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Autoantibodies to T Cell Costimulatory Molecules in Systemic Autoimmune Diseases

Toshihiro Matsui,*† Mana Kurokawa,* Tetsuji Kobata,*‡ Shinji Oki,§ Miyuki Azuma,§ Shigeto Tohma,† Tetsufumi Inoue,† Kazuhiro Yamamoto,† Kusuki Nishioka,* and Tomohiro Kato‡*

To determine whether antilymphocyte Abs to T cell costimulatory molecules are generated in patients with autoimmune diseases and, if they exist, to clarify the mechanism of their production and pathological roles, we investigated the presence of autoantibodies to CTLA-4 (CD152), CD28, B7-1 (CD80), and B7-2 (CD86) in serum samples obtained from patients with various autoimmune diseases and from normal subjects using recombinant fusion proteins. In ELISAs, anti-CD28, anti-B7-1, and anti-B7-2 Abs were rarely seen, whereas anti-CTLA-4 Abs were detected in 8.2% of the patients with systemic lupus erythematosus, 18.8% of those with rheumatoid arthritis, 3.1% of those with systemic sclerosis, 31.8% of those with Behcét’s disease, 13.3% of those with Sjögren’s syndrome, and 0% of healthy donors. This reactivity was confirmed by immunoblotting. More importantly, the purified anti-CTLA-4 Abs reacted with CTLA-4 expressed on P815 cells by flow cytometry. In addition, we found at least three epitopes on the CTLA-4 molecule. Furthermore, among the patients with Behcét’s disease, uveitis was seen significantly less frequently in the anti-CTLA-4 Ab-positive patients. Taken collectively, these data indicate that anti-CTLA-4 autoantibodies are generated in systemic autoimmune diseases by an Ag-driven mechanism and may modulate the immune response in vivo by binding to CTLA-4 on T cells.


Autoantibodies to T cell costimulatory molecules have been described in various immune conditions, such as autoimmune diseases, infections, blood transfusions, and multiple pregnancies (reviewed in Refs. 1 and 2). For instance, the presence of anti-lymphocyte Abs (ALAs) has been correlated with disease activity (3), lymphopenia (4), lymphocyte subset distortions (5, 6), and various functional abnormalities of T cells, B cells, and monocytes in systemic lupus erythematosus (SLE) (7–10). However, the pathogenetic roles of ALAs remain unclear mainly because ALAs of those with rheumatoid arthritis, SLE, and, if they exist, to clarify the mechanism of their production and pathological roles, we investigated the presence of autoantibodies to CTLA-4 (CD152), CD28, B7-1, and B7-2. Abs were rarely seen, whereas anti-CTLA-4 Abs were detected in 8.2% of the patients with systemic lupus erythematosus, 18.8% of those with rheumatoid arthritis, 3.1% of those with systemic sclerosis, 31.8% of those with Behcét’s disease, 13.3% of those with Sjögren’s syndrome, and 0% of healthy donors. This reactivity was confirmed by immunoblotting. More importantly, the purified anti-CTLA-4 Abs reacted with CTLA-4 expressed on P815 cells by flow cytometry. In addition, we found at least three epitopes on the CTLA-4 molecule. Furthermore, among the patients with Behcét’s disease, uveitis was seen significantly less frequently in the anti-CTLA-4 Ab-positive patients. Taken collectively, these data indicate that anti-CTLA-4 autoantibodies are generated in systemic autoimmune diseases by an Ag-driven mechanism and may modulate the immune response in vivo by binding to CTLA-4 on T cells.

Materials and Methods

Patients and healthy donors

Serum samples were obtained from a total of 162 patients with systemic autoimmune diseases (138 women and 24 men; mean age, 50.2 yr; age range, 18–79 yr) and 60 healthy donors. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 Abbreviations used in this paper: ALA, anti-lymphocyte antibodies; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; SSc, systemic sclerosis; Sjs, Sjögren’s syndrome.

