTLA4Ig and a control fusion protein, L6Ig, were provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA) (47). Later studies were performed with CTLA4Ig provided by Repligen Corp. (Cambridge, MA) (33) and a control human Ig purchased from Sigma Chemical Co. (St. Louis, MO). Similar results were obtained with both sets of reagents. The hamster anti-murine B7-1 mAb (16-10A1) was provided by Repligen Corp. The rat anti-murine B7-1 mAb (1G10) was generated as previously described (48). The rat anti-murine B7-2 mAb (GL1) was produced in an Acusyst Jr. bioreactor (Coons

River, MN) and was purified by passage over a protein G–coupled Sepharose column (35). FITC-coupled anti-Thy1 and anti-B220 mAbs and biotin-coupled anti-CD4, anti-CD8, and anti-CD69 were purchased from PharMingen (San Diego, CA). PE-coupled streptavidin was purchased from Southern Biotechnology Associates (Birmingham, AL).

Treatment Protocol. Groups of NOD mice were treated with CTLA4Ig, anti-B7-1 mAbs (16-10A1 and 1G10), anti-B7-2 mAb (GL1), and control Abs (L6Ig and CtIg) as follows. Male and female NOD mice between 2 and 3 wk of age were treated with 50 μg of either control Abs (L6Ig or CtIg), CTLA4Ig, anti-B7-1 mAb (1G10 or 16-10A1), or anti-B7-2 mAb (GL1) every other day for 14 d. Animals then received one additional 50-μg dose at weeks 6, 7, and 8. Animals receiving both anti-B7-1 (16-10A1) and anti-B7-2 mAb treatment received 50 μg of each mAb. To test the effects of these reagents on later stages of the disease, female NOD mice were treated with 50 μg of the above reagents three times per week starting at 80 d of age for ~10 wk, or until the animals became diabetic. Animals treated with control antibodies beginning at 2 wk of age showed a slight delay in the development of diabetes (12 wk) compared with untreated mice (10 wk) although the penetrance of disease was the same by 24 wk of age, with nearly 90% of both control treated and untreated female mice becoming diabetic by 24 wk of age.

Assessment of Diabetes. Starting at 7 wk of age, diabetes was assessed by weekly measurements of blood glucose levels by use of a glucose meter (One Touch II; Lifescan, Inc., Milpitas, CA). Glucose strips were kindly provided by Lifescan, Inc. Animals were considered diabetic after two consecutive measurements >250 mg/dl. Onset of diabetes was dated from the first of the sequential diabetic measurements.

Histological Analysis. The pancreases from killed animals were fixed in buffered formalin and embedded in paraffin. 4-μm sections were cut and hematoxylin and eosin (H&E) staining was performed. To determine the severity of insulitis in the various treatment groups, four to six animals per time point (4, 8, and 12 wk) were analyzed, and multiple tissue sections of the pancreases for each animal were scored for insulitis. At least 50 islets were counted per time point. Islets were scored blindly and found to be either free of insulitis (score = 0), exhibiting periinsulitis (lymphocytes surrounding the islets and ducts but not infiltrating the islet architecture; score = 1), exhibiting moderate insulitis (lymphocytes infiltrating <50% of the islet architecture; score = 2), or exhibiting severe insulitis (>50% of the islet tissue infiltrated by lymphocytes; score = 3). Mean clinical score = severity score × number islets in that category/number of mice.

Pancreatic Lymphocyte Isolation. Pancreases from animals within the same treatment group were minced into 2–4-mm fragments. These fragments were then digested in an enzyme mixture containing 5 mg of hyaluronidase (Sigma Chemical Co.), 1,500 U of DNase (Sigma Chemical Co.), and 50 mg of collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 50 ml of complete media (DMEM, 10% FCS, 2 mM L-glutamine, 25 μM Hepes buffer, 100 U penicillin, 100 μg/ml streptomycin, 2 mM nonessential amino acids, and 5×10^{-5} M 2-ME). This mixture was incubated at room temperature for 60 min with constant stirring, then 1 ml of FCS was added, and the mixture was incubated for an additional 60 min. A single-cell suspension was obtained by filtering through a nytex screen. The cells were washed twice, resuspended in 10 ml media, and then fractionated on a discontinuous BSA density gradient by layering 10 ml of 35% BSA (Sigma Chemical Co.) under 10 ml of 24% BSA, which had been layered under the 10 ml of complete media containing the cells. After centrifugation at 1,400 rpm for 40 min, the interface between the 35 and 24% layers was removed, washed twice, and then analyzed by FACS® (Becton Dickinson & Co., Mountain View, CA) for subsets and activation markers.

Flow Cytometric Analysis. 10^5 spleen cells or pancreatic lymphocytes were washed in FACS® buffer (0.1% BSA and 0.01% sodium azide in 1 × PBS) and then incubated with FITC or biotin-coupled staining reagents for 30 min at 4°C. The cells were then washed in FACS® buffer. Biotin-coupled reagents were developed with PE-coupled streptavidin by staining for 15 min at 4°C. The cells were then washed in FACS® buffer, and two-color flow cytometry was performed by use of a FACScan® flow cytometer (Becton Dickinson & Co.). Data were analyzed with the Lysis II software program (Becton Dickinson & Co.). Data were collected on 10^4 cells based on forward-scatter intensity and exclusion of dead cells based on staining with propidium iodide.

