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Identification and characterization of an alternative cytotoxic T lymphocyte-associated protein 4 binding molecule on B cells

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ABSTRACT **To determine whether alternative cytotoxic T lymphocyte-associated protein 4 (CTLA4) binding proteins exist on B cells, we constructed (*i*) mCTLA4hIgG consisting of the extracellular region of a mouse CTLA4 molecule and the Fc portion of a human IgG1 molecule and (*ii*) PYAAhIgG, a mutant mCTLA4hIgG, having two amino acid substitutions on the conserved MYPPPY motif in the complementarity-determining region 3-like region and lacking detectable binding to both B7-1 and B7-2 molecules. Using these fusion proteins (mCTLA4hIgG and PYAAhIgG), we demonstrated that a mouse immature B-cell line, WEHI231 cells, expressed alternative CTLA4 binding molecules (ACBMs) that were distinct from both B7-1 and B7-2. ACBMs were 130-kDa disulfide-linked proteins. More importantly, ACBMs were able to provide costimulatory signal for T-cell proliferation in the presence of anti-CD3 monoclonal antibodies. In addition, we demonstrated that more than 20% of B220$^+$ cells obtained from normal mouse spleen expressed ACBMs.**

The antigen-specific activation of T lymphocytes is dependent on two distinct signals delivered by the antigen-presenting cells (APCs) (1, 2). The first signal is antigen-specific and delivered when the T-cell receptor is ligated by antigen in the context of the major histocompatibility complex. However, for the optimal T-cell proliferation, the T cell has to receive costimulatory signals mediated by the antigen-non-specific interaction of adhesion molecules that are expressed on both T cells and APCs (3). CD28 and cytotoxic T lymphocyte-associated protein 4 (CTLA4) are examples of such critical adhesion molecules on T cells. The molecule known as B7-1 was originally described as a human B-cell-associated activation antigen (4) and was identified as a ligand for CD28 and CTLA4 (5). An alternative splicing form of B7-1, MB7-2 was also identified in lipopolysaccharide-stimulated mouse spleen cells (6). Recently, an additional ligand for CD28 and CTLA4, B7-2 that could provide costimulatory signal was identified on APCs (7–10). Herein, we demonstrated that a mouse immature B-cell line, WEHI231 cells, and the B220$^+$ population *in vivo* express alternative CTLA4 binding molecules (ACBMs). Additionally, we discussed some characteristics of ACBMs.

MATERIALS AND METHODS

Antibodies and Flow Cytometry Analysis. Anti-B7-1 [rat monoclonal antibody (mAb), 1G10], anti-B7-2 (rat mAb, GL1), fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-anti-human CD4 (mouse mAb, RPA-T4), FITC-anti-B7-1, PE-anti-B7-2, and FITC-anti-rat κ-chain mAbs were purchased from PharMingen. PE-anti-B220 mAb was purchased from Caltag (South San Francisco, CA). Anti-CD3 mAb (hamster mAb, 145-2C11), biotin-anti-B220 mAb, and hCTLA4hIgG were provided by J. A. Bluestone (University of Chicago), K. Onoe (Hokkaido University), and P. S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute), respectively. Anti-CD28 Fab mAb was prepared as described (11). FITC-anti-human Ig, FITC-avidin, and Red670-avidin were purchased from IBL (Gunma, Japan), Jackson Immuno Research, and GIBCO/BRL, respectively. For flow cytometry analysis, cells were first pretreated with anti-FcγR mAb 2.4G2 and then incubated with various mAbs or Ig fusion proteins followed by incubation with second-step regents. Propidium iodide (Sigma) was added for the last 1 min to gate out dead cells. Living cells were analyzed on a FACScan (Becton Dickinson).

Isolation of B7-2 cDNA and Transfection. Mouse B7-2 cDNA was isolated from mouse spleen by using the forward primer B7-2-5′ (5′-TGCTCGAGAACTTACGGAAGCACC-3′) and reverse primer B7-2-3′ (5′-AACTCTTTCCTCAG-GCTCTCACTG-3′). For B7-2-CHO cells, mouse B7-2 cDNA was cloned into pcDLSRα296 and transfected to CHO cells by using the LipoFectin reagent (GIBCO/BRL).

