Induction of Alloantigen-specific Hyporesponsiveness in Human T Lymphocytes by Blocking Interaction of CD28 with Its Natural Ligand B7/BB1

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Summary

The specificity of T lymphocyte activation is determined by engagement of the T cell receptor (TCR) by peptide/major histocompatibility complexes expressed on the antigen-presenting cell (APC). Lacking costimulation by accessory molecules on the APC, T cell proliferation does not occur and unresponsiveness to subsequent antigenic stimulus is induced. The B7/BB1 receptor on APCs binds CD28 and CTLA-4 on T cells, and provides a costimulus for T cell proliferation. Here, we show that prolonged, specific T cell hyporesponsiveness to antigenic restimulation is achieved by blocking the interaction between CD28 and B7/BB1 in human mixed leukocyte culture (MLC). Secondary T cell proliferative responses to specific alloantigen were inhibited by addition to the primary culture of monovalent Fab fragments of anti-CD28 monoclonal antibody (mAb) 9.3, which block interaction of CD28 with B7/BB1 without activating T cells. Hyporesponsiveness was also induced in MLC by CTLA4Ig, a chimeric immunoglobulin fusion protein incorporating the extracellular domain of CTLA-4 with high binding avidity for B7/BB1. Cells previously primed could also be made hyporesponsive, if exposed to alloantigen in the presence of CTLA4Ig. Maximal hyporesponsiveness was achieved in MLC after 2 d of incubation with CTLA4Ig, and was maintained for at least 27 d after removal of CTLA4Ig. Accumulation of interleukin 2 (IL-2) and interferon γ but not IL-4 mRNA was blocked by CTLA4Ig in T cells stimulated by alloantigen. Antigen-specific responses could be restored by addition of exogenous IL-2 at the time of the secondary stimulation. Addition to primary cultures of the intact bivalent anti-CD28 mAb 9.3, or B7/BB1$^+$ transfected CHO cells or exogenous IL-2, abrogated induction of hyporesponsiveness by CTLA4Ig. These data indicate that interaction of CD28 with B7/BB1 during TCR engagement with antigen is required to maintain T cell competence and that blocking such interaction can result in a state of T cell hyporesponsiveness.

Effective presentation of antigen to T cells requires a complex series of events to initiate the immune response. In addition to processing and presenting antigenic peptides in the context of MHC molecules to specific TCRs, APCs must provide one or more costimulatory signal(s) to fully activate T cells, and induce IL-2 release and DNA synthesis (1–7). In the absence of costimulatory signals, T cells presented with antigen may enter a state of anergy characterized by the failure to activate the IL-2 gene in response to further antigenic stimulation (4). In certain instances, lack of costimulation may lead to activation-driven cell death (8). Binding of surface receptors on T cells to their natural ligands, such as CD2 to LFA-3 (9), CD4 to MHC class II (9), LFA-1 to intercellular adhesion molecule 1 (ICAM-1) or ICAM-2 (10), and CD28 to B7/BB1 (11) have been implicated in facilitating T cell–APC interactions and inducing T cell activation. CD28 signaling stimulates cytokine production by T cells, by regulating gene transcription and also by stabilizing mRNAs (12–16). Binding of CD28 to the B7/BB1 counter receptor costimulates IL-2 mRNA accumulation and T cell proliferation (17–20). CD28-mediated signaling prevents induction of anergy in murine T cell clones (21).

CTLA-4, a molecule homologous to CD28 originally identified by screening of a murine cytolytic T cell cDNA library (22), also binds to B7/BB1 (23). Studies of the binding properties of CTLA-4 and B7/BB1 were facilitated by con-

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struction of a soluble fusion protein consisting of the extracellular domain of CTLA-4 and an IgG γ1 chain (23). CTLA4Ig has a high avidity for the B7/BB1 molecule (K_d ~12 nM) and is a potent inhibitor of immune responses in vitro and in vivo (23–25).

In this study, we have investigated the role of CD28 interactions with B7/BB1 in providing the costimulation necessary to maintain proliferative competence of human T cells. We have found that blocking the interaction of CD28 with B7/BB1 either by anti-CD28 mAb 9.3 Fab fragments or by CTLA4Ig when T cells are presented with alloantigen in a mixed leukocyte culture (MLC)[1] leads to sustained T cell hyporesponsiveness to the specific alloantigen.

Materials and Methods

Ig Fusion Proteins, mAbs, and Transfected Cell Lines. CTLA4Ig was produced by CHO cells transfected with the CTLA4Ig cDNA expression construct and was purified as described previously (23). Purified human mouse chimeric mAb L6 was a gift of Ingegerd and Karl Erik Hellstrom (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Murine mAbs 9.3 (anti-CD28, IgG2a), BB1 (anti-B7/BB1 antigen, IgM), 9E8 (anti-p15E, IgG2a), and T11D7, (anti-Thy1.1, IgM, kind gift of Irwin Bernstein, Fred Hutchinson Cancer Research Center) have been described previously and were purified from ascites before use (26–28). Preparation of Fab fragment of mAb 9.3 has been described previously (29). B7$^+$ CHO cells have been previously described (23) and CD5$^+$ CHO cells were constructed as described (19, 23) using an expressible CD5 cDNA provided by Dr. A. Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute).

