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Article **Immunology**

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GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes

See related Commentary on pages 869–870.

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Insulin-dependent type 1 diabetes is an autoimmune disease mediated by T lymphocytes recognizing pancreatic islet cell antigens. Glutamic acid decarboxylase 65 (GAD65) appears to be an important autoantigen in the disease. However, T cells from both patients with type 1 diabetes and healthy subjects vigorously proliferate in response to GAD65 stimulation ex vivo, leading us to postulate that the critical event in the onset of human diabetes is the activation of autoreactive T cells. Thus, we investigated whether GAD65-reactive T cells in patients with diabetes functioned as previously activated memory T cells, no longer requiring a second, costimulatory signal for clonal expansion. We found that in patients with new-onset type 1 diabetes, GAD65-reactive T cells were strikingly less dependent on CD28 and B7-1 costimulation to enter into cell cycle and proliferate than were equivalent cells derived from healthy controls. We hypothesize that these autoreactive T cells have been activated in vivo and have differentiated into memory cells, suggesting a pathogenic role in type 1 diabetes. In addition, we observed different effects with selective blockade of either B7-1 or B7-2 molecules; B7-1 appears to deliver a negative signal by engaging CTLA-4, while B7-2 engagement of CD28 upregulates T cell proliferation and cytokine secretion.

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Introduction

Type 1a or insulin-dependent diabetes mellitus (IDDM) is the result of a progressive T cell–mediated autoimmune destruction of insulin-producing β cells in the pancreatic islets of Langherans (1). An insulitis consisting of a mononuclear cell infiltrate is observed in the pancreatic islets at disease onset (2, 3). The role of autoreactive T cells in the pathogenesis of IDDM has been suggested by the effects of immunosuppressive drugs in delaying disease onset (4, 5) and the transfer of disease by transplantation of bone marrow of a patient with diabetes to an immunosuppressed nondiabetic recipient (6). Moreover, T cells from mice with a spontaneous onset of autoimmune diabetes, the nonobese diabetic (NOD) mouse, can transfer the disease to healthy neonatal mice, and both CD4 and CD8 T cells are required for disease occurrence (7). Thus, an inflammatory autoimmune pathogenesis has been strongly implicated in the disease's pathogenesis.

While several β cell autoantigens have been implicated in the triggering of islet autoimmunity, experiments in the NOD model predict that due to epitope spreading in the inflamed pancreas, patients at the time of disease onset will exhibit autoimmune responses to a number of islet cell antigens (8). Of these, GAD65 appears to be highly antigenic in both humans and NOD mice, because proliferative responses to the antigen can be readily measured in normal subjects without autoimmune disease (9–11). For example, while detectable T cell responses can be measured to insulin and proinsulin in normal subjects and patients with type 1 diabetes (12, 13), significantly high responses, as measured by thymidine incorporation, can be detected to the GAD antigen (14). However, the response to GAD is similar between patients with IDDM and normal subjects, using assays that measure T cell responses independent of the functional state of the T cell. This is quite similar to the response to another organ-sequestered antigen, myelin basic protein, where the T cell responses to this antigen, as determined by using proliferation assays, are the same between patients with multiple sclerosis and normal controls (15, 16). Thus, autoreactive T cells can be found in the circulation of normal subjects without autoimmune disease. Based on experiments performed in animal models of autoimmunity, it can be postulated that the activation of autoreactive T cells, most likely by cross-reactive microbial antigens in a genetically susceptible host, is the critical event leading to autoimmune destruction of β cells (17).

Optimal activation of naive T cells requires two signals (18–20). The first is mediated through the T cell receptor (TCR) recognizing antigen bound to MHC, and the second is mediated by costimulatory signals provided by the interaction of CD28 on T cells with B7 family molecules (B7-1/CD80 and B7-2/CD86) on antigen-presenting cells

(APCs) (21–25). If a second costimulatory signal through the CD28/B7 pathway is not provided, T cells enter into a state of antigen-specific nonresponsiveness or anergy (26, 27). In contrast, memory or primed T cells are significantly less dependent on a second, costimulatory signal and can proliferate with TCR engagement alone (28).

It has been shown that blocking the costimulatory signals provided via the CD28/CTLA-4/B7 pathway can influence the development of diabetes in the NOD mouse. Specifically, administration of CTLA-4 Ig or anti–B7-2 mAb to NOD mice between 5 and 7 weeks of age prevents the onset of diabetes, though it has no effects on the development or severity of insulitis (29). Similarly, CTLA-4 Ig can prevent experimental autoimmune encephalitis both during antigen priming and in the adoptive transfer model of the disease by inhibiting Th1 cells (30, 31). In contrast, treatment with anti–B7-1 or a combination of anti–B7-1 and anti–B7-2 mAb results in a more rapid and severe onset of diabetes in mice and induces the disease in normally resistant male mice (29). Similarly, studies using transgenic NOD mice either secreting high amounts of circulating CTLA-4 or with gene disruption of CD28 ($CD28^{-/-}$ NOD) showed a more rapid onset and higher severity of diabetes as compared with littermate controls (32). Thus, the CD28/B7 pathway appears to be important in the pathogenesis of autoimmune diabetes, and the manipulation of this pathway could provide a valuable mechanism to prevent T cell activation in the periphery with important implications for the treatment of autoimmune diseases.