Ig Fusion Proteins. For mB7-2hIgG, the extracellular region of mouse B7-2 was amplified using forward primer B7-2-5′-2 (TGCTCGAGAACTTACGGAAGCACC) and reverse primer B7-2-3′-2 (CTTCGGATCCGTTTGAGGAGAT-GGA). For CTLA4hIgG, the extracellular region of mouse CTLA4 was amplified by a two-step PCR using mouse CTLA4 cDNA as a template. First-step PCR was performed using forward primer CTLA-5′-1 (5′-CATCCAGTTGCCTTCTT-GGGACTGATGCTGGTGACAACCACGGCCTTCGCCA-TACAGGTGACCCAACC) and reverse primer CTLA-3′ (5′-TTTGGGCTCCGGATCCTCAGAATCCGGGCATG-GTTC). Second-step PCR was done using CTLA-5′-2 (5′-CTAGCCACTGCTCGAGCACCAATGAAGTTCCTCTC-TGCAAGAGACTTCCATCCAGTTGCCTTCTTGGGAC-TG) and reverse primer CTLA-3′. For PYAAhIgG, cDNA of mCTLA4hIgG was amplified using forward primer CTLA-5′-2 and reverse primer PYAA-3′ (CATGCCCACAAAGGCT-GCCGGGTACAT) or forward primer PYAA-5′ (ATGTAC-CCACCAGCTGCCTTTGTGGGCATG) and reverse primer CTLA-3′. Single bands were reamplified using forward primer CTLA-5′-2 and reverse primer CTLA-3′. For mCD28hIgG, the extracellular region of mouse CD28 was amplified using forward primer CD28-5′ (AACTCGAGCATCAGAACAAT-GACACTCAG) and reverse primer CD28-3′ (TTGGATC-CTTAGGAGATGACTGAGTATGA). All resulting PCR fragments were fused in-frame with cDNA of the human IgG1 heavy chain constant region in pCDM8 or pBOS expression vector. COS-7 cells were transfected with genes coding for mCTLA4hIgG, PYAAhIgG, mCD28hIgG, or mB7-2hIgG and the respective proteins were purified from culture medium as described (12, 13).

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Abbreviations: CTLA4, cytotoxic T lymphocyte-associated protein 4; ACBM, alternative CTLA4 binding molecule; APC, antigen-presenting cell; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Northern Blot Analysis. Northern blot analysis was preformed as described (13) using mRNA of WEHI231, B7-1-CHO, and B7-2-CHO cells that was prepared by the guanidine thiocyanate/CsCl gradient centrifusion and oligo(dT)-cellulose (Boehringer Mannheim).

Surface Labeling and Immunoprecipitation. Surface iodination was achieved using IodoBeads (Pierce) as described (14). ^{125}I-labeled cells were lysed with Nonidet P-40 buffer (15, 16), and then clear lysates were incubated with human IgG (Jackson Immuno Research) at 20 μg/ml or mCTLA4hIgG at 20 μg/ml plus protein A-Sepharose. Immunoprecipitates were analyzed by SDS/PAGE.

Biotinylation of PYAAhIgG and Binding Inhibition Analysis. Biotinylation of PYAAhIgG molecules was performed using sulfo-*N*-hydroxysuccinimide-biotin (Pierce). For binding inhibition analysis, WEHI231 cells were treated with anti-FcγR mAb, washed, and incubated with mCTLA4hIgG at 32 μg/ml. After washing, cells were stained with various concentrations of biotin-PYAAhIgG (0–40 μg/ml) plus avidin-FITC and then analyzed on a FACScan.