Primary MLC. PBMC were prepared by density gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in medium containing RPMI 1640, 25 mM Hepes, 1 U/ml penicillin, 1 μg/ml streptomycin, and 15% pooled human serum that had been heat inactivated at 56°C for 30 min. As indicated for certain experiments, T cell subsets were purified by negative selection using complement-dependent lysis and panning (30). Responders and stimulators were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR antigen mismatched within each pair. 5×10^4 responder cells were mixed with 5×10^4 irradiated stimulator cells (3,000 rad) in round-bottomed 96-well plates. These were incubated at 37°C in a 5% CO_2 atmosphere. Assays were performed in triplicate. Cultures were pulsed with one μCi of [^{3}H]thymidine 18 h before harvesting. 10 replicate plates were set up and one was harvested each day for 10 consecutive d. Data are reported as mean cpm of the three replicates. In selected experiments, readings were taken on day 6 of the MLC.

Restimulation Assays. 10^7 PBMC from one individual were primed with an equivalent number of irradiated (3,000 rad) PBMC from another HLA class I- and II-incompatible individual in 25 cm^2 flasks, using identical culture conditions as for primary MLC carried out in 96-well plates. For blocking experiments, cells were cultured for 7 d in the presence of human Ig fusion proteins CTLA4Ig or human–mouse chimeric mAb L6, used as control. Then cells were washed three times, recultured in medium without Ig for an additional 3 d, harvested on day 10, and then restimulated. Primed cells were restimulated with fresh stimulator cells from the original donor or from an unrelated donor. The two donors did not share HLA-DR, DQ, or DP antigens. In experiments of tertiary stimulation, a secondary culture was carried out in flask, as in the first. As indicated in certain experiments, alloantigen-primed CD4$^+$ T cell lines were generated by stimulation with cells from an EBV-transformed B line from an unrelated donor. For the assay, 2×10^4 primed responders and 5×10^4 irradiated stimulators were incubated in 96-well round-bottomed wells in medium without any Ig fusion protein. Assays were performed as detailed for primary MLC.

Generation of CTL. Fresh PBMC or primed cells were tested for CTL precursor activity by priming in MLC. Responder cells (10^7) either fresh or primed as specified for each experiment, and irradiated stimulators (10^7), were cultured for 6 d, harvested, washed twice, and tested for cytolytic effector activity in a 4-h ^{51}Cr-release assay against PHA blasts. Both autologous or stimulator cells were tested as target cells. Maximum and spontaneous release values were obtained by incubating targets with 1% Triton X-100 and medium alone, respectively. Triplicate assays were carried out at E/T ratios of 25:1, 50:1, and 100:1 in V-bottomed 96-well plates. Data are reported as mean percent specific ^{51}Cr-release.

RNA Blot Analysis. RNA was prepared from T cells (~1–3 $\times 10^7$/sample) by a rapid isolation procedure (31). RNA (10 μg) was fractionated on formaldehyde agarose gels, transferred and cross-linked to Zetaprobe membranes (Bio-Rad Laboratories, Cambridge, MA). Probes for IL-2, IL-4, IFN-γ and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) have been previously described (19, 32, 33). DNA fragments were purified and labeled with ^{32}P using a random priming kit (Boehringer Mannheim Corp., Indianapolis, IN). The prehybridized membranes were sequentially hybridized with different ^{32}P-labeled probes. Between hybridizations, each probe was stripped from the blots by boiling in a solution of SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS.

Results

Induction of Antigen-specific Hyporesponsiveness by Fab Fragments of Anti-CD28 mAb 9.3. Monovalent Fab fragments of the anti-CD28 mAb 9.3 can block T cell proliferative responses to alloantigen in primary MLC, by preventing the interaction of CD28 with its natural ligand B7/BB1 expressed on APCs (12). To evaluate whether there is a long-lasting effect of blocking the CD28 receptor during the initial exposure to antigen, we performed restimulation experiments. Lymphocytes were cultured with alloantigen for 7 d in separate flasks in medium containing 5 μg/ml Fab of 9.3 mAb or control mAb. Cells were then washed to remove mAb, cultured in fresh medium for an additional 3 d, and then restimulated with irradiated PBMC from either the original donor (Fig. 1, *left*) or from a third party donor (Fig. 1, *right*) in medium without mAb. Cells primed in the presence of control mAb and restimulated with PBMC originally used for priming showed a typical accelerated secondary proliferative response peaking on day 3. In contrast, those same primed cells showed a typical primary response, peaking on day 6, when stimulated with PBMC from a third party donor. Cells primed in the presence of 9.3 Fab, however, showed a decreased response when challenged with PBMC from the original donor, yet responded normally to PBMC from a third party donor. These results demonstrate that the secondary proliferative response of human T cells can be inhibited in

[1] *Abbreviations used in this paper:* CTLp, cytolytic precursor; MLC, mixed leukocyte culture.

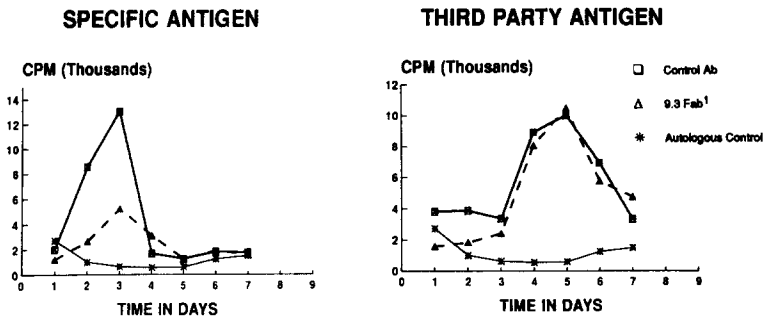

Figure 1. Fab fragments of anti-CD28 mAb 9.3 induce T cell hyporesponsiveness in MLC. For the secondary MLR, responders from a normal individual were primed with cells from an HLA-incompatible donor in the presence of 9.3 mAb Fab (*triangle*) or control (*square*). Cells were restimulated from the original donor (*left*) or a third party donor (*right*) in the absence of mAb. Cells primed in the absence of mAb were restimulated against autologous cells to define the background for the assay (*).

an antigen-specific manner by blocking CD28 during the primary exposure to alloantigen.