Since patients with type 1 diabetes as well as healthy subjects have circulating GAD65-reactive T cells, we investigated whether these autoreactive T cells functioned as previously activated memory T cells and no longer required a second costimulatory signal for clonal expansion in patients with diabetes as compared with the healthy control subjects. We found that in patients with new-onset type 1 diabetes, GAD65-reactive T cells are strikingly less dependent on CD28 and B7-1 costimulation to enter into cell cycle and proliferate as compared with those in healthy controls. These data indicate that GAD65-reactive T cells from patients have been previously activated in vivo and possess a functional memory phenotype. In addition, we observed different effects with selective blockade of either B7-1 or B7-2 molecules; B7-1 appeared to deliver a negative signal by engaging CTLA-4, while B7-2 engagement of CD28 upregulated T cell proliferation and cytokine secretion.

Methods

Patients and controls. Ten patients with recent-onset type 1a diabetes (less than 6 months from diagnosis) were used in this study (Table 1). The age range of the patients was 19–35 years, with a mean age of 28 years. Eight out of ten diabetic patients had the diabetes-associated HLA types DR3 or DR4 or both. Nine control subjects with no significant medical history and an age range of 23–36 (mean age of 31.5) were also tested (Table 2). Five normal controls had the HLA types DR3 or DR4 or both. An additional five patients (three women and two men, with an age range of 23–34, mean age of 27.6 years) who have had diabetes for more than 5 years were also examined. Peripheral venous blood samples were obtained with informed consent from patients and controls and analyzed for T cell reactivity to GAD65. All blood samples were immediately processed and analyzed.

Proliferation assay. PBMCs were purified by Ficoll-Paque (Amersham Pharmacia Biotech Europe GmbH, Uppsala, Sweden) according to the manufacturer's protocol. Within 4 hours of blood drawing, PBMCs (10^6 cells/100 μl media) were preincubated with either GAD65 (Diamyd Diagnostic AB, Stockholm, Sweden) or tetanus toxoid (TT) (Massachusetts Department of Health, Boston, Massachusetts, USA) as a control antigen for 2 hours in the presence or absence of blocking Ab's in polystyrene round-bottom tubes (Becton Dickinson and Co., Franklin Lakes, New Jersey, USA). After 2 hours of incubation at 37°C, PBMCs were plated at 1.5×10^5 cells/well into 96-well round-bottom plates (Corning-CoStar Corp., Corning, New York, USA) in

Table 1
Autoantibodies and HLA profile of type 1 diabetic patients.

Type 1 diabetes patients

			Autoantibodies			HLA haplotypes			
Patient no.	Sex	Age	GAD65	IA-2	IAA	DRB1 alleles		DQB1 alleles	
1	F	24	2.41	3.23	427.2	0301	0404	0201	0302
2	M	38	1.57	5.18	1,913.2	0401	1302	0301	05
3	M	19	0.01	0.02	2,021.9	0301	–	0201	–
4	M	35	1.09	0.12	1,286.2	0405	0701	0201	–
5	M	22	0.07	0.00	123.7	0301	–	0201	–
6	M	22	1.53	2.13	586.1	0701	–	0201	0303
7	F	29	1.59	1.55	3,611.8	0301	1104	0302	06
8	M	27	ND	ND	ND	0301	–	0201	–
9	M	31	ND	ND	ND	ND	ND	ND	ND
10	F	33	8.69	2.02	10.2	0301	–	0301	–

The normal range of autoantibodies is anti-GAD65 < 0.1 index, anti–IA-2 < 0.1 index, and anti-IAA < 39 μl/ml. ND, not detected; IAA, insulin autoantibody; NC, normal control.

Table 2
Autoantibodies and HLA profile of normal controls

Normal control subjects									
			Autoantibodies			HLA haplotypes			
NC no.	Sex	Age	GAD65	IA-2	IAA	DRB1 alleles		DQB1 alleles	
1	F	33	0.03	0.00	25.4	0701	1101	0201	0301
2	M	36	0.06	0.02	13	0301	1501	0201	06
3	F	24	ND	ND	ND	ND	ND	ND	ND
4	M	23	0.84	6.05	420	0401	1501	0302	06
5	M	35	0.00	0.04	18	ND	ND	ND	ND
6	M	36	1.32	1.38	21	0301	0401	0201	0302
7	M	34	0.00	0.00	12	0701	–	0201	–
8	M	31	0.09	0.01	21.3	0301	1104	0201	0301
9	F	32	0.09	0.00	2.9	0901	–	03032	–

Autoantibody and HLA profile of healthy controls. The normal range of autoantibodies is: anti-GAD65 < 0.1 index, anti-IA-2 < 0.1 index, and anti-IAA < 39 nU/ml. ND, not detected.