Results

Early Treatment of NOD Mice with CTLA4Ig Prevents the Development of Diabetes. The development of autoimmune disease in the NOD mouse begins at between 2 and 4 wk of age as evident by the infiltration of the islet of Langerhans by lymphocytes (5, 49). This insulitis is an essential step in the development of full-blown diabetes 8–20 wk later. We therefore treated NOD mice beginning at ~2–3 wk of age, presumably at the onset of insulitis. Animals received either CTLA4Ig or control antibody (CtIg) at a dose of 50 μg every other day for 2 wk followed by three additional 50-μg injections at weeks 6, 7, and 8. Female mice were continually monitored for the development of diabetes. While ~87% of the animals treated with CtIg developed disease between weeks 12 and 33, treatment with hCTLA4Ig blocked the development of diabetes (Fig. 1). Only 11% of the treated female mice became diabetic by 33 wk of age in contrast to the 87% of the CtIg-treated animals. Within 24 h after the last injection, we could no longer detect serum levels of CTLA4Ig due to the animals mounting a vigorous antibody response to the human protein (data not shown). These results indicate that a relatively short treatment protocol that interrupts the CD28/B7 costimulatory pathway either by directly signaling or blocking important interactions between members of the CD28/B7 family during a critical time in disease development prevents IDDM in female NOD mice.

CTLA4Ig's Interaction with B7-2 Plays an Important Role in Preventing Disease. CTLA4Ig's ability to inhibit the development of diabetes could have resulted from the binding of CTLA4Ig to either of its two natural ligands, B7-2 or B7-1. We have previously shown that while both molecules have the ability to costimulate T cell responses, under physiologic conditions, B7-2 appears to play the dominant costimulatory role both in vitro and in vivo (33, 40). Anti-B7-2 mAb, but not anti-B7-1 mAbs, inhibits an allogeneic mixed lymphocyte response and prolongs allogeneic islet graft survival (Zeng, J., et al., manuscript submitted for publication). We therefore examined directly the role of B7-2 in the development of autoimmune diabetes in the NOD mouse model.

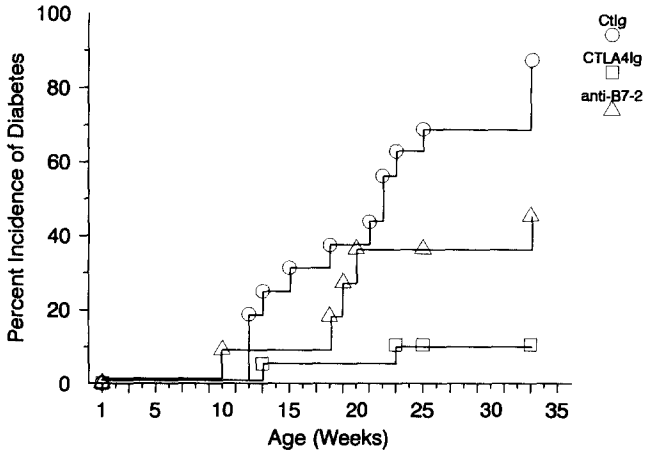

Figure 1. Early treatment of NOD mice with CTLA4Ig and anti-B7-2 mAbs prevents the development of diabetes. Female NOD mice were treated with CTLA4Ig (□; n = 19), anti-B7-2 mAb (△; n = 11), and CtIg (○; n = 16) as described in Materials and Methods. Beginning at 8 wk of age, diabetes was assessed by weekly measurements of blood glucose levels.

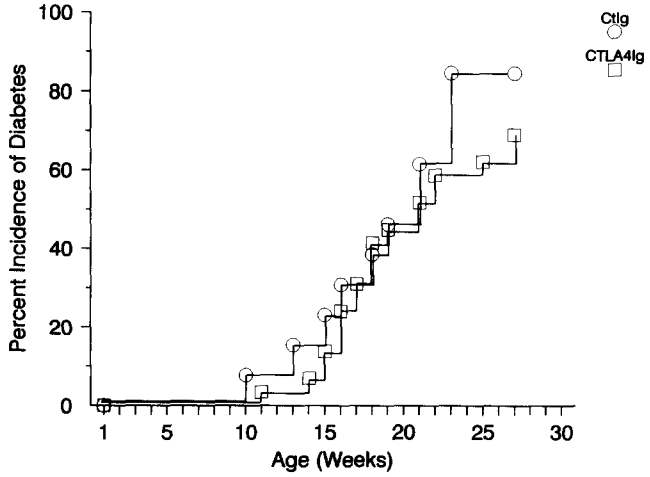

Figure 2. The late treatment of NOD mice has no effect on disease progression. NOD mice were treated with either CtIg (○; n = 13) or CTLA4Ig (□; n = 29) three times a week for at least 8 wk, or until the development of diabetes, beginning at ~10 wk of age (80 d). The development of diabetes was monitored as described.

