Binding of the B Cell Activation Antigen B7 to CD28 Costimulates T Cell Proliferation and Interleukin 2 mRNA Accumulation

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Summary

A successful immune response requires intercellular contact between T and B lymphocytes. We recently showed that CD28, a T cell surface protein that regulates an activation pathway, could mediate intercellular adhesion with activated B cells by interaction with the B7 antigen. Here we show that CD28 is the primary receptor for B7 on activated peripheral blood T cells, that CD28 binds to B7 in the absence of other accessory molecules, and that interaction between CD28 and B7 is costimulatory for T cell activation. To characterize the binding of CD28 to B7, we have produced genetic fusions of the extracellular portions of B7 and CD28, and immunoglobulin (Ig) Cy1 chains. 

It has long been known that interactions between T and B lymphocytes play a central role in regulating an immune response (1, 2). More recent studies have shown that activation and differentiation of both T and B lymphocytes is dependent upon direct intercellular (cognate) interactions between these cell types. While the specificity of T-B cell interactions is determined by interaction between the TCR/CD3 complex (3-5) and antigen associated with class II MHC molecules on B cells (6), interactions between other (accessory) cell adhesion molecules are also necessary for a full immune response (7-9). Interactions between accessory receptors and their counter-receptors may increase the avidity of cellular interactions (7); control lymphocyte localization and migration (10); and have direct signaling functions (7, 11, 12) during lymphocyte activation. Accessory receptors and their counter-receptors involved in T-B cell interactions include (reviewed in reference 7): CD2 and LFA-3, CD4 and class II MHC molecules, LFA-1, and ICAM-1 and ICAM-2. The T cell homodimer, CD28, a member of the Ig superfamily (13), has been shown in studies using mAbs to have an accessory function during T cell activation (14). Anti-CD28 mAbs have been shown to costimulate T cell proliferation induced by a number of polyclonal stimuli (reviewed in reference 14). These mAbs also inhibited alloantigen and soluble antigen-specific T cell responses (15, 16), indicating that CD28-mediated signaling may be crucial during these responses. CD28-mediated T cell activation, unlike that initiated via TCR, was resistant to inhibition by the immunosuppressive agent, cyclosporine (17). Some of the effects of anti-CD28 mAbs appear to result, in part, from the coordinate stimulation of several T cell-derived cytokines (18) through stabilization of their mRNAs (19).

We recently reported that Chinese hamster ovary (CHO) cells transfected with CD28 mediated specific adhesion with certain activated normal and malignant B cells (20). CD28-mediated adhesion was blocked by the mAb BB-1 (21), which recognizes the B cell activation antigen, termed B7, another member of the Ig superfamily (22). COS cells transfected with a cDNA clone encoding B7 adhered specifically to CD28+ CHO cells, thereby indicating that the B7 antigen is a counter-receptor for CD28.

1 Abbreviations used in this paper: CHO, Chinese hamster ovary; dhfr, dihydrofolate reductase.
The recognition of CD28 by B7 is a novel mechanism to regulate interaction of T and B lymphocytes. In this paper, we have further characterized biochemical and functional aspects of the interaction between these molecules. Fusion proteins of B7 and CD28 with human Ig Cγ1 chains were expressed and used to measure the specificity and apparent affinity of their interaction. We have also used purified B7 Ig fusion protein, as well as CHO cells transfected with B7 to investigate the functional importance of this interaction on T cell activation and cytokine production.

Materials and Methods

Plasmid Construction. Expression plasmids containing cDNAs encoding CD28 (13), CD5 (23), and B7 (22) have been described previously. For initial attempts at expression of soluble forms of encoding CD28 (13), CD5 (23), and B7 (22) have been described.