RPMI-1640 media supplemented with 2 mM L-glutamine, 5 mM HEPES, and 100 U/μg per milliliter of penicillin/streptomycin (final volume 200 μl/well; all from BioWhittaker Inc., Walkersville, Maryland, USA), with 5% heat-inactivated human serum (Gemini Bio Products, Woodland, California, USA). Ten replicate wells were established for different antigen concentrations (GAD65 used at 0.1, 1, 5, 10, 20 μg/ml, and TT used at 0.01, 0.1, 1 flocculation units/ml [Lf/ml]) in the presence of one of the following blocking Ab's: anti-CD28 (clone 3D10), anti–CTLA-4 (IgG1, clone 26B), anti–B7-1 (IgG2b, clone H1F1), anti–B7-2 (IgG2b, clone H3D1), all provided by Genetic Institute (Cambridge, Massachusetts, USA), and a purified mouse IgG F(ab′) as a control Ab (ICN Biomedicals, Aurora, Ohio, USA). All the blocking Ab's were processed into F(ab′) fragments according to the manufacturer's instructions (Pierce Chemical Co., Rockford, Illinois, USA), titrated, and used at a concentration of 5 μg/ml. Control wells were also established with no antigen in the presence or absence of each one of the blocking Ab's. On the fifth day of culture, 20 U/ml of recombinant human IL-2 (rh IL-2) (Teceleukin; National Cancer Institute, Frederick, Maryland, USA) were added to the wells, and on day 10–12, supernatants were removed for cytokine detection and 1 μCi [^{3}H]thymidine (NEN Life Science Products, Boston, Massachusetts, USA) was added to each well. The cells were harvested on day 11–13 and counts per minute per well were determined by scintillation counting (Perkin Elmer Wallac, Gaithersburg, Maryland, USA).

Solution phase radioimmunoassay for detection of anti-GAD Ab's. The human serum used in the cultures was tested for the presence of GAD65 Ab by solution phase radioimmunoassay. In each Ab analysis, ^{35}S-labeled GAD (20,000–25,000 cpm) were incubated with sera at a 1:25 dilution overnight at 4°C in buffer (20 mmol Tris, pH 7.4, 150 mmol NaCl, 1% BSA, 0.1% Tween 20, and a protease inhibitor cocktail containing benzamidine and aprotinin) in a total volume of 50 μl. This immunocomplex was then precipitated with 50 μl of a 35% protein A-Sepharose slurry in a MultiScreen-DP opaque 96-well filtration plate (Millipore Corp., Bedford, Massachusetts, USA). The plates were shaken for 45 minutes at 4°C and washed with cold washing buffer for two cycles (each cycle with three washes and with 5 minutes of shaking at 4°C between cycles) using the Millipore vacuum-operated 96-well plate washer (Millipore Corp.). After washing, 100 μl of scintillation liquid (Microscint-20; Packard Instrument Co., Meriden, Connecticut, USA) was added to each well, and radioactivity was determined directly in the 96-well plate with a Top Count scintillation counter (Packard Instrument Co.). Positive and negative control sera were included in each assay. The culture serum used for all assays was negative for the presence of GAD65 Ab's.

Insulin Ab assay. Insulin Ab assay was performed according to Vardi et al. (33). Briefly, sera were tested for specific insulin binding using a known amount of I^{125}-labeled human insulin. Diluted sera were incubated, in duplicate, for 1 hour at 4°C with either buffer or with an excess of cold (unlabeled) insulin (total of four tubes). Then, labeled human insulin was added to all tubes for 7 days at 4°C. Bound insulin was precipitated with polyethylene glycol in veronal buffer, and the pellet was counted. The competitive binding was subtracted from the tube without the cold insulin, and the results were calculated as nano-units of insulin precipitated per milliliter of serum.

Islet antigen-2 Ab assay. Islet antigen-2 (IA-2) Ab assay was performed according to Payton et al. (34). The result is expressed in an index similar to the GAD65 Ab assay. The index value (counts per minute of positive control – counts per minute of sample/counts per minute of positive control – average counts per minute of negative controls) of 0.1 represents 2 SD above the mean and is considered positive.

Measurement of cytokine concentration by ELISA. The supernatants that were removed from each well before [^{3}H]-thymidine addition were diluted and analyzed to determine the cytokine profile by ELISA. Ab pairs and standards for IFN-γ were purchased from Endogen Inc.

(Cambridge, Massachusetts, USA). Ab's for IL-13 and IL-5 were purchased from PharMingen (San Diego, California, USA) and used according to the manufacturer's protocol. Avidin-peroxidase conjugate (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) and TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) were used to develop the assay.

Statistical analysis. The mean thymidine uptake and cytokine production of ten replicate cultures were calculated for each antigen concentration with or without blocking Ab's. The response to GAD65 alone was normalized to 100, and the percentage of inhibition resulting by adding blocking Ab's was calculated for each concentration of GAD65 in both patients and normal controls. The Mann-Whitney test was used to estimate the difference in the CD28/B7-mediated costimulation requirement between diabetes patients and controls.

Results

Costimulation dependence of GAD65-specific T cells in type 1 diabetes patients and normal control subjects. To functionally evaluate the activation state of GAD65-reactive T cells from patients with new-onset type 1 diabetes ex vivo, we examined their requirement for a CD28/B7 costimulatory signal to proliferate and secrete cytokines. In essence, we differentially activated unmanipulated PBMCs ex vivo to probe their functional state. Twenty-four separate experiments were performed using blood from either normal subjects, patients with new-onset