2-wk-old NOD mice were treated with the anti-B7-2 mAb (GL1) as described above and followed for the development of diabetes. Only 45% of the anti-B7-2–treated mice became hyperglycemic by 33 wk of age, in contrast to 87% of control treated mice (Fig. 1). Of the five anti-B7-2–treated animals that did develop diabetes, four of them did not develop disease until at least 18 wk of age. Therefore, anti-B7-2 treatment was able to delay disease onset and block disease development in a subset of mice. However, its ability to induce and maintain tolerance to IDDM may not be as efficient as CTLA4Ig, in which only 11% of the treated animals became diabetic.

Late Treatment of NOD Mice with CTLA4Ig Has No Effect on Disease Outcome. While the first evidence of disease can be detected as early as 3–4 wk of age with the occurrence of insulitis, full-blown diabetes does not develop until much later. These observations have suggested that at least two different events must occur to precipitate the eventual development of diabetes. To examine what effect the CTLA4Ig treatment had when initiated well after the induction of insulitis, but before any clinical signs of disease, such as hyperglycemia, had developed, we started treatment of NOD mice when they were 80 d old. Animals were treated three times a week for 10 wk, or until the onset of disease, and monitored for the development of diabetes. Despite the ability of CTLA4Ig to inhibit disease development when administered early, it had little effect on disease outcome when treatment was initiated late in the disease process (Fig. 2). Therefore, it appears that the inhibitory effects of these reagents are exerted during early phases of the disease process, either before the initiation of disease or just subsequent to the initial antigen engagement by islet-reactive cells.

Effects of CD28 Antagonists on the Occurrence of Insulitis. To begin to address the mechanism by which CTLA4Ig and anti-B7-2 prevent disease, we examined treated animals for one of the earliest signs of diabetes, the development of insulitis. Pancreatic tissue from 8-wk-old female mice treated with CtIg, CTLA4Ig, or anti-B7-2 mAbs were prepared and examined for insulitis. Insulitis could be detected in all of the treatment groups, including the CTLA4Ig and anti-B7-2–treated mice (data not shown). To further examine the development of insulitis in these animals, male and female mice were killed at 4, 8, and 12 wk of age, and histological sections of their pancreases were prepared and scored for the presence of insulitis. Despite the ability of CTLA4Ig and anti-B7-2 mAbs to inhibit the development of disease, the presence of insulitis was readily detectable at all times. By 4 wk of age, ~30% of the islets from CtIg (mean clinical score = 7.45), CTLA4Ig (mean clinical score = 4.13), and anti-B7-2 mAb– (mean clinical score = 7.25) treated female mice showed evidence of cell infiltration although the majority of the insulitis was nondestructive periinsulitis (Fig. 3). The severity of the infiltrate continued to increase at 8 wk of age in all groups, although both the CTLA4Ig-and anti-B7-2 mAb–treated groups appeared to have a slightly more severe infiltrate at this time than did the CtIg-treated animals. By 12 wk of age, all three groups had between 40 and 60% of their islets infiltrated by lymphocytes, and the severity had increased (CtIg mean clinical score = 16.28; CTLA4Ig mean clinical score = 24.0; anti-B7-2 mAb mean clinical score = 16.75). Similar results were also observed in the treated males (data not shown). Thus, the inhibition of disease development induced by CTLA4Ig and anti-B7-2 treatment was not caused by a quantitative difference in the T cell infiltrate into the islets, and therefore these treatments must alter a later event in this disease.

Treatment of NOD Mice with a Combination of Anti-B7-1 and Anti-B7-2 mAbs Accelerates Disease Onset. Previous results in an allogeneic transplant model demonstrated that a com-

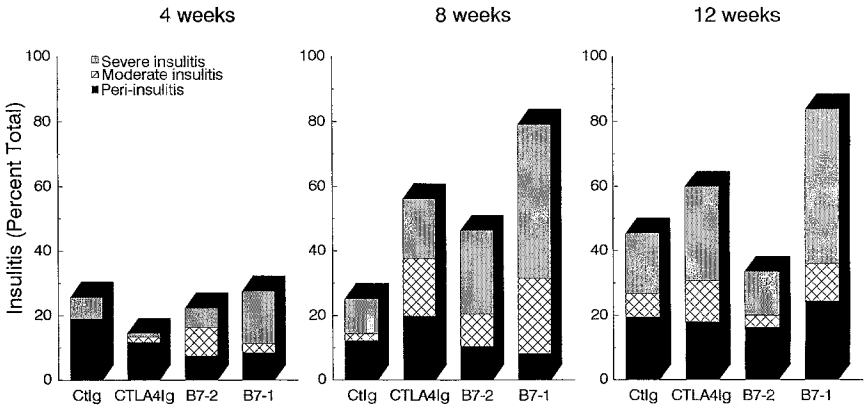

Figure 3. The incidence of insulitis in NOD mice treated with CD28 antagonists. Female animals from the various treatment groups were killed at 4, 8, and 12 wk of age. Pancreatic sections were stained with H&E and then scored for the presence of periinsulitis (*solid bars*), moderate insulitis (*cross-hatched bars*), or severe insulitis (*shaded bars*). Three to six animals (at least 50 islets) from each time point were scored. Insulitis is reported as the percentage of infiltrated islets per total islets scored for each treatment group and time point.