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range, 20–79 yr). They included 49 patients with SLE (46 women and 3 men; mean age, 42.3 yr; age range, 20–72 yr), 48 patients with rheumatoid arthritis (RA; 39 women and 9 men; mean age, 56.1 yr; age range, 22–79 yr), 32 patients with systemic sclerosis (SSc; 28 women and 4 men; mean age, 52.2 yr; age range, 29–71 yr), 22 patients with Behcet’s disease (14 women and 8 men; mean age, 50.9 yr; age range 24–78 yr), and 15 patients with Sjögren’s syndrome (SjS; 15 women; mean age, 49.4 yr; age range, 32–66 yr). Four patients with both RA and SjS were included in the respective groups. The diseases were diagnosed according to the standard criteria of each disease (28–32). Control serum samples were obtained from 82 healthy donors (68 women and 14 men; mean age, 49.5 yr; age range, 22–82 yr). The patients were being treated at the hospital of the University of Tokyo or the hospital of St. Marianna University School of Medicine.

Preparation of CTLA-4, CD28, B7-1, and B7-2 cDNA

Based on the previously reported nucleotide sequences of human CTLA-4 (33), CD28 (34), B7-1 (35), and B7-2 (36, 37), four sets of oligo-DNA primers were synthesized (Table 1), and PCR was performed. Each primer pair was used to amplify the DNA fragments that encode the full protein-coding region of CTLA-4, CD28, B7-1, and B7-2. All the PCR products were confirmed by sequencing.

Construction of the expression plasmids of CTLA-4, CD28, CD28, and B7-1

The cDNA fragments that encode CTLA-4 (672 bp) and B7-2 (972 bp) were each subcloned into pTEx7 (38), a derivative of the previously reported plasmid expression vector pEx2 (39). In this expression system, the proteins are expressed as β-galactosidase fusion proteins. The cDNA fragments that encode CD28 (663 bp) and B7-1 (867 bp) were each subcloned into pTEX7-His, which is a derivative of the plasmid expression vector pTEX7. With this plasmid, a continuous stretch of six histidines (His)6, which can be used for affinity purification, are expressed between β-galactosidase and the protein of interest.

Expression and purification of the recombinant fusion proteins

The reactivity to the fusion protein in ELISA was expressed in units according to the formula: sample (binding unit) = [ODsample* (mean ODsample* + 3 SD of normal sera)] × 100. For each sample, the OD value of β-galactosidase was subtracted from the OD value of the fusion protein to obtain ODsample*. According to this formula, 100 binding units is the cutoff point.

Immunoblotting

Immunoblotting was performed as previously described (40). Briefly, 5 µg of each purified fusion protein or β-galactosidase (as a control), was separated by 8% SDS-PAGE, transferred onto nitrocellulose membranes. After blocking with PBS containing 3% BSA and 0.1% Tween 20 for 1 h and washing in PBS with 0.1% Tween-20 for 30 min, each membrane was then incubated with the respective protein-specific Ab (goat anti-human CTLA-4 Ab, goat anti-human CD28 Ab, goat anti-human B7-1 Ab, or goat anti-human B7-2 Ab; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit

| Table I. PCR primers used to amplify the DNA fragments encoding T cell costimulatory molecules |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Molecule**    | **Primers 5′→3′** | **Reference**   |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CTLA-4          | TTGGATCCATGCTGTTGGCTGTTGGATTTCA | EcoRI         | TTTTCATTGAACTGTAATTGGAAGATTTCA | 28              |
| CD28            | TTGGATCCATGCTGTTGGCTGTTGGATTTCA | EcoRI         | TTTTCATTGAACTGTAATTGGAAGATTTCA | 29              |
| B7-1            | AAAGGATCCATGCTGTTGGCTGTTGGATTTCA | BamHI    | AAAGGATCCATGCTGTTGGCTGTTGGATTTCA | 30              |
| B7-2            | AAAGGATCCATGCTGTTGGCTGTTGGATTTCA | BamHI    | AAAGGATCCATGCTGTTGGCTGTTGGATTTCA | 31, 32          |

mM EDTA, 50 mM NaCl, and 50 mM Tris-HCl, pH 7.6. The pellets were then solubilized in 1 ml of 8 M urea.