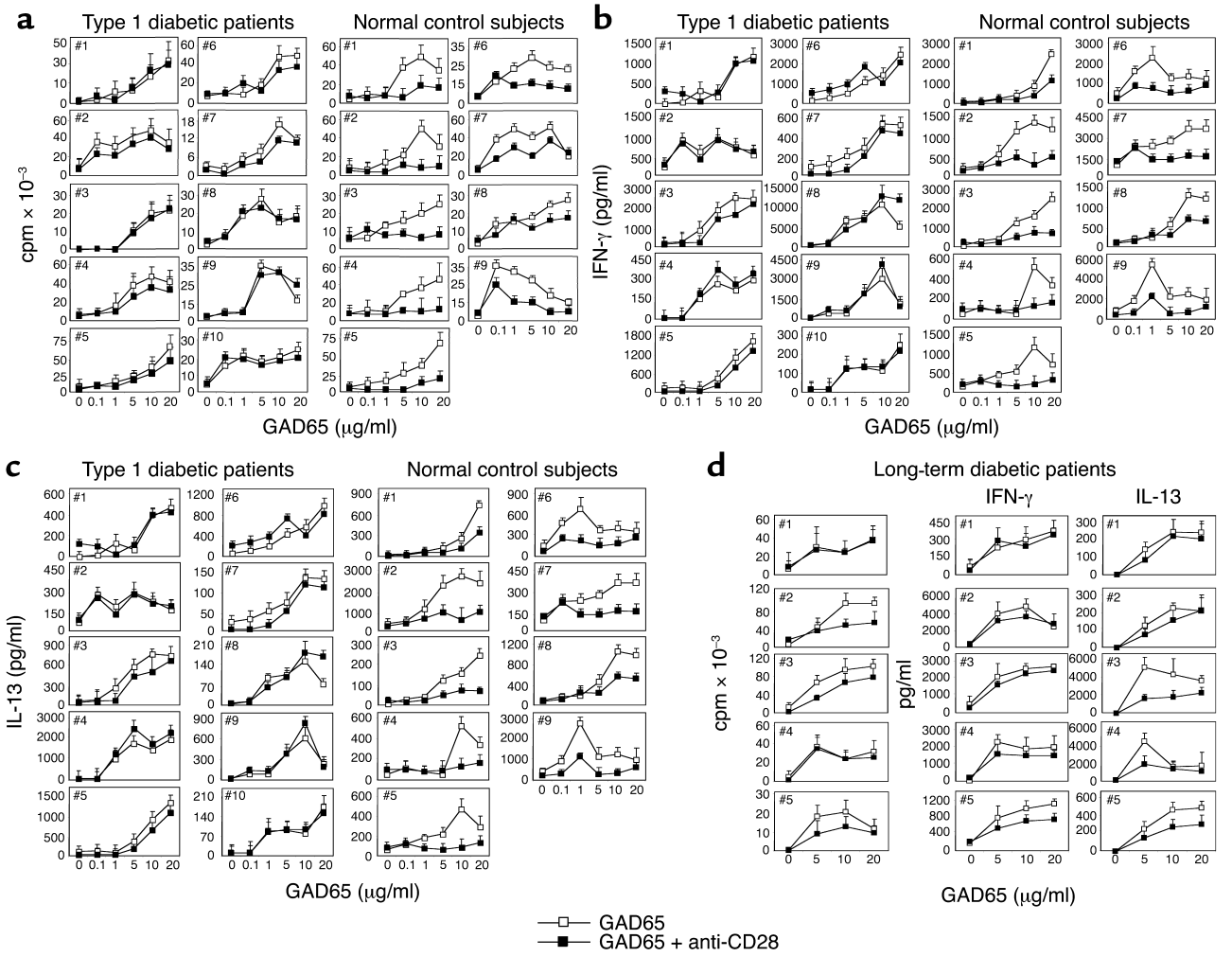

Figure 1

Response to GAD65 in individual type 1 diabetes patients and normal control subjects with blocking costimulatory signals. PBMCs from type 1 diabetes patients and normal control subjects were cultured with different concentrations of GAD65 (0.1–20 μg/ml) or TT (0.01–1 Lf/ml) in the presence or absence of anti-CD28 F(ab′) fragments. After 5 days of culture, rhIL-2 was added to each of ten replicates established for each antigen concentration with or without blocking Ab's. After an additional 5–7 days of culture, supernatants were collected for cytokine detection, and proliferation was assessed by [^{3}H]thymidine incorporation. (**a**) T cell proliferation in 19 separate experiments. Each symbol represents the mean thymidine uptake of ten replicate cultures ± SE, stimulated only with the antigen (open squares) or with antigen plus anti–CD28 F(ab′) fragment (filled squares). T cells from both patients and controls proliferated in response to GAD65, and blockade of CD28 costimulatory pathway inhibited GAD65 proliferation and (**b** and **c**) cytokine secretion in healthy subjects but not in patients with type 1 diabetes. A summary of all the new-onset patients and controls is shown in Figure 2. (**d**) Proliferation and cytokine secretion in five long-term diabetic patients.