PCR reaction using RNA from a myeloma cell line producing TGT as reverse primers, respectively. Products of the PCR reactions were cleaved with restriction endonucleases (HindIII and BclI) at sites introduced in the PCR primers and gel purified. The 3' portion of the fusion constructs corresponding to human Ig Cγ1 sequences was made by a coupled reverse transcriptase (from Avian myeloblastosis virus; Life Sciences Inc., St. Petersburg, FL) PCR reaction using RNA from a myeloma cell line producing human-mouse chimeric mAb L6 (P. Fell and M. Gayle, unpublished results) as template. The oligonucleotide, AGCAAGAGCATTTCCTGGATCAGGAGCCCAAATCTTCTGACAAAACAGTGCAGTGATCATTTGGATCCTGGCATGTGAC as reverse primer. Reactions containing the peak of an absorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28 Ig and B7 Ig, respectively, by amino acid analysis of solutions of known absorbance. The recovery of neutralized by addition of 1/10 volume of 2M Tris, pH 8. Fractional volumes (6 ml/dish). Incubation was continued for 3 days at 37°C. Transfected cells were then briefly treated (~2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. Lines expressing high levels of CD28 (CD28 CHO) or B7 (B7 CHO) were isolated by multiple rounds of fluorescence-activated cell sorting following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dfr CHO) were also isolated by fluorescence-activated cell sorting from CD28-transfected populations.

COS cells were transfected with expression plasmids using a modification of the protocol of Seed and Araullo (26). Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (~15 μg/dish) in a volume of 5 ml of serum-free DMEM containing 0.1 mM chloroquine and 600 μg/ml DEAE Dextran, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (~2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. At ~24 h after transfection, culture medium was replaced and serum-free DMEM (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded.

Purification of Ig Fusion Proteins. The first and second collections of spent-serum-free culture media from transfected COS cells were used as source for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium was applied to a column (~200-400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, MA) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column was washed with 1 M potassium phosphate, pH 8, and bound protein was eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of λabsorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28 Ig and B7 Ig, respectively, by amino acid analysis of solutions of known absorbance. The recovery of purified CD28 Ig and B7 Ig binding activities were nearly quantitative as judged by FACS analysis after indirect fluorescent staining of B7' and CD28' CHO cells.

Cell Separation and Stimulation. PBL were isolated by centrifugation of spent-serum-free culture media from transfected COS cells and used as source for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium was applied to a column (~200-400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, MA) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column was washed with 1 M potassium phosphate, pH 8, and bound protein was eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of λabsorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28 Ig and B7 Ig, respectively, by amino acid analysis of solutions of known absorbance. The recovery of purified CD28 Ig and B7 Ig binding activities were nearly quantitative as judged by FACS analysis after indirect fluorescent staining of B7' and CD28' CHO cells.

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gation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and cultured in 96-well, flat-bottomed plates (4 \times 10^4 cells/well, in a volume of 0.2 ml) in RPMI containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by uptake of [3H]thymidine during the last 5 h of a 3-d culture. PHA-activated T cells were prepared by culturing PBL with 1 \mu g/ml PHA (Wellcome, Charlotte, NC) for 5 d, and 1 d in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium before use.

**Cell Surface Iodination and Immunoprecipitation.** PHA-activated T cells were cell-surface labeled with [125I] using lactoperoxidase and 

\[ \text{H}_2\text{O}_2 \]. Nonionic detergent extracts of labeled cells were prepared and subjected to immunoprecipitation analysis as described previously (28).  

**Radio labeling of B7 Ig.** Purified B7 Ig (25 \mu g) in a volume of 0.25 ml of 0.12 M sodium phosphate, pH 6.8 was iodinated using 2 mCi [125I] and 10 \mu g of chloramine T. After 5 min at 23\degree C, the reaction was stopped by the addition of 20 \mu g sodium metabisulfite, followed by 3 mg of KI and 1 mg of BSA. Iodinated protein was separated from unreacted [125I] by chromatography on a 5-ml column of Sephadex G-10 equilibrated with PBS containing 10% FCS. Peak fractions were collected and pooled. The specific activity of [125I]-B7 Ig labeled in this fashion was 1.5 \times 10^6 cpm/\mu g.

B7 Ig was also metabolically labeled with [35S]methionine. COS cells were transfected with a plasmid encoding B7 Ig as described above. At 24 h after transfection, [35S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to concentrations of 115 \mu Ci/ml in DMEM containing 10% FCS and 10% normal levels of methionine. After incubation at 37\degree C for 3 d, medium was collected and used for purification of B7 Ig as described above. Concentrations of [35S]methionine-labeled B7 Ig were estimated by comparison of staining intensity after SDS-PAGE with intensities of known amounts of unlabeled B7 Ig. The specific activity of [35S]methionine-labeled B7 Ig was ~2 \times 10^6 cpm/\mu g.