Cell Purification and T-Cell Proliferation Assay. Lymph node cells were isolated from BALB/c mice and applied to both Sephadex G-10 and nylon wool column. The pass fraction was used as T cells. Spleen cells that passed through Sephadex G-10 column were used as a nonadherent cell population. The purified T cells were cultured at a cell density of 1.5×10^5 cells per well in 96-well microtiter plates. T cells were stimulated with phorbol 12-myristrate 13-acetate (20 ng/ml) and ionomycin (50 ng/ml) or immobilized anti-CD3 mAb (10 μg/ml, 20 min, at 37°C). Either paraformaldehyde-fixed B7-1- and B7-2-CHO cells (5×10^4 cells per well) or γ-irradiared WEHI231 cells (3×10^5 cells) were added to the culture as costimulator cells. In some experiments, mCTLA4hIgG (20 μg/ml), PYAAhIgG (20 μg/ml), anti-B7-1 (15 μg/ml), anti-B7-2 mAb (15 μg/ml), or anti-CD28Fab (7 μg/ml) was added at the start of the culture period. The plates were pulsed-labeled for the final 12 hr of the 100-hr culture with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) per well and cells were harvested.

RESULTS

WEHI231 Cells Express ACBMs. We constructed a mouse–human chimeric protein consisting of the extracellular region of mouse CTLA4 molecule and the Fc portion of human IgG1 molecule, mCTLA4hIgG and a mutant mCTLA4hIgG molecule, PYAAhIgG having two amino acid substitutions of Ala-Ala for Pro-Tyr, residues 103 and 104 in the conserved MYPPPY motif in complementarity-determining region 3-like region of mouse CTLA4 cDNA (12, 17). As shown in Fig. 1 *A*, *B*, *E*, and *F*, B7-1 and B7-2 molecules expressed on CHO cells were specifically detected by anti-B7-1 and -B7-2 mAb, respectively. mCTLA4hIgG could bind to both B7-1 and B7-2 molecules on CHO cells (Fig. 1 *C* and *G*). Although WEHI231 cells expressed very few B7-1 and B7-2 molecules, if any (Fig. 1 *I* and *J*), mCTLA4hIgG molecules could significantly bind to WEHI231 cells (Fig. 1*K*). We were well aware that WEHI231 cells express small amount of FcγR molecules (data not shown). Therefore, (*i*) WEHI231 cells were pretreated with saturating amount of anti-FcγR mAb 2.4G2 (18) to block the binding of Fc portion of mCTLA4hIgG to FcγR, and (*ii*) we used mB7-2hIgG and mB7-1hIgG molecules as controls. As shown in Fig. 1 *M* and *N*, mB7-1hIgG and mB7-2hIgG did not bind to anti-FcγR mAb-treated WEHI231 cells. To confirm the existence of ACBMs that are distinct from B7-1 and B7-2, we analyzed the binding of PYAAhIgG to B7-1-CHO, B7-2-CHO, and WEHI231 cells. PYAAhIgG significantly bound to WEHI231 cells (Fig. 1*L*) but not to B7-1- and B7-2-CHO cells (Fig. 1 *D* and *H*). In addition, mCTLA4hIgG bound to WEHI231 cells even in the presence of anti-B-1 and -B7-2 mAbs (Fig. 1*O*).

