agents for both the augmentation of immunity to microbes and tumor antigens and the inhibition of immune responses during transplantation and autoimmunity.

Note added in proof: The GL1 mAb reacts with the murine homolog of B7-2 (*14*).

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19. We thank E. Li, R. Jaenisch, and A. Abbas for providing technologies and advice and L. Berg for PCR primer sequences. L.M.N. is a consultant to Repligen Corporation. Supported by a grant from the Lucille Markey Foundation (A.H.S.), a Beckman Young Investigator Award (A.H.S.), the American Cancer Society (A.H.S. and G.J.F.), and NIH grant CA 40216 (L.M.N.).

11 August 1993; accepted 29 September 1993

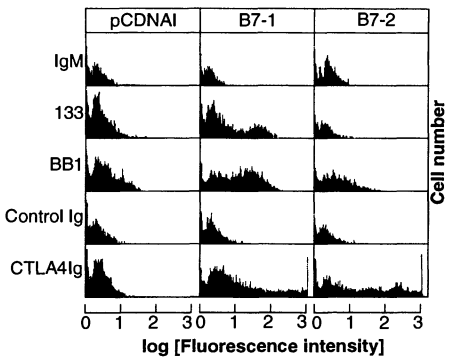

Fig. 1. B7-2 binds CTLA4Ig but not B7-1 mAb or B7-3 mAb. COS cells were transfected with cDNAs encoding B7-1 or B7-2 (clone 29) or with pCDNAI vector alone. Transfectants were stained with mAb 133 (*9*), mAb BB1 (*10*), control IgM mAb, recombinant CTLA4Ig (*12*), or control Ig fusion protein (*12*).

Cloning of B7-2: A CTLA-4 Counter-Receptor That Costimulates Human T Cell Proliferation

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Although presentation of antigen to the T cell receptor is necessary for the initiation of an immune response, additional molecules expressed on antigen-presenting cells deliver essential costimulatory signals. T cell activation, in the absence of costimulation, results in T cell anergy. The B7-1 protein is a costimulator molecule that regulates interleukin-2 (IL-2) secretion by signaling through the pathway that uses CD28 and CTLA-4 (hereafter referred to as the CD28 pathway). We have cloned a counter-receptor of CD28 and CTLA-4, termed B7-2. Although only 26 percent identical to B7-1, B7-2 also costimulates IL-2 production and T cell proliferation. Unlike B7-1, B7-2 messenger RNA is constitutively expressed in unstimulated B cells. It is likely that B7-2 provides a critical early costimulatory signal determining if the T cell will contribute to an immune response or become anergic.

After T cells receive a signal through the T cell receptor (TCR), the ligation of the CD28 and CTLA-4 receptors by the inducible counter-receptor B7-1 (originally termed B7) on antigen-presenting cells (APCs) results in IL-2 production and T cell proliferation (*1*, *2*). Because B7-1 has 20 times the affinity for CTLA-4 as it has for CD28, CTLA4Ig fusion protein is the most effective reagent for inhibiting the B7-1 CD28 costimulatory pathway (*3*–*5*). There is evidence for additional CTLA-4 counter-receptors (*6*, *7*). Activated human B lymphocytes express at least three distinct CTLA-4 counter-receptors, termed B7-1, B7-2, and B7-3 (*7*). Within 24 hours after activation, human B cells express a CTLA-4 counter-receptor (B7-2) that induces T cell proliferation (*7*). At 48 hours, two additional CTLA-4 counter-receptors are expressed including the original B7-1 identified by two monoclonal antibodies (mAbs) 133 (*8*, *9*) and BB1 (*10*) and a third CTLA-4 counter-receptor, B7-3, identified only by monoclonal antibody (mAb) BB1. These observations suggest that multiple CTLA-4 counter-receptors exist and costimulate T cells at different times after B cell activation.

A complementary DNA (cDNA) encoding a second CTLA-4 counter-receptor, termed B7-2, was isolated by cDNA expression cloning with CTLA4Ig selection from an activated human B cell cDNA library. COS cells transfected with B7-2 cDNA bound CTLA4Ig but did not bind to control immunoglobulin (Ig) fusion protein, mAb 133, or mAb BB1 (Fig. 1). In contrast, COS cells transfected with B7-1 bound to CTLA4Ig, mAb 133, and mAb BB1. Vector-transfected COS cells bound none of the above. This demonstrates that B7-2 encodes a CTLA-4 counter-receptor distinct from B7-1 and B7-3.

