 CTLA-4 is a receptor expressed on activated T cells and shares some homology with another T cell surface receptor, CD28 (1, 2). Both molecules ligate members of the B7 family on APCs. Costimulatory CD28 molecules are expressed constitutively on most CD4+ T cells and on half of CD8+ T cells (3). CTLA-4 can be detected only on CD28+ T cells, and a strong signal via TCR and/or CD28 is required for expression of CTLA-4 (4). After incubation with anti-CD3 plus IL-2, expression of CTLA-4 on activated T cells is increased both on the surface and intracellularly (5). In contrast to the costimulatory activity of CD28, CTLA-4 ligation appears to induce negative regulation of T cell activation (1, 2, 6) both in animals and humans. Indeed, soluble anti-CTLA-4 mAbs that block CTLA-4/B7 interaction augmented CTLA-4 signaling (7). Furthermore, current evidence suggests that CTLA-4 mediates Fas-independent apoptosis of activated T lymphocytes (8).

Several reports indicate that the function of CTLA-4 relates to autoimmunity. Lymphocytes of nonobese diabetic mice, an animal model of autoimmune diabetes, have reduced expression of CTLA-4 (9). CTLA-4-deficient mice show a severe lymphoproliferative disorder, and autoimmune disease, as well as early lethality (10, 11). In theory, reduced expression or function of CTLA-4 may lead to autoimmune T cell clonal proliferation and contribute to the pathogenesis of autoimmune diseases. The Journal of Immunology, 2000, 165: 6606–6611.
Materials and Methods

Patients and cells

Heparinized blood was obtained from 63 autoimmune thyroid disease patients (43 GD, 18 HT) and from 43 healthy donors (NC). GD was diagnosed on the basis of clinical and laboratory evidence of hyperthyroidism and diffuse goiter, supported by the presence of thyroglobulin and/or thyroid peroxidase Abs, or exophthalmos. HT was diagnosed by the presence of hyperthyroidism, goiter, and thyroglobulin and/or thyroid peroxidase Abs. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 2-2ME (50 μM), and antibiotics. Genomic DNA was obtained from PBMCs using DNAzol (Molecular Research Center, Cincinnati, OH).

Analysis of CTLA-4 gene polymorphism

The CTLA-4 exon 1 position 49 A/G polymorphism was typed using a PCR-restriction fragment length polymorphism method. The appropriate segment of the CTLA-4 gene was amplified using specific primers (5’-GCTTACTTCTGGAAGACCT-3’ and 5’-AGTCTCACTCACTTTG CAG-3’). PCR was performed using genomic DNA (0.2 μg), Taq polymerase (1 U), 10 pmol of each primer, and dNTPs (200 μM) under the following conditions: initial denaturation for 4 min at 94°C, annealing for 45 s at 57°C, extension for 30 s at 72°C, denaturation for 45 s at 94°C (30 cycles), and a final extension for 10 min at 72°C. The amplified products were digested with restriction enzyme Bbv1 (New England BioLabs, Beverly, MA) and analyzed on 3.5% NuSieve GTG agarose (FMC BioProducts, Rockland, ME) gels.

Proliferation assay

PBMCs (4 × 10⁶ cells/well) plus irradiated EBB (1 × 10⁶ cells/well) as activators were plated in culture medium in 96-well round bottom microplates (Nalge Nunc International, Rochester, NY) and incubated in the presence of soluble anti-human CTLA-4 mAb or isotype control Ig (PharMingen, San Diego, CA) at different concentrations. Cultures were pulsed with 1 μCi/well of [³H]thymidine (ICN, Costa Mesa, CA) after 6 days of incubation at 37°C. Pulsed plates were harvested 16 h later, and [³H]thymidine incorporation was measured in a scintillation counter (LS7500; Beckman, Fullerton, CA). All cultures were performed in triplicate.