Inhibition of T Cell Responses to Alloantigen by CTLA4Ig. To determine whether antigen-specific hyporesponsiveness could also be induced by blocking B7/BB1, the natural ligand for CD28 expressed on APC, further experiments evaluated the activity of CTLA4Ig, a fusion protein with high affinity for B7/BB1. Both responder and irradiated stimulator cells were preincubated with CTLA4Ig or control Ig for 30 min at 37°C before mixing. CTLA4Ig inhibited primary alloproliferative responses by 50–85%, and maximal inhibition was seen at or above 2.5 μg/ml of CTLA4Ig (data not shown), consistent with previous findings (23). mAb BB1 (26), a murine IgM antibody that binds to the B7/BB1 antigen with lower avidity than CTLA4Ig (23) inhibited MLR by only ~30%. Thus, CTLA4Ig inhibited primary T cell responses more efficiently than mAb BB1, although the inhibition achieved was not complete.

Previous studies had shown that $CD4^+/CD28^+$ T cells constitute 95–99.5% of $CD4^+$ peripheral blood T cells and proliferate vigorously to HLA class II determinants in MLR (34), whereas $CD4^+/CD28^-$ T cells constitute 0.5–5.0% of all $CD4^+$ T cells and respond poorly in MLR (35), and $CD8^+$ T cells do not proliferate at all in human MLR. By flow microfluorimetric analysis we found that $CD4^+$ cells constituted 79% of viable lymphocytes on day 6 of an MLR carried out in the presence of control Ig compared with 56% in the presence of CTLA4Ig, and $CD28^+$ cells constituted 72% of viable lymphocytes after an MLR carried out in the presence of control Ig compared with 56% in the presence of CTLA4Ig. Thus, CTLA4Ig blocked the increase in the proportion of $CD4^+$ and $CD28^+$ cells during MLR. Since requirements for proliferation are more stringent in naive than in memory cells, one expected MLR response of naive cells to be more susceptible to inhibition by CTLA4Ig than MLR response of memory cells. $CD4^+/CD45RA^+$ (naive) and $CD4^+/CD45RO^+$ (memory) T cell subsets were purified by negative selection, through panning of PBMC obtained from adult volunteers, and tested in MLR. CTLA4Ig inhibited thymidine uptake of $CD4^+/CD45RA^+$ cells by 84% and $CD4^+/CD45RO^+$ cells by 74%. As an alternative source of naive T cells, mononuclear cells were obtained from umbilical cord blood and tested in MLR. CTLA4Ig inhibited thymidine uptake of cord blood cells stimulated by irradiated PBMC obtained from an unrelated adult by 78%. These results indicate that CTLA4Ig can inhibit proliferative responses to HLA class II determinants in either naive or memory T cells with the $CD4^+$ and $CD28^+$ phenotype. However, in no T cell subset analyzed was the inhibition complete.

Effect of CTLA4Ig on Lymphokine Production. Proliferative T cell responses to alloantigen occurring despite the presence of CTLA4Ig might not be driven by IL-2, but rather by IL-4. Steady state message for IL-2, IL-4, and IFN-γ was measured in mRNA prepared from proliferative $CD4^+$ T cell lines stimulated by specific alloantigen in the presence or absence of CTLA4Ig (Fig. 2). Transcripts for IL-2 and IFN-γ were lower in mRNA from cells cultured with CTLA4Ig compared with control cells. In contrast, transcripts for IL-4 peaked at 4 h after stimulation and were detected at similar levels in mRNA from cells cultured with or without CTLA4Ig. Thus, IL-2 and IFN-γ transcripts do not accumulate in T cells stimulated by alloantigen when B7/BB1 is blocked by CTLA4Ig, whereas IL-4 transcripts do accumulate. Therefore, IL-4 could drive antigen-specific T cell proliferation which occurs despite blocking by CTLA4Ig.

Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. To evaluate the effect of CTLA4Ig on secondary responses,

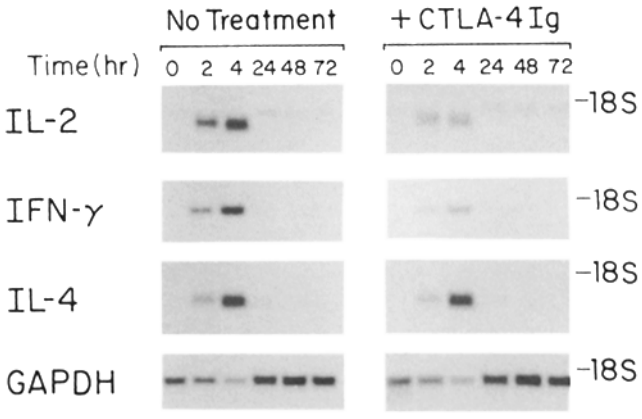

Figure 2. Regulation of lymphokine transcripts by CTLA4Ig. Resting alloantigen-primed $CD4^+$ T cells (2×10^7) were collected and restimulated with irradiated lymphoblastoid cells. Cells were harvested at the indicated times, RNA was extracted and analyzed by blot analysis. The blot was sequentially hybridized with ^{32}P-labeled probes for IL-2, IL-4, IFN-γ and GAPDH, as described in Material and Methods. Migration positions are noted of the 28S and 18S ribosomal RNA species visualized by ethidium bromide staining.