bination of anti-B7-1 and anti-B7-2 mAbs was more immunosuppressive than either drug alone (Zeng, J., et al., manuscript submitted for publication). Therefore, we examined the ability of the combined therapy to block the development of diabetes in NOD mice. 2–3-wk-old mice were treated with both anti-B7-2 and anti-B7-1 (16-10A1) mAbs as described in Materials and Methods. Despite the ability of anti-B7-2 mAb to inhibit disease onset, treatment with a combination of anti-B7-1 and anti-B7-2 mAbs resulted in an accelerated onset on disease (Fig. 4). By 12 wk of age, >65% of the combined treated female mice developed diabetes, and by 16 wk of age, all but one animal were hyperglycemic. Treatment of 2-wk-old NOD mice with the anti-B7-1 mAb (16-10A1) alone also made the disease worse (Fig. 4). In fact, hyperglycemia was detected in some mice as early as 8 wk of age in both treatment groups. More than 80% of the anti-B7-1–treated female mice were diabetic by week 12, and by 16 wk of age, 100% of the female mice were hyperglycemic. In contrast, at 16 wk of age only 20% of the CtIg-treated animals were diabetic, with only 70% developing diabetes by 24 wk of age. To eliminate the possibility that this phenomenon was due to nonspecific toxicity of the 16-10A1 mAb, these studies were repeated with another anti-B7-1 mAb, 1G10. As shown in Fig. 5 *A*, treatment of the female NOD mice with 1G10 also accelerated the development of diabetes. 100% of the 1G10-treated female mice became diabetic by week 18. Even more striking than the exacerbation of disease in the female mice was the observation that anti-B7-1 treatment induced disease in normally resistent male NOD mice (Fig. 5 *B*). Disease was first detected in these anti-B7-1– (16-

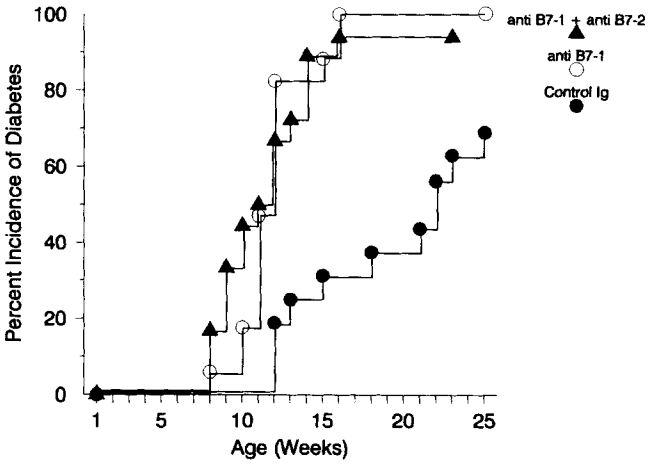

Figure 4. A combination of anti-B7-1 (16-10A1) and anti-B7-2 mAbs accelerates the onset of diabetes. Female NOD mice were treated at 2 wk of age with control Ig (●; n = 16), anti-B7-1 (○; n = 17), or both anti-B7-1 and anti-B7-2 mAbs (▲; n = 18) as described. The treated animals were then monitored for the development of diabetes.

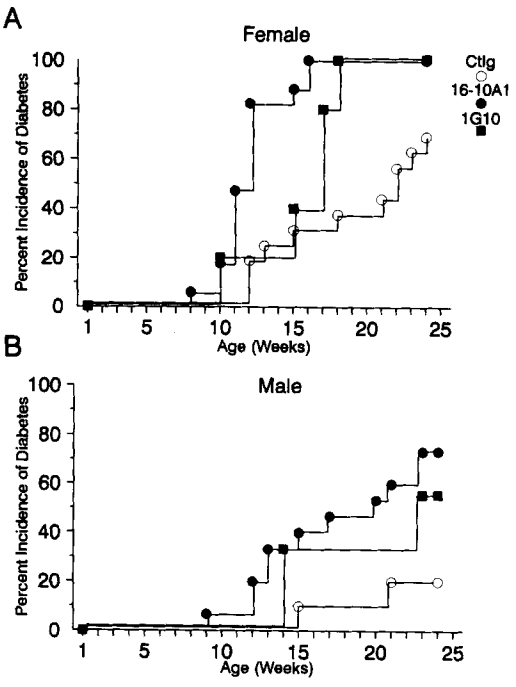

Figure 5. Anti-B7-1 therapy accelerates disease in both male and female NOD mice. Female (*A*) and male (*B*) NOD mice were treated with control Ig (○; female n = 16, male n = 10) or one of two anti-B7-1 mAbs: 16-10A1 (●; female n = 17, male n = 15) or 1G10 (■; female n = 5, male n = 9) beginning at 2 wk of age as described and followed for the development of diabetes.