Flow cytometric analysis

PBMCs were plated in 96-well round-bottom microplates and incubated at 37°C with or without EBB for different numbers of days. For detection of CTLA-4 on T cells activated with anti-CD3 mAb, PBMCs were cultured for 2 days at 37°C with a 1:10 dilution of supernatant from an anti-human CD3 mAb-producing hybridoma (American Type Culture Collection, Manassas, VA) and human IL-2 (100 U/ml). Following the incubation period, the cells were harvested and resuspended in staining buffer (1% BSA, 0.1% OVA, 0.1 M glycine, 0.1% NaN₃ in PBS) for detection of surface CTLA-4. For detection of intracellular CTLA-4, the cells were resuspended and fixed in 2% paraformaldehyde, incubated for 30 min at 4°C and then incubated in permeabilization buffer (0.04% saponin, 0.1% OVA, 0.1 M glycine, 0.1% NaN₃ in PBS) for 30 min at 4°C. PE-conjugated anti-human CD4 and Cy-Chrome-conjugated anti-human CD8 or similarly labeled isotype control Ig (PharMingen) were added and incubated for an additional 20 min at 4°C. Cells were washed and fixed with 2% paraformaldehyde. Flow cytometry was performed using FACScan (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software (Becton Dickinson).

Statistical analysis

Distributions of alleles in patients and controls were compared using Fisher’s exact test. The difference between T cell proliferation in each group was analyzed using Student’s t test. Values of p < 0.05 were regarded as significant.

Results

CTLA-4 exon 1 polymorphism in patients with GD and HT

It has been shown previously that CTLA-4 exon 1 gene polymorphism is associated with GD (14, 15). To determine whether this correlation existed in our subset of patients, we genotyped NC as well as individuals with GD and HT. The distribution of the genotypes is shown in Table I. Among patients with GD, there were more individuals with the G/G (17.8% vs 11.6% of controls) or A/G CTLA-4 exon 1 genotypes (64.4% vs 53.5% control), and significantly fewer individuals with the A/A alleles (17.8% vs 34.9% control; p = 0.049) when compared with controls. Therefore, in accordance with previously published results, the gene frequency of the G allele was higher in GD patients (50.0%) than in controls (38.4%) in our population.

Surface and intracellular expression of CTLA-4

To be able to analyze CTLA-4 function in the different groups, we first investigated whether CTLA-4 was expressed in all subgroups and genotypes of patients following T cell activation. PBMCs from GD, HT, and NC patients with the different CTLA-4 genotypes were stimulated with anti-human CD3 mAb and IL-2 for 48 h. Intact and permeabilized cells were analyzed by flow cytometry for expression of surface and intracellular CTLA-4, respectively. Unstimulated T cells expressed undetectable levels of surface or intracellular CTLA-4 (data not shown). Fig. 1, 1a and 1b show a representative example of surface and intracellular CTLA-4 expression obtained after anti-CD3 mAb stimulation. As described previously, intracellular expression of CTLA-4 was higher than surface expression. The difference in mean fluorescence intensity (ΔMFI) between CTLA-4 and isotype control Ig staining was 6 for surface staining and 84 for intracellular staining. Using this stimulation protocol, we could not detect any significant differences in the levels of expression of surface or intracellular CTLA-4 in T cells from the different subgroups or genotypes of patients (data not shown).

To be able to compare the function of CTLA-4 between different individuals, it was necessary to use a system with identical activating conditions during each stimulation. Soluble anti-CD3 mAb induced high levels of CTLA-4 expression, but has been previously shown to depend on ligation of Fc receptors on APCs to result in T cell activation (4, 24). Because of high variability in the percentage of Fc receptor-expressing APCs in the PBMCs of the different donors, we felt that this was not an optimal system. Instead, we decided to use a homogenous allogeneic cell-line (EBB) to stimulate T cells from all individuals in a MLR assay. We first performed a kinetic experiment to determine peak expression of CTLA-4 following incubation of PBMCs with irradiated EBB. Cells were collected at different time points following stimulation, permeabilized, stained with anti-CTLA-4 Ab, and analyzed by flow cytometry. Fig. 1, 2a displays the mean ΔMFI of intracellular CTLA-4 expression over time in two individuals. Levels of expression were much lower than those following anti-CD3 mAb stimulation, an expected result because only 10% of T cells are typically alloreactive. However, CTLA-4 expression was clearly detectable at day 5 after stimulation. Fig. 1, 2b and 2c show a
The expression of CTLA-4 on CD4+ T cells from normal subjects after activation with anti-CD3 or EBB. 1a, Intracellular. Surface expression after activation with anti-CD3. 1b, Intracellular expression after activation with anti-CD3. 2a, For expression of CTLA-4 after activation with EBB at different time points, PBMCs were incubated with allogeneic irradiated EBB for 1 to 6 days. The y-axis represents MFI. 2b, Surface expression after activation with EBB. 2c, Intracellular expression after activation with EBB.