lymphocytes were cultured with alloantigen for 7 d in medium containing 5 μg/ml CTLA4Ig or control Ig. Cells were then washed to remove Ig, cultured in fresh medium for an additional 3 d, and then restimulated with irradiated PBMC from either the original donor (Fig. 3, *top left*) or from a third party donor (Fig. 3, *bottom left*) in medium without Ig. Cells primed in the presence of CTLA4Ig showed a decreased response when challenged with PBMC from the original donor, yet responded normally to PBMC from a third party donor. Flow microfluorimetric analysis of $CD4^+$ cells on day 3 of the secondary cultures indicated that expression of the IL-2 receptor α chain (CD25) was lower in CTLA4Ig-treated cultures than in controls (data not shown). Antigen-specific hyporesponsiveness was achieved with as low as 1 μg/ml of CTLA4Ig in the priming culture, but there was no effect on responsiveness to third party donors even at a CTLA4Ig concentration of 10 μg/ml (data not shown). Hyporesponsiveness was demonstrated in cells cultured with alloantigen in the presence of CTLA4Ig for 7 d, and then rested in medium alone for 20 d and 27 d after initiation of the culture (Fig. 3). In six experiments using different pairs of responder and stimulator cells, primary MLC in the presence of CTLA4Ig inhibited the secondary proliferative responses to the specific alloantigens by an average (±SD) of 70 ± 13%, whereas responses to third party donors were unaffected (4 ± 3% inhibition). Secondary proliferative responses to specific alloantigen were inhibited by an identical degree, if the primary cultures were carried out in the presence of either CTLA4Ig (84% inhibition) or anti-CD28 mAb Fab (83% inhibition), but no greater inhibition was achieved by a combination of the two (84% inhibition). These results demonstrate that secondary proliferative responses can be specifically inhibited by primary exposure of T cells to alloantigen in the presence of either anti-CD28 mAb Fab fragments or CTLA4Ig, and are consistent with the model that both agents block the same pathway of T cell activation.

Effect of CTLA4Ig on Responsiveness of Primed Cells. Further experiments were designed to determine whether alloantigen-specific hyporesponsiveness could be induced by CTLA4Ig in primed cells. Cells were primed to alloantigen in medium without CTLA4Ig or control for 10 d (Fig. 4, *top left*). Cells were then washed and restimulated with irradiated cells from the original donor. Both responder and stimulator cells were incubated with CTLA4Ig or control Ig for 30 min at 37°C before mixing. The secondary proliferative response was inhibited by CTLA4Ig compared with the Ig control (Fig. 4, *top right*). In separate cultures set up in flasks, primed cells were restimulated with PBMC from the original donor in the presence of CTLA4Ig or control Ig for 7 d, washed to remove the Ig, and rested in medium for