To purify the recombinant CD28 and B7-1 proteins, the bacteria in which the recombinant proteins were induced, were lysed in 6 M guanidium buffer and sonicated. After centrifugation, the lysate was applied to a column charged with ProBond resin (NiCl2; Invitrogen, San Diego, CA) in a binding buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 7.8). The column was then developed in buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 6.0 and pH 5.3). The (His)6-tagged recombinant proteins were eluted with an elution buffer (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl, pH 4.0).

The concentration of fusion proteins was determined from absorbances at 280 and 260 nm, which was corrected for background activity at 320 nm using appropriately diluted samples.

ELISA

Each well of a multititer plate (Cook, Dynatech, Alexandria, VA) was coated with 10 µg/ml of the individual purified fusion protein or β-galactosidase (as a background) in a carbonate buffer (50 mM sodium carbonate, pH 9.6), followed by blocking with PBS containing 3% BSA and 0.1% Tween 20. To adsorb the reactivity of the serum samples to bacterial proteins and β-galactosidase, the serum samples were diluted and incubated with 20 µg/ml of bacterial lysate containing nonrecombinant pTEX7 product in PBS containing 3% BSA and 0.1% Tween-20 for 2 h at room temperature before incubation with the coated recombinants. The serum dilution that exhibited the highest specific binding was determined to be 1/1000 for CTLA-4 and B7-2 and 1/2000 for CD28 and B7-1. After reacting with the coated recombinants for 2 h, the wells were washed 10 times in PBS with 0.1% Tween-20. Next, the bound Abs were incubated with horseradish peroxidase-conjugated goat anti-human IgG (specific for γ-chain; Zymed, San Francisco, CA) at a 1/4000 dilution for 2 h at 4°C, then reacted with o-phenylenediamine as a substrate, and quantitated using a microplate photometer at 492 nm.

In the screening of autoantibodies to CTLA-4, CD28, CD28, and B7-2, serum samples from 162 patients with systemic autoimmune disease and 82 sex- and age-matched healthy donors (68 women and 14 men; mean age, 49.5 yr; age range, 22–82 yr) were prepared. In the screening of autoantibodies to CTLA-4, CD28, CD28, and B7-2, serum samples from 40 of the healthy donors (38 women and 2 men; mean age, 43.0 yr; age range, 26–66 yr) for SLE, from 40 of the healthy donors (38 women and 2 men; mean age, 43.0 yr; age range, 22–82 yr). The patients were being treated at the hospital of the University of Tokyo or the hospital of St. Marianna University School of Medicine.

Preparation of CTLA-4, CD28, B7-1, and B7-2 cDNA

Based on the previously reported nucleotide sequences of human CTLA-4 (33), CD28 (34), B7-1 (35), and B7-2 (36, 37), four sets of oligo-DNA primers were synthesized (Table 1), and PCR was performed. Each primer pair was used to amplify the DNA fragments that encode the full protein-coding region of CTLA-4, CD28, B7-1, and B7-2. All the PCR products were confirmed by sequencing.

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Construction of the expression plasmids of three cDNA fragments of CTLA-4

To map the autoantigens, we prepared three fragments of CTLA-4 (F1, F2, and F3). The pTEX7 with the F1 fragment (pTEX7-cDNA3) was constructed by removing an EcoRI/PstI fragment, nucleotides 184–672, from the pTEX7 plasmid with the cDNA of CTLA-4 (pTEX7-cDNA2). Similarly, pTEX7-cDNA2 was constructed by removing an EcoRI/EcoRI fragment, nucleotides 1–184, and an NcoI/PstI fragment, nucleotides 363–672, from pTEX7-cDNA2. The pTEX7-cDNA2 was constructed by removing an EcoRI/EcoRI/Neol fragment, nucleotides 1–363, from pTEX7-cDNA2. The reactivity to the fusion protein in ELISA was expressed in units according to the formula: sample (binding unit) = [ODsample* (mean ODsample* + 3 SD of normal sera)] × 100. For each sample, the OD value of β-galactosidase was subtracted from the OD value of the fusion protein to obtain ODsample*. According to this formula, 100 binding units is the cutoff point.