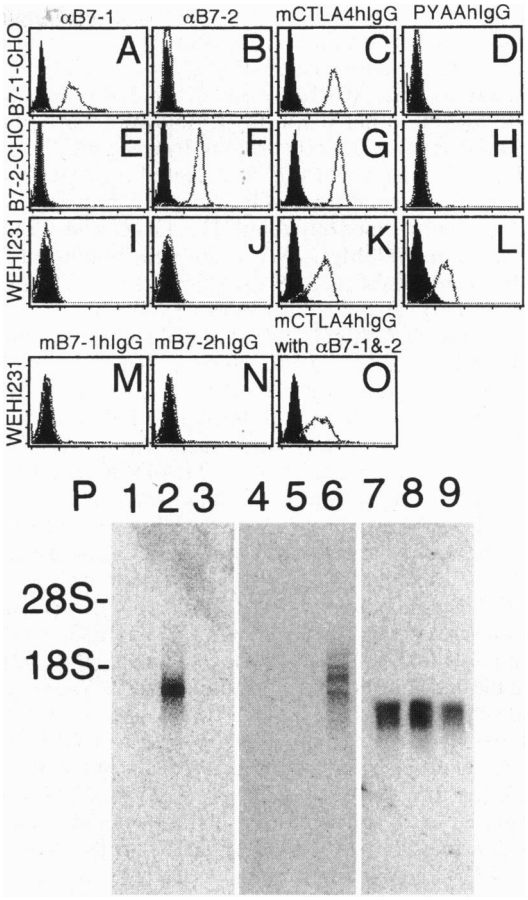

FIG. 1. ACBMs on WEHI231 cells. B7-1-CHO (*A–D*), B7-2-CHO (*E–H*), and WEHI231 cells (*I–O*) were pretreated with anti-FcγR mAb and stained with anti-B7-1 mAb at 10 μg/ml (*A*, *E*, and *I*), anti-B7-2 mAb at 10 μg/ml (*B*, *F*, and *J*), mCTLA4hIgG at 10 μg/ml (*C*, *G*, and *K*), PYAAhIgG at 10 μg/ml (*D*, *H*, and *L*), mB7-1hIgG at 10 μg/ml (*M*), mB7-2hIgG at 10 μg/ml (*N*), and mCTLA4hIgG at 10 μg/ml in the presence of anti-B7-1 or B7-2 mAb (*O*) and then incubated with FITC-anti-rat (*A*, *B*, *E*, *F*, *I*, and *J*) or FITC-anti-human Ig (*C*, *D*, *G*, *H*, *K–O*) Ab. Northern bolt analysis of WEHI231, B7-1-CHO, and B7-2-CHO cells (*P*). Two micrograms of mRNA from WEHI231 (lanes 1, 4, and 7), B7-1-CHO (lanes 2, 5, and 8), or B7-2-CHO (lanes 3, 6, and 9) cells was electrophoresed, blotted, and probed with ^{32}P-labeled B7-1 (lanes 1–3), ^{32}P-labeled B7-2 (lanes 4–6), or ^{32}P-labeled GAPDH (lanes 7–9).

There is the possibility that must be ruled out that the ACBMs that we detect are products of B7-1 and/or B7-2 genes that, by virtue of transcriptional, translational, or post-translational modification, is not recognized by mAb to B7-1 or B7-2 or chimeric proteins. To exclude the possibility described above, we performed Northern blot analysis using WEHI231, B7-1-CHO, and B7-2-CHO cells and the whole coding region of B7-1 or B7-2 cDNA as a probe. As shown in Fig. 1*P*, mRNA of B7-1 and B7-2 were detected in B7-1-CHO and B7-2-CHO cells, respectively (lanes 2 and 6), but not in WEHI231 cells (lanes 1 and 4). Additionally, although the core protein size of B7-1 and B7-2 molecules were 30–35 kDa as described (refs. 8 and 9 and data not shown), ACBMs were slightly glycosylated and the core protein size was 50–55 kDa after neuramidase and N-glycanase treatment (data not shown). All these results demonstrated that the ACBMs detected herein were not a product of B7-1 and/or B7-2 genes.

The 130-kDa Disulfide-Linked ACBMs Are Expressed on WEHI231. WEHI231, B7-1-CHO, or B7-2-CHO cells were surface-labeled with Na^{125}I, solubilized with Nonidet P-40, immunoprecipitated with mCTLA4hIgG, and analyzed by SDS/PAGE. ACBMs expressed on WEHI231 cells were ap-

proximately 130 kDa and 65 kDa under nonreducing conditions and reducing conditions, respectively (Fig. 2, lanes 1), in comparison with control lanes (Fig. 2, lanes C). These results demonstrated that ACBMs were disulfide-linked 130-kDa proteins. On the other hand, B7-1 molecules were immunoprecipitated as 65- to 75-kDa proteins by using mCTLA4hIgG, but B7-2 molecules could not be detected on CHO transfectants under both conditions (Fig. 2, lanes 2 and 3).