10A1)–treated male mice at 8 wk of age. By 24 wk of age, 73% of these male mice had developed diabetes, while there was autoimmune diabetes in only 20% of the control-treated mice. Furthermore, 1G10 treatment resulted in the development of diabetes in >50% of the male NOD mice (Fig. 5 *B*). Finally, as seen with CTLA4Ig, treatment of NOD mice after 10 wk of age with anti-B7-1 mAbs did not alter the development of diabetes (data not shown).

Treatment of NOD Mice with Anti-B7-1 mAbs Alters Early Events in Disease Development. The development of insulitis was also examined in these anti-B7-1–treated animals. While all of the treatment groups exhibited insulitis at 8 wk of age in the anti-B7-1–treated mice, the islets appeared to be more severely infiltrated, with few if any intact islets remaining (data not shown). Further analysis of these mice revealed that anti-B7-1 treatment increased the time course and severity of insulitis in both male and female mice. As seen in Fig. 3, while anti-B7-1–treated female mice did not exhibit an overall increase in the percentage of islets affected at 4 wk of age, the severity of the infiltrate was increased (CtIg [mean clinical score = 7.45] and anti-B7-1 mAb [mean clinical score = 10.35]). By 8 wk of age, the effect was even more dramatic, with >70% of the islets from anti-B7-1–treated mice demonstrating moderate to severe insulitis. In fact, >50% of the islets had a severe insulitis, resulting in a complete loss of the islet architecture, compared with 10–26% in the other treatment groups (Fig. 3). While at 12 wk of age the percentage of islets infiltrated in the other groups was nearing that of the anti-B7-1–treated group, the severity of the infiltrate in the anti-B7-1 mAb–treated mice (mean clinical score = 41.33) was much greater than either the CtIg (mean clinical score = 16.28) or the CTLA4Ig (mean clinical score = 24.0) treated mice. A similar increase in the severity of insulitis was also observed in the anti-B7-1–treated male mice (data not shown).

We also examined the effects of anti-B7-1 mAb treatment in normal B6 mice. 2-wk-old animals were treated as previously described with anti-B7-1 mAb (16-10A1). Anti-B7-1–treated male and female B6 mice were monitored for 30 wk for the development of diabetes or insulitis. None of the treated animals developed any signs of diabetes, including insulitis. 12-wk-old B6 mice treated with either CtIg (*a*) or anti-B7-1 (16-10A1) mAb (*b*) demonstrated no signs of lymphocytic infiltrates into either the islets or pancreas (Fig. 6). In contrast, the islets of a 12-wk-old NOD mouse treated with anti-B7-1 (*d*) displayed severe infiltrate into all of the islets present within the pancreas. CtIg-treated NOD mice (*c*) also exhibited signs of lymphocytic infiltrate, although once again, not as severe as in the anti-B7-1–treated animals. Therefore, the anti-B7-1 mAb did not induce disease in the absence of the genetic predisposition.

Finally, we examined the cellular makeup of the pancreatic infiltrate in the various treatment groups. Pancreases from treated animals were isolated at 11–13 wk of age, and the infiltrating lymphocytes were examined. Both T and B cells were present in all of the groups, with 12–20% of the infiltrate being composed of $B220^+$ cells and 30–42% of it composed of $Thy1^+$ cells (data not shown). The CD4/CD8 ratios were also similar between all of the groups. Analysis of T cell activation by CD69 expression (50–52) demonstrated that T cells isolated from the pancreases of all of the female treatment groups were activated to some degree (mean fluorescent intensity [MFI] of control-treated animals = 10.85) compared with either splenic T cells from the same animals (MFI of control animals = 3.39) or age-matched male mice (MFI of control animals = 4.79). However, CD69 expression of pancreatic T cells isolated from both the male and female anti-B7-1–treated mice was significantly increased above the levels observed in the CtIg-, CTLA4Ig-, or anti-B7-2–treated animals (Fig. 7). The female anti-B7-1–treated mice exhibited a mean fluorescence intensity of nearly 2.5 times that of CtIg-treated female mice, and the male mice expressed levels 1.75 times that of CtIg-treated male mice. While Fig. 7 suggests that treatment with anti-B7-2 mAb resulted in a reduced expression of CD69 in the T cells isolated from the spleen or male pancreases, this was not observed in repeated experiments. The increased expression of CD69 on the B7-1–treated pancreatic T cells together with the increased severity of insulitis indicate that anti-B7-1 treatment alters a very early event in the disease process, resulting in an accelerated onset of disease.

Discussion

One model for the induction of T cell tolerance suggests that the inability of nonconventional APC to fully activate T cells due to their lack of costimulatory molecules results in T cell inactivation (anergy) and, in some instances, cell death. While several studies have demonstrated that the ectopic expression of MHC molecules and the appropriate costimulatory molecules on nonconventional cells, such as the islets of Langerhans, can induce an autoimmune destruction of the islets (17–19), few studies have directly examined the role of costimulatory signals during the normal development of autoimmune diabetes. In this study, we examined the role of the CD28/B7 signaling pathway in the generation and propagation of autoimmune diabetes in the NOD mouse model.