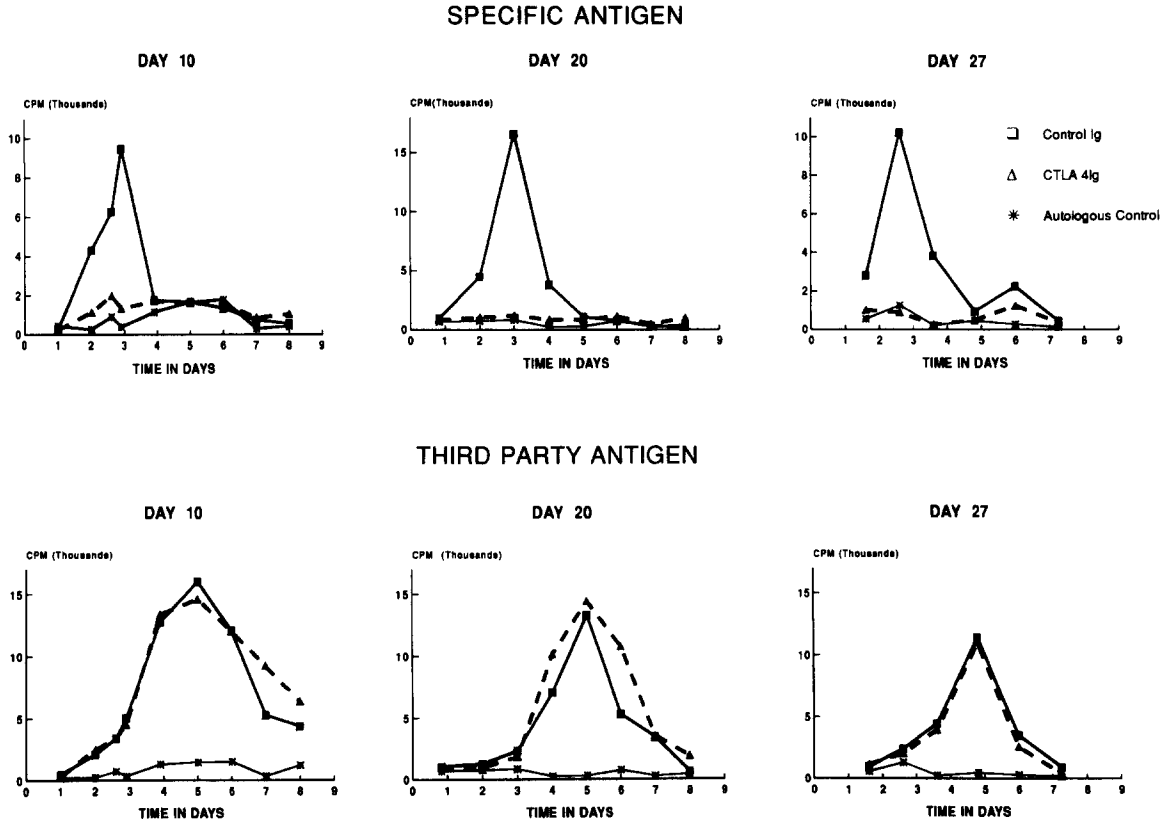

Figure 3. CTLA4Ig induces antigen-specific hyporesponsiveness in unprimed cells. Responders from a normal individual were primed with cells from an HLA-incompatible donor in the presence of CTLA4Ig (*triangle*) or control Ig (*square*). At the indicated time points, primed cells were restimulated from the original donor (*top*) or a third party donor (*bottom*) in the absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*).

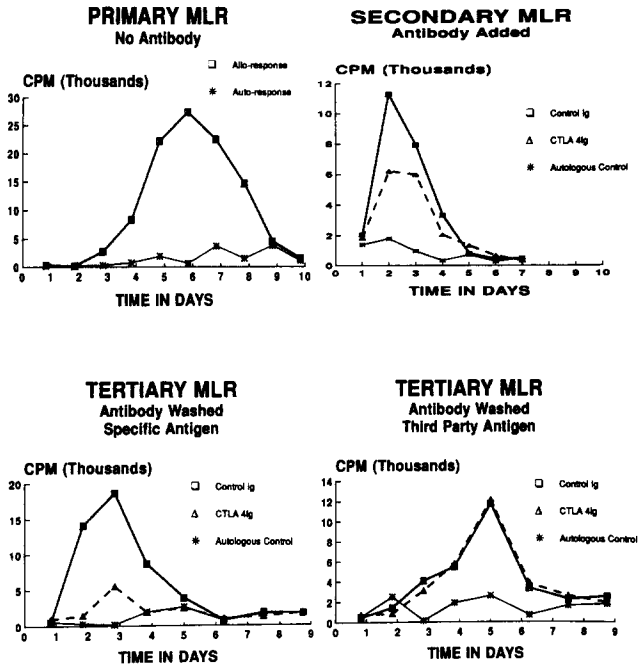

Figure 4. CTLA4Ig induces antigen-specific hyporesponsiveness in primed cells. PBMC were primed with allogeneic stimulators (*square*) in absence of Ig (*top left*; [*] autologous stimulation). Primed cells were restimulated in secondary MLR with cells from the original donor (*top right*) in the presence of CTLA4Ig (*triangle*) or control Ig (*square*). Cells from the respective cultures were washed and then restimulated in a tertiary MLR (*bottom*) with cells from the original donor (*left*) or a third party donor (*right*) in absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*).

an additional 3 d. They were then stimulated again in a tertiary culture with specific or third party alloantigen. Cells preincubated with control Ig exhibit a typical anamnestic response when restimulated with cells from the original donor. In contrast, cells preincubated with CTLA4Ig showed a diminished response to cells from the original donor (Fig. 4, *bottom left*), whereas their response to a third party donor was unaffected (Fig. 4, *bottom right*). These findings indicate that primed cells can also become hyporesponsive if exposed to alloantigen in the presence of CTLA4Ig.

Kinetics of Induction of Hyporesponsiveness by CTLA4Ig. To determine the duration of exposure to CTLA4Ig necessary for development of hyporesponsiveness, cells were washed on days 1, 2, or 3 of primary MLC, resuspended in fresh medium without Ig, and rested until day 10 when they were restimulated with irradiated PBMC from the original donor or from a third party donor. Primary MLC in the presence of CTLA4Ig for 2 or 3 d inhibited the secondary response to the original donor >80%, but had no effect on the response to third party donors. Primary MLC in the presence of CTLA4Ig for 1 d inhibited the secondary response to the original donor by only ~15%. Therefore, maximum induction of antigen-specific hyporesponsiveness is achieved in MLC after 2 d of incubation with CTLA4Ig.

Effect of IL-2 on Hyporesponsive Cells. Hyporesponsiveness in secondary MLR could be due to the death of antigen-specific T cells occurring during the primary culture or to the acquisition of a defect in one of the cellular functions that limits the rate of cell proliferation, such as IL-2 production. Addition of exogenous IL-2 to secondary cultures could help determine whether IL-2-responsive, antigen-specific T cells were still alive. Primary MLCs were set up in medium containing CTLA4Ig or control Ig. When challenged with PBMC from the original donor, cells primed in the presence of CTLA4Ig showed a lower response (Fig. 5, *center*) than cells primed in the presence of control Ig (Fig. 5, *left*), yet responded equally well to PBMC from a third party donor. Exogenous rIL-2 added at 10 IU/ml to secondary cultures restored responsiveness to specific alloantigen of cells primed in the presence of CTLA4Ig (Fig. 5, *right*). These results indicate that presentation of antigen while blocking interaction of CD28 with B7/BB1 can induce a state of T cell hyporesponsiveness to antigen which can be corrected by exogenous IL-2.