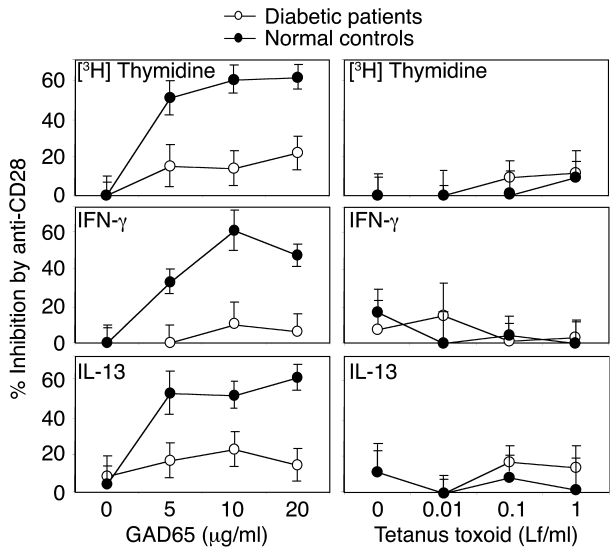

Figure 2
Proliferative response to GAD65 is not dependent on CD28/B7 costimulatory pathway in type 1 diabetes patients. The mean percentage of inhibition of GAD65-specific proliferation and cytokine secretion with CD28 blockade, as described in Figure 1, is shown. Each symbol is representative of ten diabetic patients (open circles) or nine normal controls (filled circles). The Mann-Whitney test was used to estimate the difference in the CD28 requirement between diabetic patients and controls. The difference between the two groups was significant at any concentration of GAD65 (diabetic patients vs. controls, proliferation: GAD, 5 μg/ml and 10 μg/ml, $P = 0.0019$; GAD 20 μg/ml, $P = 0.0047$; IFN-γ: GAD 5 μg/ml, $P = 0.049$; GAD 10 μg/ml, $P = 0.0019$; GAD 20 μg/ml, $P = 0.001$; IL-13: GAD 5 μg/ml, $P = 0.049$; GAD 10 μg/ml, $P = 0.03$; GAD 20 μg/ml, $P = 0.0019$) showing that antigen-specific T cells from type 1 diabetes patients are CD28 independent. No inhibition was detected in TT-specific T cells at any concentration of antigen either in the patients or in the normal controls.

type 1 diabetes, or from patients who have had diabetes for more than 5 years. PBMCs were stimulated with recombinant human GAD65 either in the presence or absence of monoclonal anti-CD28 F(ab′) Ab's. After 5 days of culture, rIL-2 was added to drive clonal expansion of the antigen-reactive T cells to better detect antigen responses. Both patients and normal subjects mounted a vigorous T cell response to the self-antigen GAD65, as shown in the 19 experiments reported in Figure 1a. As reported previously, no differences in GAD65 responsiveness (Figure 1a) or in cytokine secretion (Figure 1, b–c) were found between patients with diabetes and normal controls in the presence of costimulatory signals provided by the APCs. In striking contrast, with CD28 blockade, the proliferative response and the cytokine secretion to GAD65 were significantly inhibited at any antigen concentration in the healthy controls but not in the diabetic patients (Figure 1, a–c, and Figure 2). PBMCs derived from long-term diabetes patients also showed a strong response to the GAD65. Unlike new-onset diabetics, three of five patients were more dependent on CD28/B7-mediated costimulation as compared with patients newly diagnosed with diabetes (Figure 1d).

We then examined the nature and quantity of the cytokines secreted in response to GAD65. There were no differences in IFN-γ or IL-13 secretion to GAD65 stimulation in the presence of costimulatory signals between patients with diabetes as compared with normal controls. As with proliferative responses, the addition of anti-CD28 F(ab′) mAb's to the culture markedly impaired the secretion of both IFN-γ and IL-13 by GAD65-reactive T cells in normal subjects but not in patients with new-onset diabetes (Figure 2).

We then examined whether differences in responses to the GAD65 between patients with diabetes and healthy controls were specific for autoantigens. We chose to examine the signals required for activation to the recall antigen TT in patients and controls. In contrast to the need for costimulation for CD28 engagement by B7 for GAD65 reactivity in normal subjects, the T cell response from both patients and controls to TT was not costimulation dependent (Figure 2). These data confirm our previous work that CD28/B7 pathway is not required for T cell proliferation to recall antigens in humans (15).

Blockade of B7-1 and B7-2 inhibits proliferation similarly to anti-CD28. Both B7-1 and B7-2 are expressed on APCs and deliver a costimulatory signal to T cells through the CD28 counter-receptor. As observed with anti-CD28 blockade, anti-B7-1 F(ab′) fragments inhibited proliferation and