**Binding Assays.** For assays using immobilized CD28-Ig, 96-well plastic dishes were coated for 16–24 h with a solution containing CD28-Ig (0.5 \mu g in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were then blocked with binding buffer (DMEM containing 50 mM BES, pH 6.8, 0.1% BSA, and 10% FCS) before addition of a solution (0.09 ml) containing [125I]-B7 Ig (~3 \times 10^6 cpm) or [35S]-B7 Ig (1.5 \times 10^6 cpm) in the presence or absence of competitor. After incubation for 2–3 h at 23\degree C, wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantified by liquid scintillation or \gamma counting. When binding of [125I]-B7 to CD28 CHO cells was measured, cells were seeded (2.5 \times 10^4/well) in 96-well plates 16–24 h before the start of the experiment. Binding was otherwise measured as described above.

**SDS-PAGE.** SDS-PAGE was performed on linear acrylamide gradient gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X-ray film (Kodak XAR-5; Eastman Kodak Co., Rochester, NY).

**RNA Blot Analysis.** RNA was prepared from stimulated PHA blasts by a published procedure (29). Aliquots of RNA (20 \mu g) were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose by capillary action. RNA was crosslinked to the membrane by UV light in a Stratalinker (Stratagene), and the blot was prehybridized and hybridized with a 32P-labeled probe for human IL-2 (prepared from a ~600-bp cDNA fragment provided by Dr. S. Gillis; Immunex Corp., Seattle, WA). Equal loading of RNA samples was verified both by RNA staining and by hybridization with a rat glyceraldehyde-6-phosphate dehydrogenase probe (GAPDH, a ~1.2-kb cDNA fragment provided by Dr. A. Purch-rio, Oncogen).

**Results**

**Construction and Expression of B7 and CD28 Immunoglobulin Cy Fusion Proteins.** In initial attempts to make soluble derivative of B7 and CD28, we made cDNA constructs encoding molecules truncated at the NH2-terminal side of their transmembrane domains. In both cases, the native signal peptides were replaced with the signal peptide from oncostatin M (24), which mediates efficient release of secreted proteins in transient expression assays (J. Kallestad, P. S. Linsley, and W. Brady, unpublished observations). These cDNAs were cloned into an expression vector, transfected into COS cells, and spent culture medium was tested for secreted forms of B7 and CD28. In this fashion, we produced several soluble forms of B7, but in repeated attempts, we were unable to detect soluble CD28 molecules.

We then took the approach of making receptor Ig Cy fusion proteins. Other investigators (23, 30) have shown these molecules to be efficiently produced in transient expression systems, and easily purified and detected using standard immunochromatography techniques. The B7 and CD28 extracellular regions, preceded by the signal peptide to oncostatin M, were fused in frame to an Ig Cy1 cDNA, as shown in Fig. 1A. During construction, the Ig hinge disulfides were mutated to serine residues to abolish intrachain disulfide bonding. The resulting fusion proteins were produced in COS cells and purified by affinity chromatography on immobilized protein A as described in Materials and Methods. Yields of purified protein were typically 1.5–4.5 mg/liter of spent culture medium.

As shown in Fig. 1B, the B7 Ig fusion protein migrated during SDS-PAGE under nonreducing conditions predominantly as a single species of Mr 70,000, with a small amount of material migrating as a Mr ~150,000 species. After reduction, a single Mr ~75,000 species was observed. The nature of the Mr ~150,000 species was not investigated further. CD28 Ig migrated as a Mr ~140,000 species under nonreducing conditions and a Mr ~70,000 species after reduction, indicating that it was expressed as a homodimer. Since the Ig Cy1 hinge cysteines had been mutated, disulfide linkage probably involved cysteine residues which naturally form interchain bonds in the CD28 homodimer (25).