Effect of IL-2 on Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. CD28 signaling concurrent with TCR engagement results in IL-2 secretion, T cell activation and proliferation. Therefore, we tested whether exogenous IL-2 could provide T cells with a signal that could bypass the block provided by CTLA4Ig in the primary MLC and prevent induction of antigen-specific hyporesponsiveness. Primary MLCs were set up with CTLA4Ig, with or without rIL-2 at 10 IU/ml

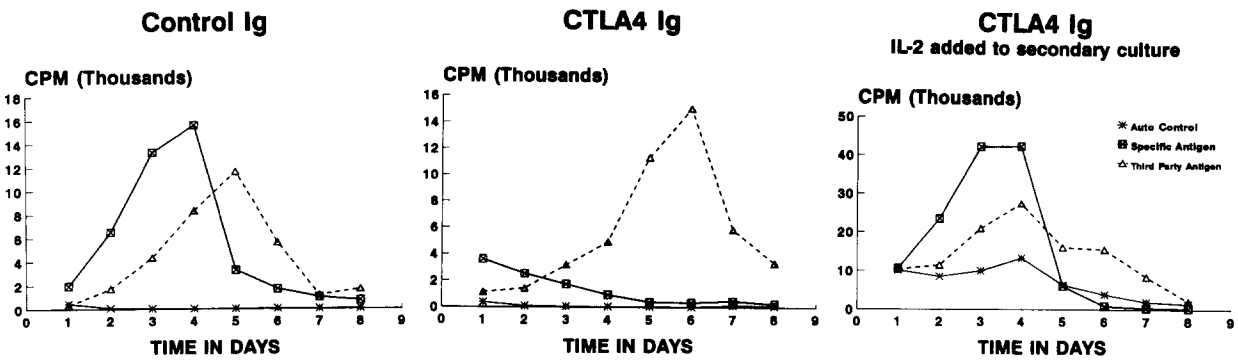

Figure 5. Exogenous IL-2 restore responsiveness to specific antigen. Cells were primed to alloantigen in the presence of control Ig (*left*) or CTLA4Ig (*center* and *right*). Primed cells were restimulated with autologous cells (*), cells from the original donor (*square*) or a third party donor (*triangle*) in the absence of Ig. Cells primed in the presence of CTLA4Ig were restimulated in medium (*center*) or 10 μ rIL-2 (*right*).

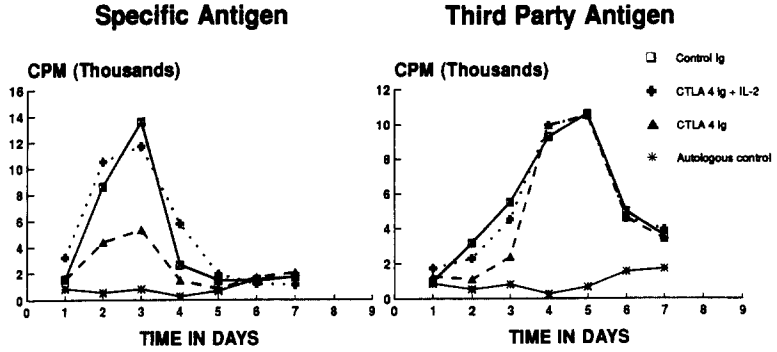

Figure 6. Exogenous IL-2 prevents induction of antigen-specific hyporesponsiveness by CTLA4Ig. Cells were primed in the presence of CTLA4Ig (*triangle*), or control Ig (*square*), or CTLA4Ig plus IL-2 (*cross*). Primed cells were restimulated with cells form the original donor (*left*) or a third party donor (*right*) in the absence of Ig or IL-2. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*).

added at the initiation of the culture or control Ig. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness when restimulated with the specific alloantigen (Fig. 6, *left*). However, cells primed in the presence of CTLA4Ig plus rIL-2 showed the same degree of secondary response to the specific stimulators as cells primed in the presence of control Ig. Neither CTLA4Ig nor rIL-2 affected secondary responses to cells from third party donors (Fig. 6, *right*). These results indicate that antigen-specific hyporesponsiveness induced by priming T cells in the presence of CTLA4Ig in MLC can be prevented by stimulation with exogenous IL-2.

Effect of Anti-CD28 mAb 9.3 or Cell-bound B7/BB1 Receptor on Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. In contrast to monovalent Fab fragments of anti-CD28 mAb 9.3, the bivalent intact mAb 9.3 can crosslink CD28 molecules and activate T cells efficiently (29). Therefore, we tested whether the intact mAb 9.3 could deliver a signal to T cells and prevent induction of hyporesponsiveness by CTLA4Ig. Primary MLCs were set up with CTLA4Ig with or without mAb 9.3 or controls. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness, compared with cells primed in the presence of control Ig alone (not shown) or control Ig plus mAb 9.3, when restimulated with the specific alloantigen (Fig. 7, *left*). However, cells primed in the presence of CTLA4Ig plus mAb 9.3 showed the same degree of secondary response to the specific stimulators as was shown by cells primed in the presence of control Ig and mAb 9.3. Neither CTLA4Ig nor mAb 9.3 affected secondary responses to cells from third party donors (Fig. 7, *right*).

Further experiments tested the effect of exogenous B7/BB1 antigen expressed on transfected CHO cells. Irradiated (10^4 rad) $B7^+$ CHO cells (19) were mixed with fresh responder PBMC at a ratio of 1:100, before addition of CTLA4Ig or control Ig and irradiated stimulator PBMC. MLC without CHO cells but with CTLA4Ig or control Ig alone were set up in parallel. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness to specific alloantigen when compared with cells primed in the presence of control Ig. Cells primed in the presence of CTLA4Ig and the negative control $CD5^+$ CHO cells also showed hyporesponsiveness. In contrast, cells primed in the presence of CTLA4Ig and $B7^+$ CHO cells showed the same degree of secondary response to the specific stimulators, as was shown by cells primed in the presence of control Ig and no CTLA4Ig (data not shown). Neither CTLA4Ig nor transfected CHO cells affected secondary responses to cells from third party donors (data not shown). These results indicate that antigen-specific hyporesponsiveness induced by priming T cells in the presence of CTLA4Ig in MLC can be prevented by stimulating CD28 with mAb 9.3 or with the natural ligand B7/BB1.