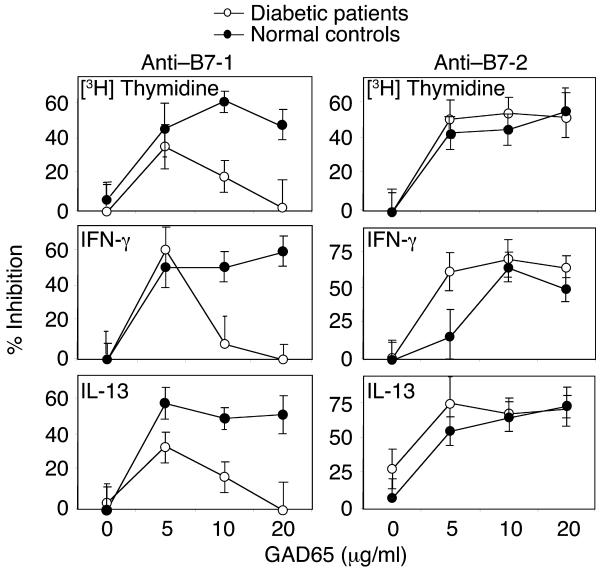

Figure 3
Differential effects of anti–B7-1 and anti–B7-2. PBMCs were cultured with different concentration of GAD65 in the presence or absence of anti–B7-1 F(ab′) or anti–B7-2 F(ab′) mAb's. Anti–B7-1 F(ab′) fragments inhibited the proliferative and cytokine response to GAD65 at all antigen concentrations in healthy subjects (filled circles), similar to anti-CD28, but only at low antigen concentration in patients with type 1 diabetes (open circles). The difference between the two groups was significant (patients vs. controls, proliferation: GAD 10 μg/ml, $P = 0.0079$; GAD 20 μg/ml, $P = 0.001$; IFN-γ: GAD 10 μg/ml, $P = 0.01$; GAD 20 μg/ml, $P = 0.001$; IL-13: GAD 10 μg/ml, $P = 0.01$; GAD 20 μg/ml, $P = 0.001$). In contrast anti–B7-2 decreased the proliferative and cytokine response in both normal controls and diabetic patients.

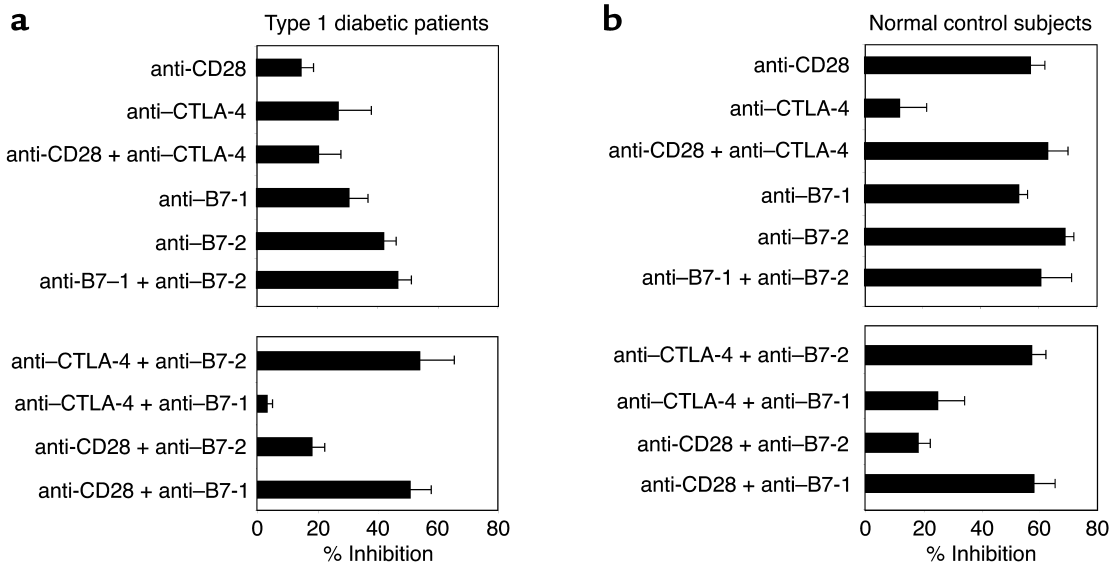

Figure 4
Blocking CD28 or CTLA-4 counter-receptors in combination with B7-1 or B7-2. PBMCs (1.5×10^5/well) from five healthy subjects and five patients with type 1 diabetes were stimulated with 10 μg/ml GAD65 in the presence or absence of each one of the blocking Ab's or with different combinations of Ab's. Ten replicates were established for each condition and harvested after 10–12 days of culture, and counts per minute per well were determined. Each column represents the mean percentage of inhibition of proliferation to GAD65 in the presence of single blocking Ab's or combinations of them in five patients with recent onset of type 1 diabetes and five normal control subjects. As shown previously in Figures 2 and 3, the blockade of CD28, B7-1, or B7-2 caused strong inhibition of proliferation in normal subjects. (**a** and **b**) Blocking both B7-1 and B7-2 molecules at the same time induced a more significant decrease of the response than blocking just one of the molecules alone in both patients and controls. While anti-CD28 in combination with anti–B7-2 had almost no effect on proliferation or cytokine secretion (data not shown), anti-CD28 in combination with anti–B7-1 induced significant decreases of responses to GAD65. In contrast, coblockade with anti–CTLA-4 and anti–B7-1 enhanced T cell proliferation, while anti–CTLA-4 in combination with anti–B7-2 suppressed GAD-specific response. While anti-CD28 induced significantly more suppression of GAD responses in the normal subjects, the effects of combining the B7-1, B7-2, CD28, and CTLA-4 F(ab′) mAb's were not different as compared with patients with diabetes.

cytokine secretion to GAD65 in healthy subjects (Figure 3). Blockade of B7-1 also inhibited GAD65 proliferative response and cytokine secretion in diabetic patients, but only at low concentrations of antigen. In contrast to B7-1 blockade, anti–B7-2 F(ab′) fragments significantly inhibited GAD65 proliferative and cytokine secretion responses both in patients with diabetes and healthy controls at any antigen concentration. Neither anti–B7-1 nor anti–B7-2 was capable of altering the response to the recall antigen TT (data not shown). The combination of anti–B7-1 and anti–B7-2 F(ab′) fragments resulted in a stronger inhibition than anti–B7-2 alone, suggesting that B7-2 rather than B7-1 provides the dominant costimulatory signal for antigen-specific activation of antigen-specific T cells in PBMC cultures (Figure 4, a and b).