**Binding Activities of B7 and CD28 Immunoglobulin Cy Fusion Proteins.** To investigate the functional activities of B7 Ig and CD28 Ig, we first tested binding to CHO cell lines expressing CD28 or B7. In early experiments, spent culture media from transfected COS cells was used as a source of fusion protein, while in later experiments, purified proteins were used (Fig. 2). Binding was detected by addition of FITC-conjugated goat anti-human Ig second step reagent. B7 Ig was bound by CD28 CHO, while CD28 Ig was bound by B7 CHO. B7 Ig also bound weakly to B7 CHO (Fig. 2), suggesting that this molecule has a tendency to form...
**Figure 1.** Construction and expression of B7 and CD28 Ig Cγ fusion proteins. (A) Maps of B7 Ig and CD28 Ig constructs. cDNA constructs encoding the indicated portions of oncostatin M (dark shaded regions), B7 and CD28 (unshaded regions), and human Ig Cγ1 (stippled regions) were constructed as described in Materials and Methods. Sequences displayed show the junctions between B7 and CD28 (capital letters), and the signal peptide (SP) of oncostatin M at their NH2 termini, and the hinge (H) of Ig Cγ1 at their COOH termini. Amino acids introduced during construction are indicated in parentheses. Asterisks denote cysteine to serine mutations introduced in the hinge. Boxes labeled V and C denote Ig superfamily-like domains present in CD28 (13) and B7 (22). The CH2 and CH3 domains of Ig Cγ1 are also indicated. (B) Purification of B7 Ig and CD28 Ig. Expression plasmid constructs encoding the fusion proteins displayed above were transfected into COS cells. Serum-free conditioned medium was collected, and Ig Cγ-containing proteins were purified by protein A affinity chromatography as described in Materials and Methods. Aliquots (1 μg) of B7 Ig (lanes 1 and 3) or CD28 Ig (lanes 2 and 4) were subjected to SDS-PAGE (4–12% acrylamide gradient) under nonreducing (−βME, lanes 1 and 2) or reducing (+βME, lanes 3 and 4) conditions. Lane 5 shows mol wt markers. Proteins were visualized by staining with Coomassie Brilliant Blue.

**Figure 2.** B7 Ig and CD28 Ig bind to transfected CHO Cells. Amplified transfected CHO cells expressing CD28, B7, or dhfr only (no surface marker) were first stained with human Ig Cγ1-containing proteins (chimeric mAb L6, CD28 Ig, B7 Ig, or CD5 Ig), or mouse mAbs (9.3 or BB-1 at 10 μg/ml), followed by FITC-conjugated anti-human or mouse Ig second step reagents. A total of 10,000 stained cells was then analyzed by FACS®.

CD28 is the Primary B7-binding Protein on PHA-activated T Cells. Although B7 Ig bound to immobilized CD28 Ig, and to CD28+ CHO cells, it was not known whether B7 Ig could bind to CD28 naturally expressed on T cells. This is
Figure 3. 125I-labeled B7 Ig binds with high affinity to immobilized CD28 Ig. (A) Competition binding analysis of 125I-B7 Ig. 96-well plastic dishes were coated with CD28 Ig as described in Materials and Methods. 125I-labeled B7 Ig (3.3 × 10^6 cpm, 2 × 10^6 cpm/pmol) was then added to a concentration of 24 nM in the presence of the indicated concentrations of unlabeled chimeric L6, mAb 9.3, mAb BB-1, or B7 Ig. Plate-bound radioactivity was determined and is expressed as a percentage of radioactivity bound to wells treated without competitor (7,800 cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by <20%. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7 Ig. (B) Scatchard analysis of unlabeled B7 Ig competition binding experiment. Data shown in Fig. 3 A was replotted in the Scatchard representation and a binding constant (K_d) was estimated from the slope of the line best fitting the experimental data (r = 0.985).