The B7-2 cDNA comprised 1120 nucleotides (GenBank accession number L25259) with a single, large open reading frame of 987 nucleotides. The NH_2-terminus of the B7-2 protein is a secretory signal peptide with a predicted cleavage between the alanines at positions 23 and 24. Processing at this site would result in a 34-kD membrane protein of 306 amino acids before modification. This protein would consist of a 220–amino acid extracellular region containing Ig superfamily V– and C–like domains, a hydrophobic transmembrane domain of 23 amino acids, and a cytoplasmic tail of 60 amino acids. The B7-2 extracellular domain contains eight potential N-linked glycosylation sites and, like B7-1, is probably heavily glycosylated. Comparison of both nucleotide and amino acid sequences of B7-2 with the GenBank and European Molecular Biology Laboratory databases has shown that only the human and murine B7-1 proteins are related. Alignment of the three B7 protein sequences shows that B7-2 has 26% identity with human B7-1 (Fig. 2). Because human B7-1, human B7-2, and murine B7-1 all bind to human CTLA-4 and CD28, conserved amino acids probably represent those necessary to form a CTLA-4 or CD28 binding site.

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Three mRNA transcripts of 1.35, 1.65, and 3.0 kb hybridized to B7-2 cDNA. RNA blot analysis demonstrated that B7-2 mRNA was expressed in unstimulated human splenic B cells and increased fourfold after activation (Fig. 3). In contrast, B7-1 mRNA was not expressed in unstimulated B cells and was transiently expressed after activation (*8*). Thus, B7-1 and B7-2 expression appears to be differentially regulated.

COS cells transfected with either B7-1 or B7-2 costimulated equivalent amounts of IL-2 production and T cell proliferation when tested at various stimulator to responder ratios (Fig. 4). In contrast, vector-transfected COS cells did not costimulate. B7-2 effectively costimulated with either phorbol 12-myristate 13-acetate (PMA) or CD3 mAb as a first signal. To distinguish B7-2 from B7-1 and B7-3, we used mAbs to B7-1 and B7-3 to inhibit proliferation and IL-2 production of submitogenically activated human CD28$^+$ T cells (Fig. 5). The mAbs 133 (9) and BB1 (*7*, *10*) completely inhibited proliferation and IL-2 secretion induced by B7-1 but had no effect on costimulation by B7-2. Isotype-matched control mAb, B5, had no effect. To determine whether B7-2 signals by means of the CD28 pathway, we tested anti-CD28 Fab and CTLA4Ig fusion protein to determine whether they inhibited B7-2 costimulation. Both anti-CD28 Fab and CTLA4Ig inhibited proliferation and IL-2 production induced by either B7-1 or B7-2 COS transfectants, whereas control Ig had no effect. The CTLA4Ig inhibited B7-2 costimulation of proliferation by more than 90%. None of the blocking agents inhibited T cell proliferation or IL-2 production induced by the combination of PMA and phytohemagglutinin (*11*).

Like B7-1, B7-2 is a counter-receptor for the CD28 and CTLA-4 T cell surface molecules. Both proteins (i) are expressed on

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hB7-1    1 M..GHTRRQGTSPSKCPYLNFFQLLV.LAGLSHFCSGV.IHVTKEVKEVA  46
hB7-2    1 M.....DPQCTMGLSN......ILFVMAFLLSGA...APLKIQAYFNETA  36
mB7      1 MACNCQLMQDTPLLKFPCPRLILLFVLLIRLSQVSSDVDEQLSKSVKDKV  50

hB7-1   47 TLSCGHNVSVEE.LAQTRIYWQKEKKMVLT.MMSGDMNI...WPEYKNRT  91
hB7-2   37 DLPCQFANSQNQSLSELVVFWQDQENLVLNEVYLGKEKFDSVHSKYMGRT  86
mB7     51 LLPCRY.NSPHEDESEDRIYWQKHDKVVLS.VIAGKLKV...WPEYKNRT  95