Soluble anti-human CTLA-4 mAb augments proliferation of T cells activated with EBB in a dose-dependent manner

Low levels of CTLA-4 expression were induced on T cells in our MLR system. To determine whether CTLA-4 exerted a function in the MLR, we attempted to prevent binding of CTLA-4 to its ligands by including a blocking anti-human CTLA-4 mAb in the culture. Several studies have shown that soluble anti-CTLA-4 mAbs augment the proliferation of stimulated T cells, whereas cross-linked anti-CTLA-4 mAbs inhibit T cell proliferation (8, 24, 25). A dose titration of soluble anti-CTLA-4 mAb or of control Ig was added at the initiation of the culture of PBMCs with irradiated EBB. Anti-CTLA-4 mAb but not control Ig augmented proliferation of T cells stimulated by irradiated EBB in a dose-dependent manner. Fig. 2 shows the mean ± SD of proliferation data from the whole population of individuals tested. The addition of 250 ng/ml of anti-CTLA-4 induced a highly significant increase in [3H]thymidine incorporation as compared with control Ig (p < 0.001). Therefore, blockade of CTLA-4 binding to B7 family members results in a significant increase in T cell proliferation, indicating that, despite the low levels of CTLA-4 expression induced by the MLR conditions, CTLA-4 ligation reduces T cell responses to allogeneic EBB in this model.

Proliferation of T cells from subjects with the G/G alleles is higher than that from individuals with the A/A alleles in both GD and NC groups

We next compared the function of T cells between the different groups of patients as well as between the different CTLA-4 genotypes. First, T cell proliferation to EBB in the absence of anti-CTLA-4 mAb was analyzed in patients with GD, HT, and in NC individuals. There was no significant difference in proliferation between groups (Fig. 3A). This result is similar to previous reports that used other stimulating conditions (26–28). Two possibilities may explain this phenomenon. This T cell response represents a global response, not specific for the CTLA-4 pathway or CTLA-4 polymorphism. Presumably, the distribution of CTLA-4 polymorphisms between subgroups (although statistically significant) was not enough to differentially affect this global T cell response.

To determine whether expression of specific CTLA-4 alleles affects T cell function, we categorized these populations with respect to the CTLA-4 exon 1 polymorphism and compared T cell proliferation. Interestingly, T cells from subjects with the G/G genotype showed a significantly higher proliferation to EBB than did T cells from subjects with the A/A alleles (p = 0.047) (Fig. 3B). This result indicates that the CTLA-4 polymorphism correlates with differential proliferation of T cells.

CTLA-4 polymorphism does affect CTLA-4 function

The expression of the G/G alleles in exon 1 of the CTLA-4 gene correlated with increased EBB-induced T cell proliferation as compared with that observed with T cells expressing the A/A alleles. To determine whether this increase in proliferation was due to a functional difference exerted by the CTLA-4 proteins, T cell proliferation between the different genotypic groups was analyzed under conditions of CTLA-4/B7 blockade by anti-CTLA-4 mAb. Results obtained with the highest concentration of anti-CTLA-4
mAb (250 ng/ml) previously shown to induce significant augmentation of T cell proliferation as compared with control Ig (see Fig. 2) were used for this purpose. We calculated the percentage of augmentation after CTLA-4 blockade using the following formula: % of augmentation = \( \frac{[T \text{ cell proliferation with anti-CTLA-4 mAb (CPM)]} - [T \text{ cell proliferation with control Ig (CPM)]}}{[T \text{ cell proliferation with control Ig (CPM)]}} \times 100\% \). There was a significant difference between proliferation from subjects with G/G and A/A alleles. * \( p \) (G/G vs A/A) = 0.047