Immunoblotting

Immunoblotting was performed as previously described (40). Briefly, 5 µg of each purified fusion protein or β-galactosidase (as a control), was separated by 8% SDS-PAGE, transferred onto nitrocellulose membranes. After blocking with PBS containing 3% BSA and 0.1% Tween 20 for 1 h and washing in PBS with 0.1% Tween-20 for 30 min, each membrane was then incubated with the respective protein-specific Ab (goat anti-human CTLA-4 Ab, goat anti-human CD28 Ab, goat anti-human B7-1 Ab, or goat anti-human B7-2 Ab; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit
Affinity purification of autoantibodies to fusion proteins

Adsorption and affinity purification were performed essentially according to the method of Olinstede (41). The partially purified recombinant proteins were separated by SDS-PAGE, then transferred onto nitrocellulose membranes. Next, the recombinant protein bands, visualized with a ponceau S solution (Sigma, St. Louis, MO), were cut out and used for affinity purification. Each serum sample that had been preincubated with bacterial lysate containing nonrecombinant pTEX7 product for 2 h was diluted in PBS containing 3% BSA and 0.1% Tween 20 (1/500 dilution) and incubated for 2 h with the proteins fixed on the nitrocellulose membrane, after which the membrane was recovered. The bound Abs were eluted with 4 ml of 0.2 M glycine HCl buffer, pH 2.5, containing 0.15 M NaCl and 3% BSA, followed by neutralization with 600 μl of 2 M Tris base.

Establishment of stable transfectant

Human CTLA-4 cDNA was obtained from anti-CD3-activated PBMC by PCR using the sense primer (GATCTCGAGAGTGGCTGCTTG GATTTGA) and the antisense primer (GATGCGCAGCGCTCAATT GATGGGAAATAG), where the recognition sites for the restriction enzymes used are underlined. The cDNA was cloned into the XhoI- and SalI-digested BCGMNSneo expression vector containing a neomycin gene (42). Transfection of murine mastocytoma P815 cells was achieved by electroporation as described by Azuma et al. (43). The drug-resistant cells were harvested and cloned, and cells with the highest cell surface expression of CTLA-4 were selected by flow cytometry.

Indirect immunofluorescence and flow cytometry

Binding of the purified anti-CTLA-4 Abs to native CTLA-4 was detected by indirect immunofluorescence. To block the binding of Igs to mouse Fc receptors, the P815 cells and the CTLA-4-transfected P815 cells were preincubated with mouse serum diluted 1/10 for 1 h at 4°C. After washing the cells in a staining buffer (PBS containing 2% BSA), the cells were suspended in the staining buffer and reacted with Abs purified from the patients’ sera or anti-CTLA-4 mAb (Biostride, Palo Alto, CA) for 30 min on ice. After washing in the staining buffer, the cells were incubated with phycoerythrin-conjugated goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) or phycoerythrin-conjugated rabbit antimouse Ig (Southern Biotechnology Associates), respectively, for 30 min on ice. The fluorescence intensity was measured by FACSCalibur (Becton Dickinson, Mountain View, CA).

Statistical analysis

Laboratory data are expressed as the mean ± SEM. The Mann-Whitney U test was used to examine differences in the clinical parameters of patients with or without anti-CTLA-4 Ab. Fisher’s exact test was used to examine the correlation between clinical features of the patients and the presence of anti-CTLA-4 Ab.

Results

Expression of four recombinant costimulatory molecules and their antigenicities

DNA fragments encoding each of the human CTLA-4, CD28, B7-1, and B7-2 molecules were obtained by PCR from cDNA of a healthy donor PBL and then expressed as a β-galactosidase fusion protein. Thus, we obtained sufficient amounts of each fusion protein that had the expected m.w. (Fig. 1). We first confirmed the antigenicity of each of these recombinant proteins by immunoblotting using the respective protein-specific Abs. As shown in Fig. 1, the recombinant CTLA-4, B7-1, and B7-2 proteins were each stained as a clear band. In the case of CD28, staining with its specific Ab produced two major bands. Since the upper band had the expected m.w., the lower band was thought to be a degraded product of the full-length CD28 fusion protein. In addition, since approximately half the purified recombinant CD28 was the full-length CD28 fusion protein, the protein was thought to be of sufficient quality for detecting autoantibodies in ELISA.
Reactivity of the serum of patients with various systemic autoimmune diseases with the recombinant CTLA-4, CD28, B7-1, and B7-2 proteins