ACBMs Bound Not Only to mCTLA4hIgG and PYAAhIgG But Also to mCD28hIgG. We examined binding ability of ACBMs to mCTLA4hIgG, PYAAhIgG, or mCD28hIgG by using WEHI231 cells. As shown in Fig. 3*A*. the dose-dependent binding of mCTLA4hIgG, PYAAhIgG, and mCD28hIgG to ACBMs were detected in the range of 0.6–17, 1.9–17, and 5.7–17 μg/ml, respectively; binding ability of ACBMs to mCTLA4hIgG was 10- and 10- to 30-fold better than that to PYAAhIgG and to mCD28hIgG, respectively. As in human cases (12), the binding ability of mCTLA4hIgG to either B7-1 or B7-2 was 20- to 30-fold better than that of mCD28hIgG; however, PYAAhIgG binding was hardly detected (Fig. 3 *B* and *C*). Next, we carried out binding inhibition assay using biotin-PYAAhIgG and mCTLA4hIgG. Without mCTLA4hIgG, biotin-PYAAhIgG bound to WEHI231 in dose-dependent manner; however, in the presence of mCTLA4hIgG, binding of biotin-PYAAhIgG was significantly inhibited (Fig. 3*D*). This result indicated that both mCTLA4hIgG and PYAAhIgG recognized same binding sites on WEHI231 cells.

ACBMs Provide Costimulatory Signal for T-Cell Proliferation. Resting T cells were cocultured with γ-irradiated WEHI231 in the presence or absence of anti-CD3 mAb. As shown in Fig. 4*A*, T-cell proliferation was detected when T cells were cultured with γ-irradiated WEHI231 cells and immobilized anti-CD3 mAb. A saturating amount (15 μg/ml) of mAb reacting with B7-1 or B7-2 could inhibit B7-1-CHO- or B7-2-CHO-induced T-cell proliferation (Fig. 4*B*). These mAbs had no inhibitory effect on WEHI231-induced T-cell proliferation. However, mCTLA4hIgG and PYAAhIgG had significant inhibitory effect (Fig. 4*A*). Also, this proliferation was significantly inhibited by anti-CD28 mAb Fab fragments (Fig. 4*A*).

ACBMs Are Expressed on the B220$^+$ Population. *In vivo* expression of ACBMs was analyzed. mB7-2hIgG did not bind significantly to B220$^+$ cells (Fig. 5*A*); however, mCTLA4hIgG bound to B220$^+$ cells (31.6% of B220$^+$ cells) (Fig. 5*B*). mCD28hIgG and PYAAhIgG bound to 20.5 and 32.3% of B220$^+$ cells, respectively (Fig. 5 *C* and *D*). It should be noted that the detection of ACBMs on B220$^+$ cells depends upon the type of reagents used; mCTLA4hIgG versus hCTLA4hIgG (both reagents contained Fc portion of human IgG1). mCTLA4hIgG significantly bound to B220$^+$ population,

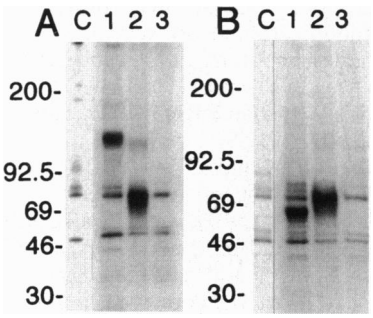

FIG. 2. Immunoprecipitation of ACBMs from WEHI231 cells. WEHI231 (2 × 10^7 cells), B7-1-CHO (1 × 10^7 cells), and B7-2 (1 × 10^7 cells) cells were surface-iodinated with 2.5 mCi of Na[^{125}I], lysed in Nonidet P-40 buffer, and immunoprecipitated with human IgG (20 μg/ml; lane C) or mCTLA4hIgG (20 μg/ml; lanes 1–3) plus protein A-Sepharose. Samples were analyzed under nonreducing (*A*) and reducing (*B*) conditions by SDS/PAGE. Lanes: C and 1, WEHI231; 2, B7-1-CHO; 3, B7-2-CHO cells.