FIGURE 4. Effect of the genotype on CTLA-4 function in the whole population. PBMCs were incubated with allogeneic irradiated EBB plus anti-CTLA-4 mAb or control IgG for 6 days. Proliferation was assessed by \(^{3}H\)thymidine incorporation for 16 h. Percent augmentation of proliferation by blocking the CTLA-4 pathway was calculated as follows: % of augmentation = \( \frac{[T \text{ cell proliferation with anti-CTLA-4 mAb (CPM)]}}{[T \text{ cell proliferation with control Ig (CPM)]}} \times 100\% \). There was a significant difference between proliferation from subjects with G/G and A/A alleles. * \( p \) (G/G vs A/A) = 0.019

Discussion

Three CTLA-4 gene polymorphisms (in the promoter at position 318 from the ATG start codon (29), at position 49 in exon 1 (30), and in an (AT)n repeat within the 3′-untranslated region of exon 3 (31)) have been published in humans. We initially reported an association between the 106-bp repeat in the 3′ untranslated region and GD (12) and confirm in this paper an association between polymorphism at position 49 in exon 1 and GD. Many subsequent studies have supported a correlation between CTLA-4 gene polymorphisms and autoimmune diseases (13–23). However, an effect of CTLA-4 gene polymorphism on T cell function could never be demonstrated. In this study, we show for the first time a direct correlation between CTLA-4 gene polymorphism and T cell function, as well as between the CTLA-4 genotype and the function of the CTLA-4 protein in T cells. The distribution of CTLA-4 polymorphism at position 49 in exon 1 in patients with GD, HT, and NC was similar to previously reported data (14–17, 19), with a higher frequency of G/G alleles and lower frequency of A/A alleles in patients with GD. The presence of G/G alleles correlated with increased T cell proliferation following EBB stimulation compared with that of T cells expressing A/A alleles. In addition, blockade of CTLA-4 ligation using soluble anti-CTLA-4 mAb did not augment proliferation as much in T cells from G/G-expressing individuals as from A/A-expressing ones. Therefore, CTLA-4 exerted less profound inhibitory effects on T cell proliferation in subjects bearing the G/G rather than the A/A genotype.

We found that the CTLA-4 G/G genotype correlated with reduced function of the CTLA-4 protein in the three populations tested, regardless of the presence of an autoimmune disease. However, the G/G genotype was more frequent in the population with GD and HT than in the NC population. The fact that some NC individuals bearing the G/G genotype do not develop autoimmune diseases whereas some patients with the A/A genotype suffer from autoimmunity underlines the multifactorial etiology of autoimmune diseases. Indeed, in most autoimmune diseases, susceptibility is also linked closely with MHC class II alleles. Presumably this is because T cells recognize the Ag presented by MHC class II on APCs, and this interaction makes the MHC class II region a strong candidate for involvement in T cell-mediated autoimmune diseases (32). For example, insulin-dependent diabetes mellitus is associated with MHC class II DR3 and DR4 (33), GD with DR3 (34), and HT with DR3 and DR4 (35). In addition, thyroid follicular cells, which express MHC class II Ags, are the target for the autoimmune process in GD. Weetman suggested that the autoreactive T cells initially stimulated by conventional APC may be stimulated by class II-positive thyroid follicular cells in the presence of B7 expressing APC and exacerbate disease (36).