Effect of CTLA-4 blockade on proliferation and cytokine secretion. CTLA-4 is a CD28 homologue expressed on activated T cells that binds with high affinity to B7-1 and B7-2 (35, 36) and functions as a negative regulator of T cell activation (37, 38). However, blockade of CTLA-4/B7 interactions in humans can result in either enhancement or suppression of T cell responses, depending upon the strength of signal delivered through the T cell receptor (39). As CTLA-4 polymorphisms link to risk of developing diabetes (40), we examined the hypothesis that defects in CTLA-4 signaling underlie the immunoregulatory defects in patients with the disease.

Blocking the T cell responses to GAD65 using anti–CTLA-4 F(ab′) did not cause a significant enhancement of the T cell response to GAD65 but only a slight increase of proliferation and cytokine release in both patients and normal controls. The same trend was seen when T cells were stimulated with the recall antigen TT in the two groups studied. These results confirmed previous studies indicating that CTLA-4 expression after T cell activation is responsible for delivering a negative signal and its interaction with B7 family ligands results in the downregulation of T cell response.

T cell proliferation is regulated by different interactions of CD28/CTLA-4 and B7-1/B7-2. The role of B7-1 versus B7-2 engagement of CD28 and CTLA-4 in regulation of the immune response is not well defined. As B7-2 blockade inhibited responses in both patients with diabetes and healthy controls at any antigen concentration, we examined the effects of blocking either the CD28 or CTLA-4 counter-receptors in combination with B7-1 or B7-2 in five patients with type 1 diabetes and five normal control subjects. Anti-CD28, anti–B7-1, and anti–B7-2 have the capability to reduce significantly the specific response to GAD65 in healthy subjects, and anti–B7-2 is also able to inhibit the antigen response in patients with type 1 diabetes. As expected, blocking both B7-1 and B7-2 pathways at the same time induced a more significant decrease of the response than blocking just one of

the molecules alone (Figure 4a). When anti-CD28 was used in combination with anti–B7-1, the degree of inhibition was enhanced further than by using each Ab alone. In contrast, the combination of anti-CD28 with anti–B7-2 either did not have any additional inhibitory effect or slightly increased the proliferation as well as the cytokine secretion of GAD65-reactive T cells (Figure 4b). When anti–B7-1 was used in combination with anti–CTLA-4, the result was an enhancement of T cell proliferation and cytokine secretion, while the combination of anti–CTLA-4 and anti–B7-2 suppressed antigen-specific response. The blockade of costimulatory molecules on T cells by anti-CD28 and anti–CTLA-4 together caused a decrease of both proliferation and cytokine production. While anti-CD28 induced significantly more suppression of GAD responses in the normal subjects, the effects of combining the B7-1, B7-2, CD28, and CTLA-4 F(ab′) mAb's were not different as compared with patients with diabetes.

Discussion

The role of autoreactive T cells in the pathogenesis of type 1 diabetes is not known, since autoreactive T cells are also present in the circulation of healthy individuals. We chose to study GAD65 as a model islet cell autoantigen and hypothesized that GAD65-reactive T cells in diabetic patients have encountered the antigen in vivo, and this promotes a different state of activation. We specifically examined the dependence of GAD65-reactive T cells on the CD28/CTLA-4/B7 costimulatory pathway to differentiate cells with an activated or memory phenotype from naive T cells according to their requirement for a costimulatory signal. Although T cells from both patients and controls proliferated to GAD65, we found that only type 1 diabetes patients but not healthy controls had autoreactive T cells capable of proliferation and cytokine production in the absence of CD28/B7 costimulatory signals. Thus, the response to GAD65 in patients with type 1 diabetes showed the features of a memory T cell response: GAD65-reactive T cells derived from patients were less dependent on costimulation, suggesting that they have been activated in vivo and differentiated into memory cells.

We did not observe any correlation between the presence of GAD65 autoantibodies and T cell proliferative response to GAD65. Perhaps this is not surprising since both normal subjects with no anti-GAD Ab's and patients with diabetes similarly proliferated to the antigen. Additionally, GAD65 autoantibodies were as common in patients with the DR3/DR4 haplotype as compared with other haplotypes. We also examined normal subjects with circulating anti-GAD autoantibodies, one with no family history of diabetes and the other with a first-degree relative with diabetes. Their response to GAD65 and costimulatory blockade was similar to that of the other normal subjects, raising the issue that the activated GAD-reactive T cells may correlate with tissue destruction. However, it is clear that long-term serial studies are required to address this important question.

Several studies have shown that resting and activated T cells have a different requirement for CD28/B7 costimulatory signals (41). To confirm that memory T cells have a lower requirement for accessory molecules than naive cells, TT has been used as a control antigen since virtually all healthy subjects have been immunized to this antigen and show a significant recall response in proliferation assays. The addition of anti-CD28 in the cultures stimulated with TT did not inhibit the proliferative response or cytokine secretion to the antigen, suggesting that reactivation of memory T cells is not CD28 costimulation dependent. In patients with type 1 diabetes the response to GAD65 has characteristics similar to the response to TT.