CTLA4Ig, a soluble CD28/B7 antagonist, has been shown to inhibit a variety of responses, including allogeneic and xenogeneic transplant rejection (45, 46), antibody responses (44), and autoimmune disease (53). In this study, CTLA4Ig treatment of NOD mice also resulted in a profound inhibition of disease onset when administered just before or at the onset of insulitis. Only 11% of the CTLA4Ig-treated mice became diabetic. Similar results were observed with an anti-B7-2 mAb, GL1, previously shown to be the dominant costimulatory CD28 ligand in allogeneic responses (33). Anti-B7-2 mAb treatment of NOD mice inhibited diabetes when initiated at 2 wk of age, with only 45% of the anti-B7-2–treated mice becoming diabetic compared with 87% of control treated mice. In both instances, the CD28 antagonists had no effect on the development of disease if administered late (>10 wk of age). These results suggest that these inhibitory reagents block early events in disease development. The

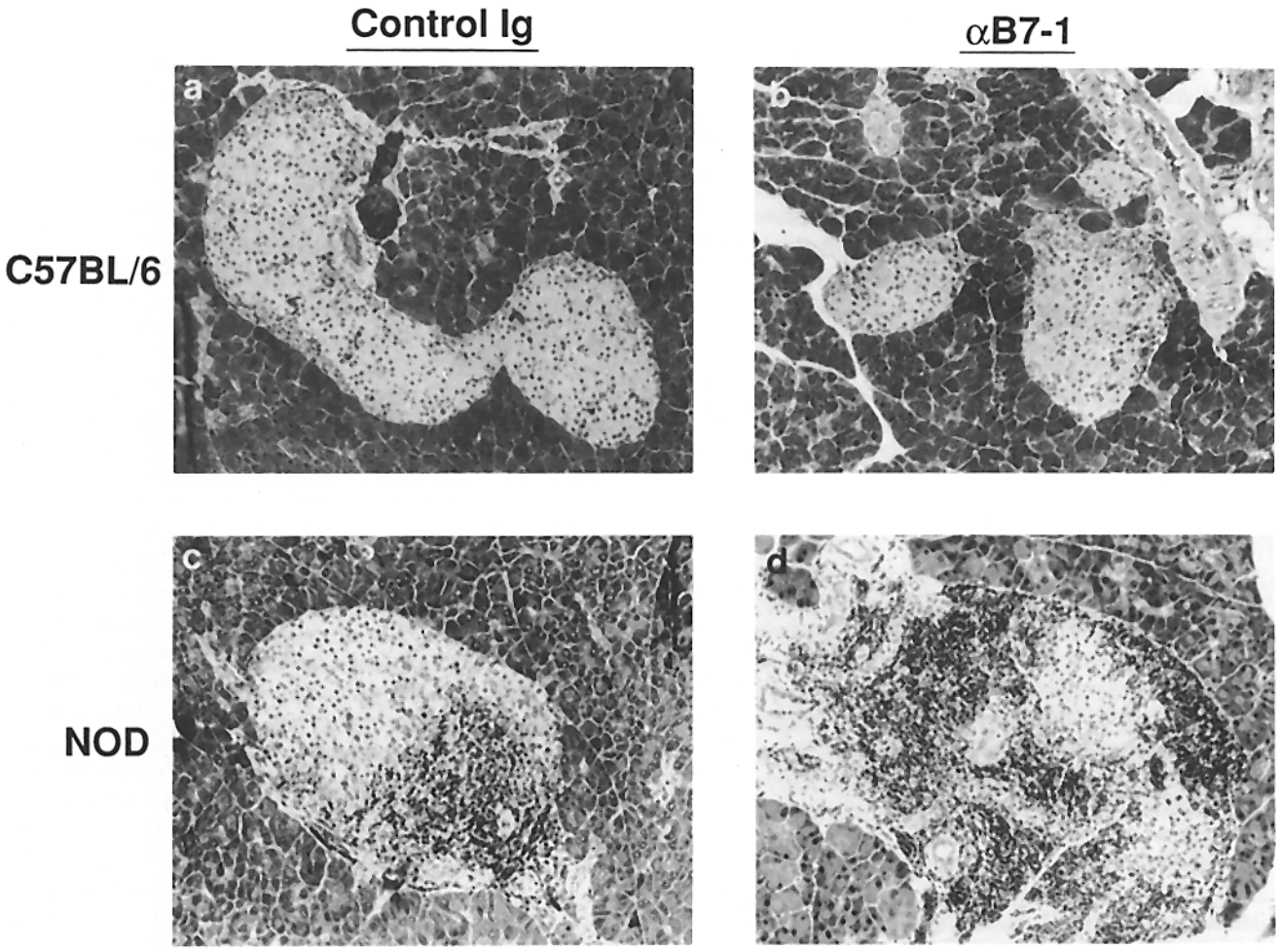

Figure 6. Anti-B7-1 treatment only induces insulitis in genetically susceptible strains of mice. NOD and B6 mice were treated with either anti-B7-1 (16-10A1) or a CtIg beginning at 2 wk of age as described in Materials and Methods. Representative H&E sections of 12-wk-old mice are shown. (*a*) CtIg-treated B6, (*b*) anti-B7-1–treated B6, (*c*) CtIg-treated NOD, and (*d*) anti-B7-1–treated NOD. ×200.