CTLA-4 has been shown in multiple in vitro and in vivo systems to inhibit T cell responses. How this inhibitory effect is mediated...
Our study indicates that the inhibitory effect of CTLA-4 on T cell responses in the absence of CD28 is still unclear. Because CTLA-4 binds B7 family members with much higher affinity than CD28, expression of CTLA-4 upon T cell stimulation may result in CTLA-4 molecules scavenging B7 ligands away from CD28, therefore reducing the costimulatory effects of CD28. In addition, the cytoplasmic tail of CTLA-4 shares some common binding motifs with CD28, such as a phosphatidylinositol 3-kinase binding domain, suggesting that CTLA-4 activation may lead to sequestering of intracellular enzymes away from the CD28 cytoplasmic tail, resulting in dampening of CD28 signaling. However, CTLA-4 has also been shown to mediate down-regulation of T cell responses in the absence of CD28 (37, 38), indicating that CTLA-4 must have signaling properties independent of its effects on CD28. In particular, CTLA-4 cross-linking has been shown to reduce activation of the mitogen-activated protein kinase pathway induced following TCR stimulation in the absence of a second signal (39).

Other hypotheses to explain reduced function of CTLA-4 in subjects with a G/G genotype have been proposed. Several hypotheses can explain these results. First, it is possible that a CTLA-4 gene polymorphism in the leader sequence (exon 1) may influence the level or pattern of expression of the protein. Moreover, we have compared the percentage of apoptosis induced by different means in T cells from patients expressing the G/G vs the A/A alleles and have not found any significant differences (data not shown).

An association between CTLA-4 gene polymorphism and CTLA-4 protein function has not been reported previously. It was important that the conditions used to stimulate T cells to detect differences in CTLA-4 function were as close to physiological as possible. Indeed, the stimuli used in mouse experiments to stimulate T cells for CTLA-4 expression are often very potent (anti-CD3 plus anti-CD28 mAbs (5) or PMA plus ionomycin (40)). It is possible that under those conditions CTLA-4 expression is maximal and differences in CTLA-4 function may therefore not be detectable if variations in expression depending on genotype are subtle. To be able to compare CTLA-4 function between patients, we felt it was necessary to ligate CTLA-4 with its natural ligand B7 rather than with cross-linking Abs. In addition, a system in which the stimulus is always the same would ensure reduced variability. To this end, we used irradiated EBB that express allogeneic MHC class II and B7-1 to ligate TCR and CTLA-4, respectively. The proliferative response was largely the result of CD4 T cell recognition of MHC class II gene products.

Under suboptimal activation conditions, CTLA-4 might serve to attenuate weak signals mediated by the TCR and CD28 (41). Recently, Brunner et al. showed that cross-linked anti-CTLA-4 mAb suppressed IL-2 mRNA accumulation at 4 h after activation, a time at which CTLA-4 was undetectable on the cell surface, and suppressed T cell proliferation (42). We described how T cells activated by EBB showed submaximal expression of CTLA-4 compared with the response using anti-CD3. We used soluble anti-CTLA-4 mAb to block the negative signal between B7 and CTLA-4 and showed suppression of T cell activation via the CTLA-4 pathway. These observations suggest that CTLA-4 is induced at sufficient levels to regulate T cell responses.

We cannot exclude the effect of other cells, such as monocytes and B cells present in our system, on cytokine production or T cell proliferation. This possibility can be eliminated by studies using purified T cells, T cell lines, or CTLA-4-transfected T cells, which are in progress. However, the lower suppression associated with the G/G allele is seen in all subgroups (GD, HT, and NC) indicating that the concept is applicable in general to immune responses and is not specific for the cell types present in patients with GD or HT.
GD is an autoimmune disease and is thought to be caused by multiple genetic factors. CTLA-4 gene polymorphism is one of these factors. CTLA-4 encoded by the G7 alleles appears to cause less suppression of T cells perhaps allowing expansion of autoimmune clones. This reduced CTLA-4 function may also explain the relation of the CTLA-4 polymorphism to several other autoimmune illnesses.