Studies have indicated that CTLA-4 has a negative regulatory function in T cell activation. CTLA-4 binds to B7 molecules with higher affinity than CD28. It has been shown that soluble anti–CTLA-4 mAb, as well as its F(ab′) fragments, increased T cell proliferation in murine mixed lymphocyte reaction responses (37), while the blockade of CTLA-4/B7 interaction results in the enhancement of T cell activation. Other studies demonstrated that cross-linking of CTLA-4 after the ligation of CD3 and CD28 strongly inhibited proliferation and IL-2 production (42). In vivo treatment of mice with anti–CTLA-4 enhanced clonal expansion of antigen-specific T cells after immunization (43) and increased antitumor immunity. Similarly, mice that had disruption of the CTLA-4 gene develop a lymphoproliferative disorder leading to a massive lymphocyte infiltration of different organs and to early death (44). Moreover, polymorphisms in the CTLA-4 gene have been linked to the onset of diabetes (45, 46). Thus, it was of interest to examine whether functional defects in CTLA-4 signaling were found in patients with diabetes. Therefore, we cultured PBMCs with GAD65 in the presence of CTLA-4 F(ab′) Ab. The response to GAD65 was either not affected or just slightly increased with the addition of anti–CTLA-4 F(ab′) fragments to the cultures, and there were no differences in the responses of patients as compared with control subjects. These data suggest that the blockade of CTLA-4 signaling may not underlie the immune dysregulation in type 1 diabetes in the presence of functional B7/CD28 interactions.

Several studies have attempted to determine whether B7-1 and B7-2 have redundant roles in regulating the T cell activation or if they can deliver distinct signals necessary for T cell functions. Although B7-1 and B7-2 bind to CD28 and CTLA-4, these molecules might mediate different functions, since differences exist in their distribution, kinetics, and expression after cell activation (24, 47). The affinities of the B7 molecules and their interaction with CD28 or CTLA-4 differ substantially; B7-1 binds CTLA-4 with the highest affinity, B7-2 binds CD28 with the lowest affinity, and B7-1-CD28 and B7-2-CTLA-4 bind with intermediate affinity. In mice, B7-2 is constitutively expressed on B cells, dendritic cells, and macrophages and is upregulated within

hours, whereas B7-1 is absent on resting cells and is induced 3–4 days after activation. Interestingly, we have observed recently the expression of B7-1 on approximately 29% of B cells in humans (48). Together these observations suggest that B7-2 is necessary for initial T cell costimulation, whereas the B7-1-CD28 interaction plays a role in sustained T cell activation.

A large number of studies have been performed on animal models to examine the in vivo functional effect of B7 blockade. The anti–B7-2 mAb inhibited the onset of diabetes in NOD mice when administered before or at the onset of insulitis, though its effect was less pronounced than CTLA-4 Ig and it did not prevent the occurrence of insulitis. In contrast, treatment of NOD mice with anti–B7-1 mAb resulted in a more severe infiltrate and a rapid onset of disease. In our studies, we examined the role of B7-1 versus B7-2 engagement of CD28/CTLA-4 counter-receptors on APCs presenting the GAD antigen. Similar to the NOD mouse, anti–B7-1 mAb's were unable to inhibit the T cell response to GAD65 while anti–B7-2 mAb's strongly diminished T cell proliferation and cytokine secretion in patients with type 1 diabetes. These data provide the first human in vitro correlate in patients with type 1 diabetes with the murine NOD model regarding the critical role of B7 costimulatory molecules in the disease.

Considering the differences we observed between B7-1 versus B7-2 blockade, it was of interest to investigate further the specific interactions of B7-1 and B7-2 with their counter-receptors, CD28 and CTLA-4. T cell proliferation and cytokine secretion in response to GAD65 were inhibited by the combination of anti–B7-1 with anti-CD28 mAb's, while the use of anti–B7-1 and anti–CTLA-4 together increased the proliferative and cytokine responses. In contrast, the combination of anti–B7-2 and anti–CTLA-4 mAb's strongly inhibited GAD65 T cell response while the coblockade of B7-2 and CD28 induced only a small decrease of the GAD response. These data led us to hypothesize that there is preferential engagement of CTLA-4 with B7-1 and their interaction may function to downregulate the immune response while T cell proliferation and cytokine production is upregulated by CD28-B7-2 engagement. These data confirm recent studies performed in an animal model of transplant demonstrating that the blockade of CTLA-4 or B7-1 significantly accelerated cardiac allograft rejection in transplanted CD28-deficient mice, while B7-2 blockade prolonged graft survival, suggesting that B7-1 is the dominant ligand for CTLA-4–mediated downregulation of alloimmune response in vivo. In contrast, B7-2 provides a positive costimulatory signal for T cells (49).

In summary, autoreactive T cells from patients with type 1 diabetes are not dependent on CD28/B7 costimulation, suggesting that they have been activated in vivo and have developed a memory phenotype. Although the clinical onset of type 1 diabetes is likely to be late in the disease process, newly diagnosed patients appeared to be independent of costimulation through the CD28/B7 pathway, while long-term diabetic patients examined here required a second signal for GAD65-specific T cell activation. B7-2 appears to be the primary costimulatory molecule engaging CD28 in T cell activation of GAD65-reactive T cells, and its engagement with CTLA-4 appears to deliver a negative signal. These findings strongly indicate that the activation state of antigen-specific cells plays a role in the autoimmune process and selected costimulatory molecules may represent the target of future therapies.

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