Figure 4. CD28 is the primary B7 Ig binding protein on PHA-activated T cells. (A) FACS® profiles of B7 Ig binding to PHA blasts. PHA-stimulated PBL were stained without or with B7 Ig at 10 μg/ml. Where indicated mAbs 9.3 or BB-1 were added (also at 10 μg/ml) to cells simultaneously with B7 Ig. Bound mAb was detected with a FITC-conjugated goat anti-human Ig Cy reagent, and stained cells were analyzed by FACS®. (B) Autoradiogram of 125I-labeled proteins immunoprecipitated by B7 Ig. PHA-stimulated PBL were surface labeled with 125I as described in Materials and Methods. Aliquots of a nonionic detergent extract of labeled cells (~3 × 10^6 cpm in a volume of 0.12 ml) were subjected to immunoprecipitation analysis with no addition (lane 1), mAb 9.3 (5 μg, lane 2), B7 Ig (10 μg, lane 3), or chimeric L6 (10 μg, lane 4). After precipitation of antigen-antibody complexes using formalin-fixed Staphylococcus aureus, extracts were then subjected to a second immunoprecipitation analysis with no addition (lane 5), mAb 9.3 (5 μg, lane 6), or B7 Ig (10 μg, lane 7). Washed immunoprecipitates were analyzed by SDS-PAGE (5–15% acrylamide gradient) under reducing (+ β-ME, lanes 1–7) or non-reducing conditions (– β-ME, lanes 8 and 9), the gel was dried, and subjected to autoradiography.

an important consideration since the level of CD28 on transfected cells was ~10-fold higher than that found on PHA-activated T cells (20). We therefore tested PHA-activated T cells for binding of B7 Ig by FACS® analysis after indirect immunofluorescent staining. As shown in Fig. 4 A, these cells bound significant levels of B7 Ig, and binding was inhibited by mAbs 9.3 and BB-1. We also determined the identity of B7 Ig–binding proteins by immunoprecipitation analysis of 125I-surface-labeled cells (Fig. 4 B). Both mAb 9.3 and B7 Ig immunoprecipitated a M_r ~45,000 protein under reducing conditions and M_r ~45,000 and ~90,000 proteins
under nonreducing conditions, with the latter form being more prominent. The $M_r \approx 45,000$ protein found in the sample precipitated with chimeric mAb L6 was due to spill-over and was not seen in other experiments. mAb 9.3 was more effective at immunoprecipitation than B7 Ig, in agreement with the greater affinity of the mAb (Fig. 3). Identical results were obtained when CD28 + CHO cells were used for immunoprecipitation analysis. Pre-clearing of CD28 by immunoprecipitation with mAb 9.3 also removed B7 Ig-precipitable material, indicating that both mAb 9.3 and B7 Ig bound the same $^{125}$I-labeled protein. Taken together, the results in this section indicate that CD28 is the major receptor for B7 Ig on PHA-activated T cells.

**B7 Binding to CD28 Blocks CD28-mediated Adhesion.** mAbs to CD28 have potent biological activities on T cells, suggesting that interaction of CD28 with its natural ligand(s) may also have important functional consequences. As a first step in determining functional consequences of interaction between B7 and CD28, we asked whether B7 Ig could block the CD28-mediated adhesion assay (20). As shown in Fig. 5, B7 Ig blocked CD28-mediated adhesion somewhat less effectively than mAb 9.3 (half-maximal inhibition at 200 nM as compared with 10 nM for mAb 9.3). The relative effectiveness of these molecules at inhibiting CD28-mediated adhesion was similar to their relative binding affinities in competition binding experiments (Fig. 3 A). CD28 Ig failed to inhibit CD28-mediated adhesion at concentrations of up to 950 nM (data not shown), suggesting that much higher levels of CD28 Ig were required to compete with the high local concentrations of CD28 present on transfected cells.