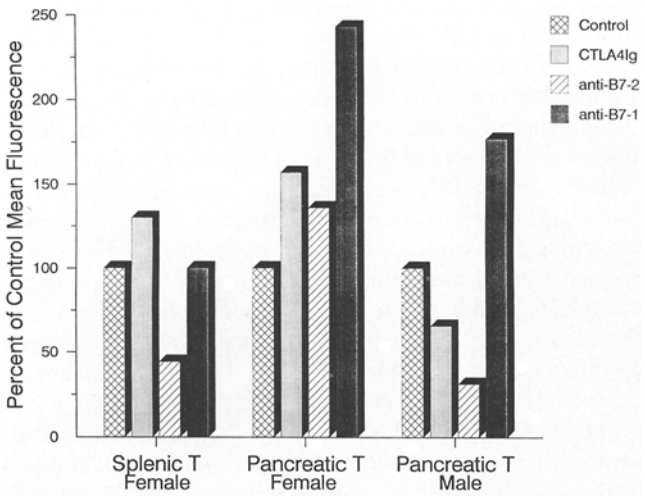

Figure 7. Phenotypic analysis of lymphocytes infiltrating the pancreas of treated animals. T cells isolated from either the spleen or pancreas of 11–13-wk-old treated mice were analyzed for surface expression of CD69 by FACS® analysis. The percentage of control mean fluorescence = (MFI of treatment group/MFI of control group) × 100. The MFI for the control-treated animals are female splenic T cells = 3.39, female pancreatic T cells = 10.85, and male pancreatic T cells = 4.79. Each group is composed of five mice, and the data are representative of two experiments.

inability of CTLA4Ig to block the development of diabetes when administered late in the disease process differs from the observations of Finck et al. (53) that late treatment could suppress an active autoimmune response in a model for murine lupus. This difference may be due to the more predominant role of antibodies in the lupus model or a difference in reagents, since in these studies only murine and not human CTLA4Ig had a beneficial effect.

While both CTLA4Ig and anti-B7-2 mAb treatment were able to decrease the incidence of diabetes, they had little effect

on the occurrence of insulitis. CTLA4Ig- and anti-B7-2–treated animals developed an equivalent lymphocytic infiltrate to that of CtIg-treated animals, so that by 12 wk of age, the mean clinical scores were essentially equivalent. Similar results were also obtained in treated male mice, although a significant degree of infiltration (>40% of the islets) was not detected until 12 wk of age (data not shown). Isolation of the lymphocytes infiltrating the pancreas demonstrated that equivalent numbers of T cells (CD4 and CD8) and B cells were present in all three groups. Moreover, the degree of T cell activation, as assessed by the expression of CD69, was equivalent in the CtIg-, CTLA4Ig-, and anti-B7-2–treated groups. Therefore, quantitatively, the infiltrate appears to be quite similar between CtIg-treated animals that develop disease and CTLA4Ig- or anti-B7-2–treated animals that do not. However, qualitative differences in the infiltrate may exist. One possibility is that the interruption of critical interactions between CD28 and its costimulatory ligands may result in the induction of anergy to islet antigens such as GAD65. These antigens have been shown to play an important role in the early phase of the development of disease (11, 12). Alternatively, CTLA4Ig and anti-B7-2 treatment may inhibit disease, not by inducing anergy to these islet antigens, but by altering the balance of Th1 and Th2 cells that infiltrate the islets and respond to the autoantigens (54). In fact, recent data from Kuchroo et al. (54a) have suggested that the in vivo functional effects of anti-B7-2 mAbs in an experimental autoimmune encephalomyelitis (EAE) model are a result of changes in the balance of Th1 and Th2 subsets in these animals.

While anti-B7-2 treatment inhibited diabetes development in NOD mice, its effects were not as profound as CTLA4Ig treatment. This could be due to the differences in affinity of the mAb and CTLA4Ig for B7-2 or CTLA4Ig's ability to bind to alternative ligands, such as B7-1. Therefore, NOD mice were treated with either a combination of anti-B7-1 plus anti-B7-2 mAbs or anti-B7-1 alone. In contrast to the immunosuppression of NOD disease observed after anti-B7-2 or CTLA4Ig therapy, treatment of NOD mice with anti-B7-1 mAbs at the onset of insulitis resulted in a more severe infiltrate and a rapid onset of disease in both male and female mice. This effect was observed with two different anti-B7-1 mAbs, 16-10A1 and 1G10, even though 1G10 has a 40-fold lower avidity for B7-1 than does 16-10A1 (33). Unlike the CTLA4Ig or anti-B7-2 treatment, which had little effect on the development of insulitis, the treatment of NOD mice with anti-B7-1 mAbs resulted in a more severe and rapid onset of insulitis. Furthermore, the T cells isolated from the anti-B7-1–treated female and male mice expressed higher levels of CD69, indicating they were more highly activated than T cells isolated from the other treatment groups.