Effect of CTLA4Ig on CTL Generation. CTLA4Ig did not inhibit the effector phase of the cytolytic reaction by activated CTL against allogeneic target T cell blasts (data not shown). To determine whether CTLA4Ig added to the primary MLC could block the generation of CTL activity, MLCs were set up in medium containing CTLA4Ig or control Ig for 5 d. Cells primed in the presence of CTLA4Ig showed a fourfold decrease in cytolytic activity against allogeneic target T cell blasts when compared with cells primed in the presence of control Ig (data not shown). To determine whether the block in the generation of CTL activity by CTLA4Ig was specific, MLCs were set up in medium containing

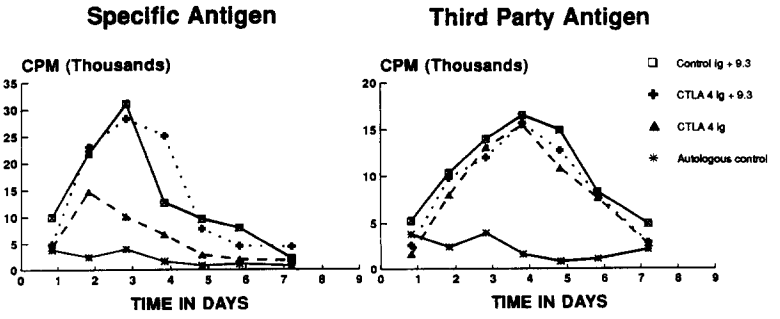

Figure 7. Intact mAb 9.3 blocks induction of antigen-specific hyporesponsiveness by CTLA4Ig. Cells were primed in the presence of CTLA4Ig (*triangle*), or control Ig plus mAb 9.3 (*square*), or CTLA4Ig plus mAb 9.3 (*cross*). Primed lymphocytes were restimulated with cells from the original donor (*left*) or a third party donor (*right*) in the absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*). This experiment is representative of two other experiments of similar design that achieved identical results.

CTLA4Ig or control Ig for 7 d. Cells were washed and recultured in fresh medium without CTLA4Ig for 3 d. Cells were then restimulated with irradiated PBMC from the original donor or from a third party donor for 3 d and then tested for cytolytic activity. Cells previously primed in the presence of CTLA4Ig again showed a fourfold decrease in cytolytic activity against specific alloantigen (Fig. 8, *left*) when compared with cells primed in the presence of control Ig. In contrast, cells previously cultured in the presence of CTLA4Ig were able to generate cytotoxic activity against a third party donor to the same degree as cells cultured with control Ig (Fig. 8, *right*). Thus, CTLA4Ig inhibited the generation of specific CTL.

Discussion

Our study demonstrates that long-lasting, antigen-specific hyporesponsiveness can be induced in T cells by exposure to alloantigen while blocking the interaction of CD28 on T cells with B7/BB1 on allogeneic APC. Effective blockade could be achieved using either monovalent anti-CD28 mAb 9.3 fragments or CTLA4Ig, a soluble recombinant fusion product of human CTLA-4 and IgG γ1 chain, the binds to B7/BB1 with high avidity. Previous reports had shown that anti-CD28 mAbs augment proliferation of human T cells in the presence of specific antigen and defective APCs (16). Further work showed that interaction of CD28 with B7/BB1 provides a costimulatory signal for T cell activation (17–20). Data from Schwartz and other investigators (4–7, 36) indicated that in the absence of costimulatory signals provided by the APC, T cells encountering specific antigen enter a state of anergy characterized by an IL-2 production defect. Harding et al. (21) demonstrated that CD28 signaling can prevent anergy in murine T cell clones. Our data are consistent with the model that the CD28–B7/BB1 interaction can provide a costimulus required for T cell activation. CD28 ligation is required for IL-2 gene activation in antigen-specific responses. Blocking CD28 ligation by either 9.3 mAb Fab or CTLA4Ig may inhibit IL-2 expression and elicit a state of T cell hyporesponsiveness.

Human MLR experiments allowed us to study the requirements for antigen-specific responses of $CD4^+/CD28^+$ cells, since proliferation of $CD4^+/CD28^-$ cells and $CD8^+$ cells cannot be detected in this model system. CTLA4Ig blocked proliferation of $CD4^+/CD28^+$ in primary MLC and achieved a similar degree of inhibition in naive and memory T cells. A state of antigen-specific hyporesponsiveness could be induced by CTLA4Ig in primed as well as in unprimed cells. Secondary proliferative responses to the specific alloantigen were decreased, but not abolished by the presence of either anti-CD28 mAb 9.3 Fab or CTLA4Ig in primary cultures. Residual T cell responsiveness hardly could be explained by incomplete blocking of the CD28 or B7/BB1 receptors, since anti-CD28 mAb Fab and CTLA4Ig used together did not demonstrate additive inhibitory activity. T cell proliferation could be induced through CD28-independent activation pathways initiated by other accessory receptors, such as ICAM-1 (10). The heat-stable antigen also functions as a costimulatory molecule and regulates T cell responsiveness (37). CTLA4Ig blocked antigen-mediated activation of IL-2 and IFN-γ expression, but did not block IL-4 expression. Thus, IL-4 might be responsible for driving T cell proliferation in primary or secondary MLR. Further experiments will need to address whether neutralization of IL-4 in culture can achieve complete T cell unresponsiveness in this model.