**B7 Costimulates T Cell Proliferation.** We next investigated whether triggering of CD28 by B7 was costimulatory for T cell proliferation. The ability of B7 Ig to costimulate proliferation of PBL together with anti-CD3 was first explored. In several experiments, B7 Ig in solution at concentrations of 1–10 μg/ml showed only a modest enhancement of proliferation of PBL. Anti-CD3 stimulation was with mAb G19-4 at 1 μg/ml in solution. For CD28 stimulation, mAb 9.3 or B7 Ig were added in solution at 1 μg/ml, or after immobilization on the culture wells by pre-incubation of proteins at 10 μg/ml in PBS for 3 h at 23°C and then washing the culture wells. B7 + CHO and control dhfr + CHO cells were irradiated with 1,000 rad before mixing with PBL at a 4:1 ratio of PBL/CHO cells. After culture for 3 d, proliferation was measured by uptake of $[^3H]$thymidine for 5 h. Values shown are means of determinations from quadruplicate cultures (SEM <15%).

| Exp. | CD28 stimulation | - Anti-CD3 | + Anti-CD3 |
|------|------------------|------------|------------|
| 1    | None             | 0.1        | 26.0       |
|      | mAb 9.3 (solution)| 0.3        | 156.1      |
|      | mAb 9.3 (immobilized)| 0.1        | 137.4      |
|      | B7 Ig (immobilized)| 0.1        | 174.5      |
| 2    | None             | 0.2        | 19.3       |
|      | mAb 9.3 (solution)| 0.4        | 75.8       |
|      | B7 + CHO cells   | 9.4        | 113.9      |
|      | dhfr + CHO cells | 23.8       | 22.1       |

Table 1. B7 Ig Is Costimulatory with Anti-CD3 for Proliferation of Peripheral Blood Lymphocytes

PHLA blasts were cultured at 50,000 cells/well with varying amounts of irradiated CHO cell transfecants. After 2 d of culture, proliferation was measured by a 5-h pulse of $[^3H]$thymidine. Shown are means of quadruplicate determinations (SEM <15%). Background proliferation of PHA blasts without added CHO cells was 11,200 cpm. $[^3H]$Thymidine incorporation by irradiated B7 + CHO and CD5 + CHO cells alone was <1,800 cpm at each cell concentration and has been subtracted from the values shown.

| T cells/CHO cells | + B7 + CHO | + CD5 + CHO |
|-------------------|------------|------------|
| 25:1              | 92.7       | 15.5       |
| 50:1              | 135.4      | 19.5       |
| 100:1             | 104.8      | 16.8       |
| 200:1             | 90.3       | 17.7       |
| 400:1             | 57.0       | 13.7       |
| 800:1             | 42.3       | 17.6       |

Table 2. B7 is Directly Stimulatory for Proliferation of PHA Blasts

PHLA blasts were cultured at 50,000 cells/well with varying amounts of irradiated CHO cell transfecants. After 2 d of culture, proliferation was measured by a 5-h pulse of $[^3H]$thymidine. Shown are means of quadruplicate determinations (SEM <15%). Background proliferation of PHA blasts without added CHO cells was 11,200 cpm. $[^3H]$Thymidine incorporation by irradiated B7 + CHO and CD5 + CHO cells alone was <1,800 cpm at each cell concentration and has been subtracted from the values shown.
Table 3. Stimulation of Proliferation of PHA Blasts by B7+ CHO Cells Is Inhibited by CD28- or B7-specific mAbs

| Stimulation | mAb     | [3H]-T incorporation |
|-------------|---------|----------------------|
| None        | None    | 10.8                 |
| B7+ CHO     | None    | 180                  |
| B7+ CHO     | 9.3 Fab | 132                  |
| B7+ CHO     | BB-1    | 98.3                 |
| B7+ CHO     | LB-1    | 196                  |
| CD7+ CHO    | None    | 11.5                 |
| CD7+ CHO    | 9.3 Fab | 10.0                 |
| CD7+ CHO    | BB-1    | 10.0                 |
| CD7+ CHO    | LB-1    | 11.3                 |

PHA blasts were stimulated as described in Table 2 with irradiated CHO cells at a ratio of 100:1 T cells/CHO cells. mAbs were added at 10 μg/ml at the beginning of culture. mAb LB-1 (21) is an isotype-matched control for mAb BB-1. Proliferation was measured by uptake of [3H]-thymidine during a 5-h pulse after 2 d of culture. Values represent means of quadruplicate cultures (SEM <15%).