These results indicate that B7-1 plays a direct role in controlling this autoimmune response by directly signaling through the B7-1 molecule, interrupting a critical interaction between B7-1 and one of its ligands, or interacting with a distinct population of APC during the development of disease. Interestingly, transfectants of both B7-1 and B7-2 are capable of providing the necessary costimulatory signals to the T cell (37–39). However, differences in both the expression and function of these two molecules have been observed. The expression of B7-2 occurs much more rapidly than B7-1 after B cell activation (33, 41). Furthermore, Ig cross-linking only induces significant levels of B7-2 and not B7-1 (40). Thus, it is possible that B7-2 is expressed on APC essential for initiating full-blown diabetes, while B7-1 is expressed on cells that regulate the development of insulitis. For instance, B7-1 is expressed on activated T cells (55). Thus, the anti-B7-1 mAb might deliver a signal to the T cells that alters effector cell function, such as lymphokine production, resulting in a potential shift in the balance of Th1 and Th2 subsets. Alternatively, the interaction of the anti-B7-1 mAb with conventional APC could increase the antigen presentation or costimulation capabilities of the cells, resulting in a more potent T cell response. Finally, anti-B7-1 treatment may mediate its effects by blocking the interaction of B7-1 with one of its counter-receptors, CTLA-4 (43). Recent data from our laboratory suggest that the signals delivered to the T cell by CD28 and CTLA-4 may be different. F(ab) fragments of anti-CTLA-4 antibodies augment T cell proliferation in an allogeneic MLR by blocking an off signal presumably delivered by a CTLA-4 ligand (42). These results suggest the possibility that while the CD28 molecule provides important costimulatory signals to the T cell, CTLA-4/B7-1 interactions may actually function to downregulate an immune response. The interruption of such a negative signal by anti-B7-1 mAbs would prevent the downregulation of an autoimmune response and result in a more severe disease.

Despite the ability of anti-B7-2 mAb to inhibit costimulation and prevent diabetes, a combination of anti-B7-1 and anti-B7-2 mAbs increased the onset of diabetes in both female and male mice (data not shown), similar to anti-B7-1 mAb treatment alone. These results raise the possibility that B7-1 and B7-2 function at different time points during the development and propagation of this autoimmune response. In this regard, there is good evidence that this disease progresses in at least two stages (56). The first event results in the development of insulitis, and later events are responsible for the progression to full-blown diabetes. While both CTLA4Ig and anti-B7-2 treatment inhibited the development of diabetes, neither treatment prevented the occurrence of insulitis. Furthermore, animals not receiving the additional three doses at weeks 6, 7, and 8 were not protected from developing diabetes (data not shown). Together, these results suggest that anti-B7-2 and CTLA4Ig treatment act late in disease development. In contrast, anti-B7-1 mAbs increased both the rate and severity of insulitis, and the additional three doses at weeks 6, 7, and 8 were not necessary for exacerbation of disease (data not shown), suggesting that this therapy altered the initial stages of the disease process. There are several possible explanations for the exacerbation of disease observed with a combination of anti-B7-1 and anti-B7-2 mAbs. First, it is possible that the initial activation event may be CD28 independent. If this is the case, then this event would rely on alternative costimulatory pathways and would therefore not be affected by blockade of the CD28 ligands, B7-1 or B7-2.

In this regard, it is interesting to note that early alloantigen responses are largely unaltered in vivo or in vitro in CD28-deficient mice (57). By comparison, the later events of disease progression would appear to be exclusively CD28 dependent. Alternatively, all of the stages of autoimmune diabetes may be CD28 dependent, and the initiation of treatment at 2 wk of age is not early enough to prevent the development of insulitis, but would inhibit the later events responsible for disease progression. In either case, autoreactive T cells would be activated and express both CTLA-4 and B7-1. Therefore, the exacerbation of disease mediated by the anti-B7-1 mAb would dominate the inhibitory effects of anti-B7-2 treatment by either directly signaling through the B7-1 molecule or interrupting a critical interaction responsible for shutting down the immune response. Future experiments with Fab and F(ab)'2 fragments of the anti-B7-1 and anti-B7-2 mAbs, as well as genetically altered B7-1 and B7-2 knockout mice, will allow us to determine the mechanism by which B7-1 treatment exacerbates disease.

Finally, these observations do not appear to be restricted to the NOD autoimmune mouse model. Preliminary studies performed in collaboration with Dr. Steve Miller (Northwestern University, Chicago, IL) in an EAE model have shown that treatment of mice with anti-B7-1 during the primary response to proteolipid protein resulted in more rapid and severe secondary relapses (Miller, S., C. Vanderlugt, D. J. Lenschow, and J. A. Bluestone, unpublished observations). Therefore, the mechanism responsible for the anti-B7-1–mediated acceleration of disease in the NOD mouse model may be similar for other autoimmune diseases.

In conclusion, these results clearly demonstrate that T cell costimulation is an essential component of the in vivo activation of autoreactive T cells and the development of autoimmune diabetes. Thus, the manipulation of this costimulation pathway may provide a powerful new target for the development of future therapies for diabetes and other autoimmune diseases.

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