The recombinant CTLA-4, CD28, B7-1, and B7-2 proteins were used to detect the presence of the respective autoantibodies in the sera of patients with various systemic autoimmune diseases by ELISA. As shown in Fig. 2, IgG-type anti-CTLA-4 Ab was detected in the sera of 21 of the 162 patients (13.0%); however, the sera of only three patients were positive for anti-B7-2 Ab, and no patient was positive for anti-CD28 or for anti-B7-1 Ab. Autoantibodies to each of the four molecules were not detected in the sera of any of the healthy donors. In addition, the presence of IgM-type anti-CTLA-4 Ab was determined in a similar way and was detected in the sera of only three patients (data not shown).

The prevalence of the anti-CTLA-4 autoantibody in each disease is shown in Fig. 3. The anti-CTLA-4 Ab was detected in the sera of 4 of 49 patients with SLE (8.2%), 9 of 48 patients with RA (18.8%), 1 of 32 patients with SSc (3.1%), 7 of 22 patients with Behçet’s disease (31.8%), and 2 of 15 patients with Sjs (13.3%). The positive serum samples were further tested by immunoblotting. Of the 21 samples that were positive by ELISA, 19 reacted to CTLA-4. The results of 5 representative autoimmune disease patients are shown in Fig. 4.

Recognition of native CTLA-4 by the autoantibodies

Our findings showed that a considerable percentage of the tested autoimmune disease sera contained anti-CTLA-4 Abs. However, the recombinant CTLA-4 produced in E. coli and solubilized in 8 M urea is denatured, and thus would not be expected to have the native conformation.

Therefore, we further investigated whether the anti-CTLA-4 autoantibodies in the patients could bind to the native form of CTLA-4 molecules. Specifically, we purified the Abs bound to the recombinant CTLA-4 and then examined whether these Abs bind to CTLA-4 molecules expressed on mammalian cells (P815) by

**FIGURE 3.** Prevalence of autoantibodies to recombinant CTLA-4 in sera from patients with SLE, RA, Behçet’s disease, SSc, and Sjs and from healthy donors (normals). The serum dilution was 1/700. The OD of each sample is indicated in binding units (see Materials and Methods). The dotted line represents the positive cutoff point, which was calculated as the mean binding units of 40 sex- and age-matched healthy donors + 3 SD in the respective diseases. ●, patient with both RA and Sjs.

**FIGURE 4.** Immunoreactivity of recombinant CTLA-4. Recombinant CTLA-4 and β-galactosidase (βG) were separated by 8% SDS-PAGE and were transferred onto nitrocellulose membranes. Serum samples that were anti-CTLA-4 Ab positive by ELISA were tested by immunoblotting. The control membranes were stained with Ponceau S. Five representative samples are shown. SLE6, SLE patient; RA5, RA patient; ns-27, 31, 51, Behçet’s disease patients.

**FIGURE 5.** Reactivity of anti-CTLA-4 Abs with CTLA-4-transfected P815 cells. P815 cells and CTLA-4-transfected P815 cells were examined by indirect immunofluorescence for reactivity with murine mAb to CTLA-4 (A and B) and with Abs eluted from the recombinant CTLA-4 of blots (C and D).
immunofluorescent staining followed by FACS analysis. The purified anti-CTLA-4 fusion protein Ab was able to bind to the TLA-4-transfected P815 cells, but not to P815 cells. A representative case is shown in Fig. 5. These data provide direct evidence that the anti-CTLA-4 autoantibodies of patients react with the native form of the CTLA-4 molecules expressed on the mammalian cell surface.