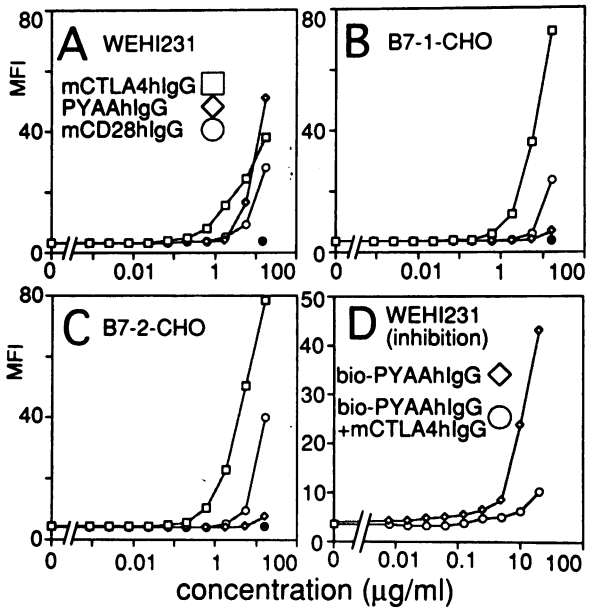

FIG. 3. Comparison of mCTLA4hIgG, PYAAhIgG, and mCD28hIgG binding to ACBM, B7-1, and B7-2 molecules. Anti-FcγR mAb pretreated-WEHI231 (*A*), -B7-1-CHO (*B*), and -B7-2-CHO (*C*) cells (5 × 10^5 cells per well) were incubated with various concentrations of mCTLA4hIgG (open squares), PYAAhIgG (open circles), mCD28hIgG (open diamonds), and mB7-2hIgG (solid circles) and incubated with FITC-anti-human Ig Ab. Approximately 10,000 viable cells were analyzed, and the mean fluorescence intensity (MFI) was determined. (*D*) Binding inhibition assay. WEHI231 cells were first incubated with anti-FcγR mAb and pretreated with (open circles) or without (open diamonds) mCTLA4hIgG (32 μg/ml). Cells were then incubated with biotin-PYAAhIgG, followed by avidin-FITC.

whereas hCTLA4hIgG failed to do so (Fig. 5 *B* and *E*). Furthermore, the binding of mCTLA4hIgG to B220$^+$ cells was clearly detected even in the presence of a saturating amount (15 μg/ml) of anti-B7-1 and B7-2 mAbs (Fig. 5*F*). Using a nonadherent cell population, derived from BALB/c mouse spleen cells, a two-color flow cytometry analysis was performed (Fig. 6 *A–D*). As shown in Fig. 6 *B* and *D*, both B7-1 and B7-2 molecules were not expressed on B220$^+$ cells. On the other hand, B7-2 molecules were expressed on B220$^-$ cells (Fig. 6*D*). Thus, all these results demonstrated that ACBMs are distinct from B7-1 and B7-2 and are expressed not only on a cultured B-cell line but also on a part of B220$^+$ cells *in vivo*.

DISCUSSION

In the present report, we demonstrated that a mouse immature B-cell line, WEHI231 cells, and a part of B220$^+$ cells expressed ACBMs that are distinct from B7-1 and B7-2. This conclusion was based on following observations. First, we detected ACBMs by using mouse–human fusion proteins consisting of the extracellular region of mouse CTLA4 or a mutant mouse CTLA4 molecule, PYAA, and the Fc portion of human IgG1 molecule. These regents allowed us to examine the species-specific ligand–receptor binding (19) and to avoid the system in which binding occurred between endogenous mouse Ig and FITC-conjugated anti-mouse Ig antibody. It was reported that complementarity-determining region 3-like region of both CD28 and CTLA4 are important for their binding to B7-1 and B7-2, because (*i*) Y104A mutant of CTLA4IgG and both P103A and Y104A mutants of CD28IgG showed an almost complete loss of binding to B7-1 molecules, and (*ii*) Y100A mutant of CTLA4IgG did not bind to B7-2 molecules but, however, showed reduced, but still significant, binding to B7-1 (12, 17). Therefore, we decided to generate a mutant mCTLA4hIgG, PYAAhIgG,