Discussion

We have used soluble Ig G fusions of both CD28 and B7 to measure the strength of their interaction. The apparent...
binding. The relative contribution of different adhesion systems to the overall strength of T cell-B cell interactions is not easily gauged, but is likely a function of both affinity/avidity and the densities on apposing cell surfaces of the different receptors and counter-receptors involved. Since both CD28 and B7 are found at relatively low levels on resting lymphoid cells (16, 22), they may be less involved than other adhesion systems in initiating intercellular interactions. The primary role of CD28/B7 interactions may be to maintain or amplify a response subsequent to induction of these counter-receptors on their respective cell types.

While occupancy or crosslinking of the T cell antigen receptor is sufficient to initiate a T cell immune response, full activation requires additional costimulatory signals. In some cases, soluble molecules such as IL-1 can provide costimulatory activity, but in other cases the nature of the molecule(s) involved is unclear (8, 9). Schwartz (9) has recently reviewed evidence for a costimulatory activity on antigen-presenting cells which may determine whether TCR occupancy leads to a productive or anergic response in murine T cell clones. This activity appears to require cell-cell contact, leads to increased IL-2 production, and may operate through a different signal-transducing pathway than the TCR (9). All of these characteristics are shared with the CD28 pathway (reviewed in reference 14), leading to the intriguing possibility that CD28 may be one of these functionally defined costimulatory molecules. In agreement with this possibility, is the finding by Ledbetter et al. (31) that anti-CD28 mAbs can partially overcome inhibition of T cell proliferation induced by internalization of the TCR. Studies of the involvement of CD28/B7 interactions in various experimental systems will be required to fully evaluate the role of these costimulatory interactions.

Binding of B7 to CD28 on T cells was costimulatory for T cell proliferation (Tables 1–3), suggesting that some of the biological effects of anti-CD28 mAbs result from their ability to mimic T cell activation resulting from natural interaction between CD28 and its counter-receptor, B7. mAb 9.3 has greater affinity for CD28 than does B7 Ig (Figs. 4 and 5), which may account for the extremely potent biological effects of this mAb (14) in costimulating polyclonal T cell responses. Surprisingly, however, anti-CD28 mAbs are inhibitory for antigen-specific T cell responses (15, 16). This may indicate that antigen-specific T cell responses are dependent upon costimulation via CD28/B7 interactions, and that inhibition therefore results from blocking of CD28 stimulation. Despite the inhibition, CD28 must be bound by mAb under these conditions, implying that triggering by mAb is not always equivalent to triggering by B7. Although mAb 9.3 has higher apparent affinity for CD28 than B7 (Fig. 3), it may be unable under these circumstances to induce the optimal degree of CD28 clustering (31) for stimulation.

CD28/B7 interactions may also be important for B cell activation and/or differentiation. We have recently observed that mAbs 9.3 and BB-1 block T<sub>α</sub> cell-induced Ig production by B cells (N. Damle, P. S. Linsley, and J. A. Ledbetter, unpublished results). This blocking effect may be due in part to inhibition by these mAbs of production of T<sub>α</sub>-derived B cell–directed cytokines, but may also involve inhibition of B cell activation by interfering with direct signal transduction via B7. These results suggest that cognate activation of B lymphocytes, as well as T<sub>α</sub> lymphocytes, is dependent upon interaction between CD28 and B7.

The mechanism of IL-2 transcript induction in T cells by B7<sup>+</sup> CHO cells (Fig. 6) is not currently known. It is well known that mRNAs for cytokines and other inflammatory mediators are characterized by the presence in their 3' untranslated regions of AU-rich motifs (37), which control mRNA stability (38). Studies by Lindsten et al. (19) showed that anti-CD28 mAbs stimulated production of several T cell–derived cytokines by stabilizing transcripts for these molecules. However, as discussed by June et al. (14), anti-CD28 mAbs may also enhance transcription of IL-2 mRNA. Whatever the mechanism of transcript accumulation by CD28/B7 interactions, regulation of cytokine production by cell-cell contact has also been shown in other systems (39). Many effectors functions of T lymphocytes are cytokine mediated (40), and control points in the synthetic pathways of these molecules are therefore extremely important. As dysfunctional cytokine production has been implicated as a contributing factor in several human diseases (40), it will be important to determine how B7/CD28 interactions regulate T cell cytokine production in various pathological states.
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