**Epitope mapping of CTLA-4**

To map the autoepitopes on the CTLA-4 protein, three pTEX7 plasmids with cDNA fragments F1, F2, and F3 (pTEX7-cDNA F1, pTEX7-cDNA F2, and pTEX7-cDNA F3) were constructed (Fig. 6A). We obtained the respective proteins shown in Fig. 6B. The reactivity of sera from 18 anti-CTLA-4 Ab-positive patients and 82 healthy donors to each fragment was examined by ELISA. Table II shows that the sera of nine of the 18 (50%) patients reacted to all fragments, the sera of seven (38.8%) patients reacted to two fragments, and the sera of two (11.1%) patients reacted specifically to either F1 or F2. In total, the F1, F2, and F3 proteins were recognized by 14 (77.8%), 15 (83.3%), and 14 (77.8%), respectively, of 18 patients’ sera.

To confirm these reactivities, serially diluted serum samples were similarly tested by ELISA. Representative results (RA-5, ns-65, and ns-31) are shown in Fig. 7. Thus, most of the serum samples recognized multiple epitopes on CTLA-4.

**Laboratory data and clinical features of Behcét’s disease patients whose sera do and do not contain anti-CTLA-4 Abs**

Since anti-CTLA-4 Abs were detected most frequently in the sera of patients with Behcét’s disease, we compared the laboratory data and the clinical features, including past history of the anti-CTLA-4 Ab-positive and -negative Behcét’s disease patients. There was no significant difference in the laboratory data (erythrocyte sedimentation rate, peripheral lymphocyte count, and serum levels of IgG, IgA, and IgM) of the two groups of Behcét’s disease patients (Table III). Similarly, most of the clinical features of the two groups did not differ significantly. However, uveitis was detected in 13 of the 15 (86.7%) Ab-negative patients, but in only two of the seven (28.6%) Ab-positive patients, and this difference is statistically significant (Table IV).

**Discussion**

We have demonstrated for the first time that CTLA-4, a costimulatory molecule of T cell activation, is a target of ALAs. Furthermore, we showed that the autoantibody to CTLA-4 reacts with the native form of human CTLA-4 molecule expressed on the mammalian cell surface.

In our study, anti-CTLA-4 Abs were detected more frequently among patients with Behcét’s disease and RA than among those with SLE. This was unexpected, since ALAs are detected in a higher percentage of patients with SLE than in patients with Behcét’s disease and RA (2). This may be because CTLA-4 is a minor target of ALA (discussed below), and thus the prevalence of anti-CTLA-4 Ab would be different from the prevalence of total ALA previously reported (2). Alternatively, the discrepancy may be related to the detection method. The standard method for detection of ALAs is the microcytotoxicity test, in which the presence of ALA-mediated complement cytolysis is tested using PBL of healthy donors. This method may miss the ALAs to Ags that are
temporarily expressed or induced by activation, such as CTLA-4. Thus, ALA detection using recombinant proteins may produce different results from those obtained by the conventional method. Screening of a larger number of serum samples than that reported here would be needed to resolve this issue.

The laboratory data and clinical features of patients did not correlate with the presence or the absence of anti-CTLA-4 Ab among patients with Behçet’s disease (Table II), those with RA, and those with SLE (data not shown). Previous reports indicated that the presence of ALAs correlates with lymphopenia (4); however, our data showed that the presence of anti-CTLA-4 Ab did not correlate with the lymphocyte count. As to clinical features, it is interesting that a negative correlation was found between uveitis and the presence of anti-CTLA-4 Ab in Behçet’s disease patients. Although the pathogenesis of Behçet’s disease remains unclear, immunological abnormalities, in particular infiltration of predominantly T lymphocytes in erythema nodosum-like lesions (44), oral ulcers (44), terminal ileum (45), and ocular lesions (46), have been reported. Thus, T cells play a role in the affected lesions. In this regard, the anti-CTLA-4 Ab detected in the Behçet’s disease patients may act to suppress the proliferation of uveitis-driving T cells. Decreased function of suppressor T cells and subsequent increased B cell function have been implicated in Behçet’s disease, and Sakane et al. suggested that ALAs might be responsible for the loss of suppressor T cell function (47). From this viewpoint, anti-CTLA-4 Abs may suppress the function of such suppressor T cells and dysregulate B cell function. In all patients with uveitis in our study, the uveitis was inactive at the time their serum samples were obtained. Therefore, further studies comparing disease activity with the titer of anti-CTLA-4 Ab may give us some clues to understanding the role of anti-CTLA-4 Ab in vivo. In RA, Verwilghen et al. reported that anti-CTLA-4 mAb had an inhibitory effect on the mixed lymphocyte cytotoxicity test when synovial fluid T cells from RA patients were used as stimulatory cells (48). These findings suggest that anti-CTLA-4 Ab in RA acts to suppress arthritis-driving T cell function, similar to its role in suppressing uveitis in Behçet’s disease.