hB7-1   92 IFDITNNLSIVILALRPSDEGTYECVVLKYEKDAFKREHLAEVTLSVKAD 141
hB7-2   87 SFD.SDSWTLRLHNLQIKDKGLYQCIIHHKKPTGMIRIHQMNSELSVLAN 135
mB7     96 LYDNTT.YSLIILGLVLSDRGTYSCVVQKKERGTYEVKHLALVKLSIKAD 144

hB7-1  142 FPTPSISDFEIPTSNI.RRIICSTSGGFPEPH....LSWLENGEELNAIN 186
hB7-2  136 FSQPEIVPISNITENVYINLTCSSIHGYPEPKKMSVLLRTKNSTIEYDGI 185
mB7    145 FSTPNITESGNPSADT.KRITCFASGGFPKPR....FSWLENGRELPGIN 189

hB7-1  187 TTVSQDPETELYAVSSKLDFN...MTTNHSFMCLIKYGHLRVNQTFNWNT 233
hB7-2  186 MQKSQDNVTELYDVSISLSVSFPDVTSNMTIFCILETDKTRLLSSPFSIE 235
mB7    190 TTISQDPESELYTISSQLDFN...TTRNHTIKCLIKYGDAHVSEDFTWEK 236

hB7-1  234 TKQEHF.PDNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRR 282
hB7-2  236 .LEDPQPPPDHIPWITAVLP....TVIICVMVFCLILWKWKKKRPRNSY 280
mB7    237 PPEDPPDSKNTLVLFGAGFGAVITVVVIVVIIKCFCKHRSCFRRNEA.SR 285

hB7-1  283 ESVRPV*                                          288
hB7-2  281 KCG...TNTMEREESEQTKKREKIHIPERSDEAQRVFKSSKTSSCDKSDT 327
mB7    286 ETNNSLTFGPEEALAEQTVFL*                            306

hB7-2  328 CF*                                               329
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Fig. 2. Predicted protein sequence of B7-2 and comparison with B7-1. A cDNA library was prepared in the pCDM8 expression vector with polyadenylated RNA from human splenic B cells activated by antibody to Ig (anti-Ig) (10 min to 96 hours). COS cells were transfected with the activated B cell cDNA library DNA by DEAE dextran transfection (*8*). Cells were harvested after 47 hours and incubated with CTLA4Ig (10 μg/ml) and CD28-Ig, and binding cells were isolated by panning on plates coated with goat antibody to human IgG (*8*). Episomal DNA was isolated, transformed into *Escherichia coli* DH10B/P3, and the plasmid DNA re-introduced into COS cells by means of spheroplast fusion. Cells expressing B7-1 were removed by incubation with B7-1 mAbs [133 and B1.1 (*6*)] and immunomagnetic bead depletion. Transfectants were selected by panning with CTLA4Ig and CD28-Ig as described above. A third round of selection, identical to the second round, was done. Four of 48 isolated plasmids contained a 1.2-kb cDNA insert. COS cells transfected with these plasmids bound CTLA4Ig but not control Ig fusion protein. The deduced amino acid sequence of B7-2 is shown and compared with murine and human B7-1. The nucleotide sequence was deposited in GenBank (accession number L25259). Vertical lines show identical amino acids between human B7-2 and human B7-1 or human B7-2 and murine B7.