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References

1. Chambers, C. A., M. F. Krummel, B. Boitel, A. Hurwitz, T. J. Sullivan, S. Fournier, D. Cassell, M. Brunner, and J. P. Allison. 1996. The role of CTLA-4 in the regulation and initiation of T-cell responses. Immunol. Rev. 153:27.
2. Linsley, P. S. 1995. Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation? J. Exp. Med. 182:289.
3. Stilikas, P. P., and C. S. Vai. 1997. Expression of CD28, CTLA-4, CD80, and CD86 molecules in patients with autoimmune rheumatic diseases: implications for autoimmunopathology. Clin. Immunol. Immunopathol. 83:195.
4. Linsley, P. S., J. G. Greene, P. Tan, J. Bradshaw, J. A. Ledbetter, C. Anasetti, and N. K. Damle. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J. Exp. Med. 176:1593.
5. Alegre, M.-L., P. J. Noel, B. J. Eifsfeld, E. Chuang, M. R. Clark, S. L. Reiner, and C. B. Thompson. 1996. Regulation of surface and intracellular expression of CTLA-4 on mouse T cells. J. Immunol. 157:4762.
6. Oosterwegel, M. A., R. J. Greenwald, D. A. Mandelbrot, R. B. Lorsbach, and A. H. Sharpe. 1999. CTLA-4 and T cell activation. Curr. Opin. Immunol. 11:294.
7. Tivol, E. A., A. A. Schweitzer, and A. H. Sharpe. 1996. Costimulation and autoimmunity. Curr. Opin. Immunol. 8:822.
8. Scheipers, P., and H. Reiser. 1998. Fas-independent death of activated CD4 T lymphocytes induced by CTLA-4 crosslinking. Proc. Natl. Acad. Sci. USA 95:10083.
9. Colucci, F., M. L. Bergman, C. Penna-Goncalves, C. M. Cilio, and D. Holmberg. 1997. Apoptosis resistance of nonobese diabetic peripheral lymphocytes linked to the IDd5 diabetes susceptibility region. Proc. Natl. Acad. Sci. USA 94:8670.
10. Tivol, E. A., F. Borriello, A. A. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multigorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 3:541.
11. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, B. C. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science 270:985.
12. Yangagawa, T., Y. Hidaka, V. Guimarães, M. Soliman, and L. J. DeGroot. 1995. CTLA-4 gene polymorphism associated with Graves’ disease in a Caucasian population. J. Clin. Endocrinol. Metab. 80:41.
13. Kouri, K., P. F. Watson, and A. P. Weetman. 1997. A CTLA-4 gene polymorphism is associated with both Graves disease and autoimmune hypothyroidism. Clin. Endocrinol. 46:551.
14. Donner, H., H. Rau, P. G. Wallish, J. Braun, T. Siegmund, R. Finke, J. Herwig, K. H. Usadel, and K. Badenhoop. 1997. CTLA-4 alanine-17 confers genetic susceptibility to Graves’ disease in Japanese. Thyroid 7:843.
15. Badenhoop, K., H. Donner, J. Braun, T. Siegmund, H. Rau, and K. H. Usadel. 1996. Genetic markers in diagnosis and prediction of relapse in Graves’ disease. Exp. Clin. Endocrinol. Diabetes 104 (Suppl. 4):98.
16. Awata, T., S. Kashiwara, M. Itaka, S. Takii, I. Ione, C. Ishii, K. Negishi, and T. Iizumida. 1998. Association of CTLA-4 gene A-G polymorphism (IDDM12 locus) with acute-onset and insulin-depleted IDDM as well as autoimmune thyroid disease (Graves’ disease and Hashimoto’s thyroiditis) in the Japanese population. Diabetes 47:128.
17. Braun, J., H. Donner, T. Siegmund, P. G. Wallish, K. H. Usadel, and K. Badenhoop. 1998. CTLA-4 promoter variants in patients with Graves’ disease and Hashimoto’s thyroiditis. Tissue Antigens 51:563.
18. Donner, H., J. Braun, C. Seidl, H. Rau, R. Finke, M. Ventz, P. G. Wallish, and K. H. Usadel. 1997. Codon 17 polymorphism of the cytotoxic T lymphocyte antigen 4 gene in Hashimoto’s thyroiditis and Addison’s disease. J. Clin. Endocrinol. Metab. 82:4130.