Epitope mapping using three recombinant CTLA-4 fragments revealed that the CTLA-4 molecule contains at least three epitopes recognized by the IgG type of the anti-CTLA-4 Ab. Most of the anti-CTLA-4 Ab-positive sera reacted to more than one CTLA-4 fragment. This result indicates that the production of autoantibodies to CTLA-4 is induced by an Ag-driven mechanism, which is the main mode of production of many antinuclear Abs (49, 50). In addition, this diversity of anti-CTLA-4 Abs may indicate the existence of several kinds of anti-CTLA-4 Abs with different functions.

Among the four T cell costimulatory molecules tested, autoantibodies to CTLA-4 were frequently detected in patients with autoimmune disease, but autoantibodies to CD28, B7-1, and B7-2 were rare. In particular, CD28 shares 31% amino acid identity with CTLA-4 (51), but anti-CD28 Ab was not detected in any of the

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**Table III. Laboratory parameters in the anti-CTLA-4 Ab-positive and -negative Behçet’s disease patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive (n)</th>
<th>Negative (n)</th>
</tr>
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<tbody>
<tr>
<td>ESR* (mm/h)</td>
<td>13 ± 3 (7)‡</td>
<td>24 ± 6 (15)</td>
</tr>
<tr>
<td>Lymphocyte (/μm³)</td>
<td>2127 ± 199 (7)</td>
<td>1869 ± 191 (15)</td>
</tr>
<tr>
<td>IgG (mg/dl)</td>
<td>1370 ± 36 (6)</td>
<td>197 ± 86 (15)</td>
</tr>
<tr>
<td>IgA (mg/dl)</td>
<td>204 ± 30 (6)</td>
<td>252 ± 33 (14)</td>
</tr>
<tr>
<td>IgM (mg/dl)</td>
<td>141 ± 18 (6)</td>
<td>133 ± 14 (14)</td>
</tr>
</tbody>
</table>

*All values are not significant.

†p values calculated by Mann-Whitney U test.

‡Mean ± SEM.

*ESR: erythrocyte sedimentation rate
patients. This fact suggests the existence of a CTLA-4-specific immune response among the four costimulatory molecules tested in this study. This may be related to the difference in the manner in which the molecules are expressed, i.e., activation-induced or constitutive expression. Further studies are needed to elucidate this mechanism.

To screen autoantibodies to the costimulatory molecules, we used proteins produced in bacteria. Therefore, autoantibodies to the natural form of the molecules could have been missed. To detect such kinds of Abs, transfected cells can be useful. We tried to detect anti-CTLA-4 Abs with the CTLA-4-transfected P815 cells using whole sera instead of purified anti-CTLA-4 Abs from patients. However, this was not effective, since a considerable number of serum samples strongly reacted to the transfектs and also to the control P815 cells (data not shown). It is possible that other kinds of autoantibodies in the sera cross-reacted with the surface molecules of the P815 cells. Thus, the transfected cells were useful in determining whether an Ab specifically purified from sera could react to the natural form of the molecule, but was inadequate for screening of ALA using whole sera.

Several studies in mice and humans have indicated that a lack or modification of negative signals through CTLA-4 is implicated in autoimmune diseases (21–27). In addition, CTLA-4 was reported to be essential for peripheral tolerance of CD4+ T cells (52). From these findings, it is very likely that CTLA-4 is involved in the pathogenesis or abnormal immune responses in autoimmune diseases. The autoantibodies to CTLA-4 demonstrated here may mediate the immune response in patients with autoimmune disease.

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