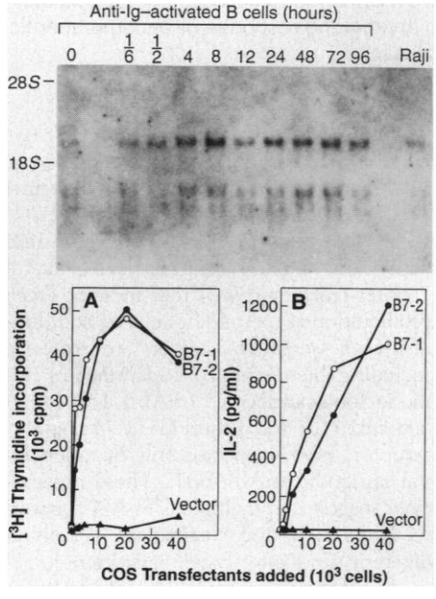

Fig. 3 (top). RNA blot analysis of B7-2 expression in unstimulated and activated human B cells. Human splenic B cells were isolated and activated with anti-Ig beads, and RNA was prepared at the indicated times (*9*). Equal amounts of RNA (20 μg) were electrophoresed on an agarose gel, blotted, and hybridized to ^{32}P-labeled B7-2 cDNA. RNA blot analyses of unstimulated and anti-Ig–activated human splenic B cells and of Raji cell line (B cell Burkitt's lymphoma) are shown. **Fig. 4 (bottom).** B7-1 and B7-2 COS transfectants costimulate T cell proliferation and IL-2 production. Human CD28$^+$ T cells were isolated (*1*). B7-1–, B7-2–, and vector-transfected COS cells were harvested 72 hours after transfection, incubated with mitomycin C (25 μg/ml) for 1 hour, and then extensively washed. We incubated CD28$^+$ T cells (10^5) with PMA (1 ng/ml) and the indicated number of COS transfectants. (**A**) Proliferation was measured by [^{3}H]thymidine (1 μCi) incorporation for the last 12 hours of a 72-hour incubation. (**B**) IL-2 production was measured by enzyme-linked immunosorbent assay (Biosource, Camarillo, California) with supernatants harvested 24 hours after the initiation of culture.

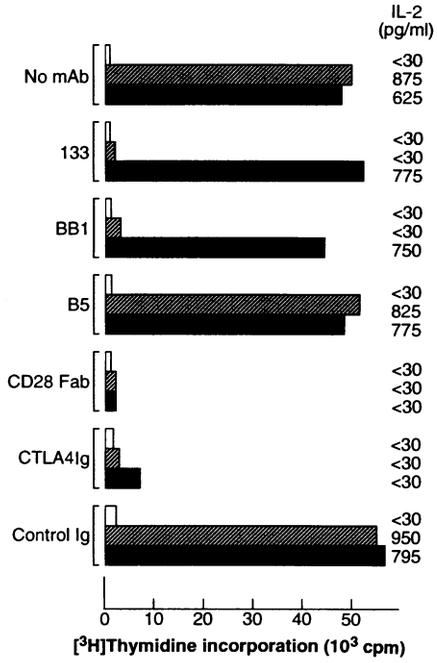

Fig. 5. B7-2 costimulation is not blocked by B7-1 and B7-3 mAbs but is blocked by CTLA4Ig and anti-CD28 Fab. Human CD28$^+$ T cells and B7-1–, B7-2–, and vector-transfected COS cells were prepared as described in Fig. 4. CD28$^+$ T cells (10^5) were incubated with PMA (1 ng/ml) and 2 × 10^4 COS transfectants. Blocking agents (10 μg/ml) are indicated. Proliferation and IL-2 production were measured as in Fig. 4.

the surface of APCs, (ii) are structurally related to the Ig supergene family with an Ig V– and Ig C–like domain, and (iii) costimulate T cells to produce IL-2 and proliferate. However, B7-1 and B7-2 differ in that B7-2 mRNA is constitutively expressed in unstimulated B cells, whereas B7-1 mRNA does not appear until 4 hours and cell surface protein is not detected until 24 hours (*8*, *9*). Unstimulated human B cells do not express CTLA-4 counter-receptors on the cell surface and do not costimulate T cell proliferation (*7*). Therefore, expression of B7-2 mRNA in unstimulated B cells would allow rapid expression of B7-2 protein on the cell surface after activation, presumably from stored mRNA or protein. Expression of B7-1 and B7-2 in cell lines and human B cell neoplasms differ (*11*), suggesting they may have biologically distinct functions.

The functional necessity for multiple CD28 and CTLA-4 counter-receptors is unknown. Indeed, whereas CD28 can clearly transduce a costimulatory signal, the signaling function of CTLA-4 is currently unknown. A T cell, which has received a first signal from the TCR, must commit within 12 to 24 hours to either activation or anergy (*12–15*). Although B7-1 (*12*, *13*) or cross-linking by CD28 mAb (*15*) can provide a costimulatory signal that prevents the induction of anergy in vitro, the late appearance (between 24 and 48 hours) of B7-1 on B cells (*9*) and macrophages (*16*) after activation suggests that B7-1 is not the critical costimulatory molecule that regulates whether a T cell becomes activated or anergic. We postulate that the earlier appearance of B7-2 provides this critical costimulatory signal (*17*, *18*). If confirmed, then B7-1 costimulation is more likely to provide a signal that results in T cell clonal expansion. Identification and characterization of B7-2 will enable further manipulation of the CD28 pathway, potentially leading to therapeutic strategies for the control of autoimmunity, transplantation tolerance, and the stimulation of viral and tumor immunity.