Exogenous IL-2 could restore antigen-specific proliferative responses in secondary cultures, suggesting that hyporesponsiveness was not the result of T cell death but, instead, was likely the result of an acquired T cell defect in IL-2 production. Whether blocking alloantigen-mediated T cell activation by CTLA4Ig can induce a sustained defect in IL-2 production remains to be verified. Exogenous IL-2 added at the beginning of the primary MLC prevented induction of antigen-specific hyporesponsiveness by CTLA4Ig in this study. This finding contrasts with the observation in the model using murine T cell clones stimulated in absence of accessory cells, where exogenous IL-2 cannot prevent hyporesponsiveness. As opposed to the murine model, MLR cultures contain accessory cells and with them an indefinite number of stimuli that could make less stringent the requirements for T cell activation.

Both CD28 and CTLA-4 are natural ligands for B7/BB1, a receptor expressed on activated B lymphocytes and other APCs (38–41). Once expressed, B7/BB1 interacts with CD28 and CTLA-4 to provide a stimulus for T cell activation (18, 42). The time required for B7 expression, 16–24 h after B cell activation (39), can explain why T cell hyporesponsive-

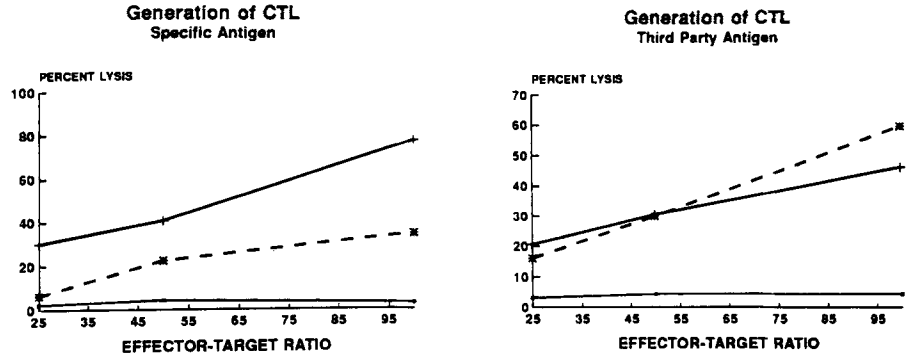

Figure 8. CTLA4Ig inhibits CTL generation. Responder cells were primed in the presence of CTLA4Ig (*) or control Ig (*cross*). Primed cells were restimulated with cells from the original donor (*left*) or from a third party donor (*right*) in the absence of Ig and cytotoxic activity was assayed on day 3 of the secondary cultures against PHA stimulated T lymphoblasts from the respective donors. Lysis of autologous targets (*square*) by cells primed in absence of Ig define the background for the assay. This experiment is representative of two other experiments of similar design that achieved identical results.

ness is not completely achieved in MLC by 24 h but requires 48 h of incubation with CTLA4Ig. CD28 ligation by the whole anti-CD28 mAb 9.3 could prevent induction of hyporesponsiveness by CTLA4Ig in MLC. Hyporesponsiveness was also prevented by mixing irradiated $B7^+$ CHO cells with responder lymphocytes at the initiation of the MLC before adding CTLA4Ig. Prevention of hyporesponsiveness by $B7^+$ CHO cells may be achieved by direct stimulation of T cells through CD28 or by neutralization of soluble CTLA4Ig. We favor the former hypothesis since $B7^+$CHO cells were used at a very low frequency in the culture (1:100:100, $B7^+$ CHO cells/responders/stimulators). Under these experimental conditions, we calculated that the concentration of CTLA4Ig in the culture exceeded the concentration of the B7/BB1 receptor on the surface of CHO cells by at least 100-fold on a molar basis. Thus, it was unlikely that CTLA4Ig could be neutralized by $B7^+$ CHO cells. The role of the CTLA-4 receptor in the achievement of T cell hyporesponsiveness has not been addressed directly in our studies. However, since blocking CD28 by 9.3 mAb Fabs induced a level of hyporesponsiveness comparable to blocking B7/BB1 by CTLA4Ig, and since triggering CD28 by the intact 9.3 mAb could completely overcome hyporesponsiveness induced by CTLA4Ig, it is unlikely that signaling by CTLA-4 per se is of major importance in regulating T cell responses.

CTLA4Ig not only blocked primary and secondary proliferative responses but also blocked activation of cytolytic precursors (CTLp). Cells exposed to alloantigen in the presence of CTLA4Ig were found to generate markedly diminished specific cytolytic activity. These results suggest that generation of cytolytic activity in the primary culture in the presence of CTLA4Ig was an unlikely explanation for the hyporesponsiveness in secondary culture. Recent findings indicate that CD28 interaction with B7/BB1 can amplify T cell–mediated cytolysis at the effector phase (43). In our experiments, however, there was no interference of CTLA4Ig at the lytic stage, probably because activated T cell targets do not express B7/BB1. It remains to be determined whether CTLA4Ig blocked CTLp activation directly or indirectly by inhibiting Th cell functions.

Defining the role of the interaction between CD28 and B7/BB1 and between other T cell accessory receptors and their natural ligands will help understand the mechanisms for self-tolerance, propose new strategies to manipulate the immune response, and achieve transplantation tolerance (24, 25, 44).

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