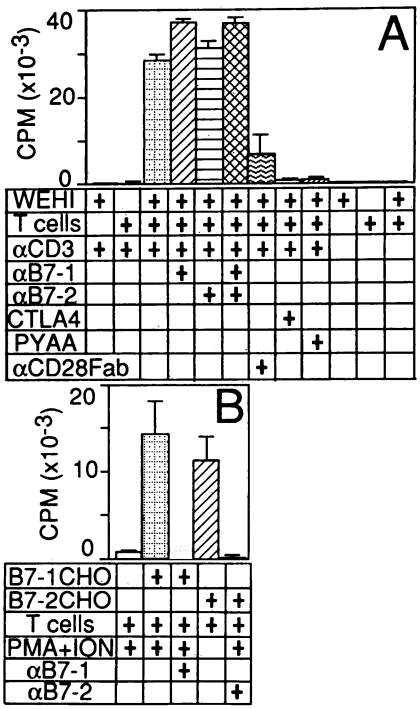

FIG. 4. Response of T cells to costimulation provided by WEHI231 cells. T cells were cocultured with γ-irradiated-WEHI231 (*A*) or paraformaldehyde-fixed B7-1-CHO and B7-2-CHO cells (*B*) in the presence of anti-CD3 mAb (*A*) or phorbol 12-myristrate 13-acetate and ionomycin (*B*). Anti-B7-1 and -B7-2 mAb (15 μg/ml), anti-CD28Fab (7 μg/ml), or fusion proteins (20 μg/ml) are included in the culture as a inhibitor. T-cell proliferative responses were measured by incorporation of [^{3}H]thymidine.

that possessed two amino acid substitutions of Ala-Ala for Pro-Tyr, residues 103 and 104. According to our expectation, PYAAhIgG molecules did not bind significantly to both B7-1 and B7-2 but could still bind to ACBMs (Figs. 1 and 3). It is unlikely that the ACBMs described herein binds to the Fc portion of mCTLA4IgG because of the lack of reactivity to ACBM with other Fc proteins (Fig. 1 and data not shown). More importantly, as shown in Fig. 4, ACBMs could interact

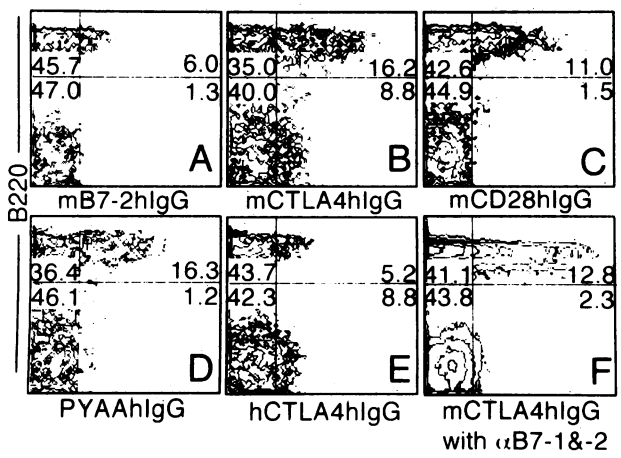

FIG. 5. Two-color staining for ACBM on spleen B cells. Nonadherent cell fraction of spleen was incubated with anti-FcγR mAb and stained with PE-anti-B220 mAb (5 μg/ml) (*A–E*) and treated with mB7-2hIgG (15 μg/ml) (*A*), mCTLA4hIgG (15 μg/ml) (*B*), mCD28hIgG (15 μg/ml) (*C*), PYAAhIgG (15 μg/ml) (*D*), hCTLA4hIgG (15 μg/ml) (*E*), and mCTLA4hIgG (15 μg/ml) in the presence of anti-B7-1 and B7-2 mAb (*F*) and then incubated with FITC-anti-human Ig Ab.

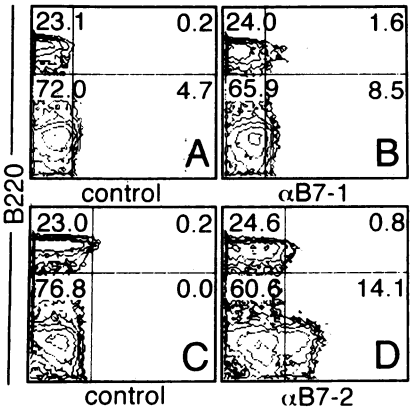

FIG. 6. Reactivity of anti-B7-1 and anti-B7-2 mAb on spleen B cells. Nonadherent cell population of spleen was incubated with anti-FcγR mAb, washed, stained with FITC-anti-human CD4 mAb (5 μg/ml) (*A*), FITC-anti-B7-1 mAb (5 μg/ml) (*B*), PE-anti-human CD4 (5 μg/ml) (*C*), and PE-anti-B7-2 mAb (5 μg/ml) (*D*), and incubated with biotin-anti-B220 mAb plus avidin-Red670 (*A–D*).