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19. Supported by NIH grant CA 40216 to L.M.N. L.M.N. is a consultant to Repligen Corporation.

11 August 1993; accepted 29 September 1993

Gene Replacement in *Toxoplasma gondii* with Chloramphenicol Acetyltransferase as Selectable Marker

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A system for stable transformation of *Toxoplasma gondii* tachyzoites was developed that exploited the susceptibility of *Toxoplasma* to chloramphenicol. Introduction of the chloramphenicol acetyltransferase (CAT) gene fused to *Toxoplasma* flanking sequences followed by chloramphenicol selection resulted in parasites stably expressing CAT. A construct incorporating the tandemly repeated gene, *B1*, targeted efficiently to its homologous chromosomal locus. Knockout of the single-copy gene, *ROP1*, was also successful. Stable transformation should permit the identification and analysis of *Toxoplasma* genes important in the interaction of this opportunistic parasite with its host.

Toxoplasma gondii is an obligate intracellular parasite that can infect most warm-blooded vertebrates. In humans, infection can result in severe congenital defects and life-threatening infection in immunocompromised hosts. Recently, the parasite has received increased attention as an opportunistic pathogen affecting up to 25% of patients with acquired immunodeficiency syndrome (AIDS) (*1*). Detailed study of the basic biology of the host-parasite interaction has been hampered by the absence of a methodology to introduce and stably express or knockout genes in this organism. Despite intensive efforts, transfection of intracellular eukaryotic parasites was unsuccessful until recently when transient CAT expression driven by the upstream regions of three *Toxoplasma* genes was achieved (*2*).

Efforts to stably transform *Toxoplasma* were complicated by the inability of these parasites to replicate outside host cells. Neomycin and hygromycin, drugs commonly used for selection of stable transformants in systems such as protozoan parasites from the kinetoplastida order (*3–5*), kill host cells as efficiently as they kill parasites. We have developed an alternative system in which CAT is used as an efficient and easily detectable selectable marker in *Toxoplasma*.

Chloramphenicol and other antibiotics that inhibit prokaryotic translation have potent effects against *Plasmodium* and are currently used for malaria chemotherapy. It has been thought that *Toxoplasma* tachyzoites are not susceptible to chloramphenicol in vitro. We have found, however, that chloramphenicol, like the mechanistically similar antibiotic clindamycin (*6*), has a potent parasiticidal effect, but one that is delayed in onset: Parasites complete two to three cycles of host cell lysis (approximately 7 days or 20 to 25 divisions) before any effect of drug is evident. Daily visual inspection of cultures indicated that 10 μM chloramphenicol killed more than 90% of parasites, but had no obvious effects on the host monolayer. These results indicate that *Toxoplasma gondii* has a highly specific susceptibility to drugs inhibiting prokaryotic translation. This may reflect a unique target for chemotherapy in these coccidian parasites, for example, targets in mitochondria or other organelles as suggested for *Plasmodium* (*7*, *8*).

Our initial constructs (Fig. 1A) utilized the upstream and downstream regions of a gene, *SAG1*, that encodes the major surface antigen p30. These sequences have been previously shown to drive efficient CAT expression (*2*). Stable transfection, if by means of homologous recombination with replacement of *SAG1*, seemed likely to be a deleterious event given that *Toxoplasma* tachyzoites are haploid and *SAG1* is single-copy. [Indeed, Sag1$^-$ mutants are impaired in virulence and intracellular growth (*9*, *10*).] We therefore added to the construct a segment of *B1*, a gene of unknown function that is tandemly reiterated approximately 35 times within the genome (*11*). In transient transfection, this *B1*-containing construct induced CAT activity similar to that induced by a construct lacking *B1* (*12*).

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