with CD28 molecules expressed on resting T cells, since proliferation of resting T cells in the presence of WEHI231 cells and anti-CD3 mAb was inhibited with anti-CD28 Fab. This data strongly suggested that native CD28 is a likely receptor for ACBMs in the early stage of the T-cell response. Thus, it is conceivable that in the late stage of the T-cell response, ACBMs bind to naive CTLA4 molecules. However, it would be of interest to confirm that ACBMs bind to not only CTLA4Ig but also native CTLA4 molecules. Second, ACBMs were immunoprecipitated as 130-kDa disulfide-linked molecules using mCTLA4hIgG (Fig. 2*A*). Because ACBMs were about 65 kDa under reducing condition (Fig. 2*B*), those molecules might exist as homodimers. However, further analysis is required to conclude those molecules form homodimers or heterodimers. The molecular weight of alternative CTLA4 binding molecules was distinct from that of B7-1. Although B7-2 molecules were not immunoprecipitated with mCTLA4hIgG, it was due to that mCTLA4hIgG dissociate more rapidly from B7-2 than from B7-1 (12). In any event, the molecular weight of ACBM was different from those of B7-1 and B7-2 (9, 10), and there is no report describing the dimerization of B7-1 and B7-2 molecule. However, as shown in Fig. 2*A*, lane 2 (B7-1-CHO) appears to have a high molecular weight band, but the molecular weight is slightly lower than the ACBM in WEHI231 cells. This band disappeared under reducing condition (Fig. 2*B*, lane 2). Thus, this high molecular weight band may be a disulfide-linked B7-1 dimer or a B7-1 specific associated molecule on B7-1-CHO cells. Finally, analysis on the function and expression of B7-1 and B7-2 indicated that the expression of those molecules are regulated differently, B7-2 is rapidly induced on the surface of activated B cells or monocytes and is expressed at high levels on dendritic cells. Additionally, it was suggest that B7-1 and B7-2 account for nearly all of the CTLA4 binding capacity and costimulatory activity of APCs including activated B cells and dendritic cells (7–10). In contrast, ACBMs were expressed not only on WEHI231 cells but also on a part of B220$^+$ cells in spleen, bone marrow, and lymph node (Fig. 5 and data not shown). Although a part of B220$^-$ cells that may be dendritic or macrophage cells expressed B7-2 molecules, majority of B220$^+$ cells did not significantly express both B7-1 and B7-2 (Fig. 6 *C* and *D*). The absence of significant B7-1 and B7-2 expression on the resting B-cell population (B220$^+$ cells in spleen) was in good agreement with recent reports (7–10). The expression of ACBM on the resting B-cell population is in sharp contrast to previous reports (7, 20). The discrepancy between our data and others can be explained by the affinity differences between

hCTLA4hIgG/mCTLA4hIgG and their counter receptor, ACBM, on resting B cells. As shown in Fig. 5 *B* and *E*, hCTLA4hIgG bound to B220$^+$ resting B cells significantly lesser than mCTLA4hIgG did.

ACBMs on WEHI231 cells could provide costimulatory signals for T-cell proliferation, whereas B cells, which also express ACBM, were repeatedly shown not to provide such costimulatory signals (21). Further analysis of ACBMs might provide a reasonable explanation for this discrepancy. However, it is safe to state that ACBMs can provide costimulatory signals for T-cell proliferation under certain conditions. In this regard, it should be pointed out that an additional CTLA4 binding molecule on a human B lymphoblastoma reported by Naddler and coworkers (22) could induce apoptosis of a T-cell clone through interaction with CTLA4, only when the function of coexpressed B7-1 and B7-2 molecules were blocked by a mAb reacting with both B7-1 and B7-2.

It is important to note that the binding region of CTLA4 to ACBM and to B7-1 and B7-2 were different, since PYAAhIgG bound to ACBM but not to B7-1 and B7-2. A significant population of resting B220$^+$ B cells expressed alternative CTLA4 binding molecules (Fig. 5). Those resting B220$^+$ cells expressed very few, if any, B7-1 and B7-2 molecules (Fig. 6). Thus, it is reasonable to speculate that ACBMs exert functionally unique activity.

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