B7 But Not Intercellular Adhesion Molecule-1 Costimulation Prevents the Induction of Human Alloantigen-specific Tolerance

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Summary

Presentation of antigen by the major histocompatibility complex to T lymphocytes without the requisite costimulatory signals does not induce an immune response but rather results in a state of antigen-specific unresponsiveness, termed anergy. To determine which costimulatory signals are critical for the T cell commitment to activation or anergy, we developed an in vitro model system that isolated the contributions of alloantigen and each candidate costimulatory molecule. Here, we show that transfectants expressing HLA-DR7 and either B7 or intercellular adhesion molecule 1 (ICAM-1) deliver independent costimulatory signals resulting in alloantigen-induced proliferation of CD4-positive T lymphocytes. Although equivalent in their ability to costimulate maximal proliferation of alloreactive T cells, B7 but not ICAM-1 induced detectable interleukin 2 secretion and prevented the induction of alloantigen-specific anergy. These results are consistent with the hypothesis that blockade of the ICAM-1:lymphocyte function–associated 1 pathway results in immunosuppression, whereas blockade of the B7:CD28/CTLA4 pathway results in alloantigen-specific anergy. This approach, using this model system, should facilitate the identification of critical costimulatory pathways which must be inhibited in order to induce alloantigen-specific tolerance before human organ transplantation.

The success of human allogeneic organ transplantation, which is presently dependent upon toxic nonspecific immunosuppression, would be improved substantially if it were possible to induce long-lasting specific tolerance to alloantigen. One approach to achieve this objective is to present alloantigen to T lymphocytes in the absence of requisite costimulatory signal(s). This therapeutic concept is based on considerable evidence that demonstrates that T lymphocytes must receive two signals from APCs to induce an antigen-specific response (1–5). The first signal, which confers antigen specificity, requires the TCR complex to recognize foreign antigenic peptide presented in association with the MHC complex on the surface of APCs. This interaction between the TCR complex and antigen MHC is necessary but not sufficient for T cell activation (6). The outcome of TCR engagement is determined by additional signals, termed costimulatory, provided by one or more cell surface molecules expressed on APCs (7). These costimulatory signals are neither antigen specific, nor MHC restricted, yet they are critical for the induction of maximal T cell proliferation, cytokine secretion, and effector function. Therefore, when antigen is presented by MHC without appropriate costimulation, functional unresponsiveness of antigen-specific T lymphocytes is induced (1, 2, 4, 8).

A number of ligands expressed on APCs and their receptors expressed on T cells are candidates for critical costimulatory signals including: B7:CD28/CTLA4 (9–14); intercellular adhesion molecule 1 (ICAM-1)[1] (CD54), -2, -3: LFA-1 (CD11a/CD18) (15–20); LFA-3 (CD58): CD2 (21–23); CD40: CD40 ligand (24, 25); and heat stable antigen (CD24): heat stable antigen (CD24) (26). Although each of these ligand: receptor pairs have been reported to have costimulatory potential, their relative importance in regulating alloantigen-mediated T cell proliferation, lymphokine production, and/or in preventing the induction of tolerance remains unresolved. Previous studies (17, 19, 27) have demonstrated that the ICAM-1:LFA-1 ligand-receptor pair can costimulate T cell proliferation and that inhibition of this pathway can prolong murine allograft survival (28). In contrast, recent studies demonstrate the

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[1] *Abbreviations used in this paper:* allo-APC, alloantigen-presenting cell; CsA, Cylosporin A; ICAM-1, intercellular adhesion molecule 1; IM, calcium ionophore Ionomycin; LBL, lymphoblastoid B cell line; mito-C, mitomycin-C; MLR, mixed lymphocyte reaction.

B7:CD28/CTLA4 ligand-receptor pair not only induces T cell proliferation and IL-2 production (10, 11), but also prevents the induction of antigen-specific tolerance (29, 30). Two recent murine in vivo experiments corroborate the apparent significance of this costimulatory pathway since blockade of the B7:CD28/CTLA4 pathway results in long-lasting tolerance to human xenoantigen and prolongation of murine cardiac allograft survival (31, 32).

In light of the large number of candidate costimulatory molecules and their potential to prevent the induction of tolerance, we have developed an in vitro system that evaluates the capacity of ligands to costimulate alloantigen-induced T cell recognition and, more importantly, to determine whether these costimulatory molecules can prevent the induction of alloantigen-specific tolerance. In the studies reported below, we demonstrate that although both B7 and ICAM-1 are equally potent costimulators of T cell proliferation, only B7 costimulation prevents the induction of alloantigen-specific anergy. These results are consistent with the notion that blockade of the ICAM-1:LFA-1 pathway results in inhibition of primary response to alloantigen, whereas blocking the B7:CD28/CTLA4 costimulatory pathway results both in inhibition of primary response and in specific tolerance to alloantigen.

Materials and Methods

Human T Cells. PBMC were isolated from HLA-DR7 and DR1-negative healthy donors by density gradient centrifugation on Ficoll-Hypaque. Monocytes were depleted by adherence on plastic. The CD4$^+$ T cell population was further enriched by separation from residual monocytes, B cells, NK cells, and CD8$^+$ T cells by mAb and complement lysis, using anti-CD14 (Mo2), anti-CD11b (Mo1), anti-CD20 (B1), anti-CD16 (3G8), and anti-CD8 (7PT 3F9) mAbs. The efficiency of the purification was analyzed in each case by flow cytometry (Epics Elite; Coulter Electronics, Hialeah, FL), using anti-CD3, anti-CD4, anti-CD8, and anti-CD14 mAbs followed by FITC-conjugated goat anti-mouse Ig (Fisher Scientific Co., Pittsburgh, PA). The final cell preparation was always >99% CD3$^+$, >99% CD4$^+$, <1% CD8$^+$, and <1% CD14$^+$. Before use, cells were resuspended in complete culture medium consisting of RPMI 1640 with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and gentamicin sulfate (5 μg/ml).

Monoclonal Antibodies and Fusion Proteins. mAbs were used as purified Ig unless indicated otherwise: anti-B7 (mAb 133): IgM (33); anti-CD20: B1, IgG2a (34); anti-B5: IgM (35); anti-CD19: B4, IgG1 (36); anti-CD8: 7PT 3F9, IgG2a; anti-CD11b: Mo1, IgM and anti-CD14: Mo2, IgM (37); anti-MHC class II: 9–49, IgG2a (Dr. R. Todd, University of Michigan, Ann Arbor, MI) (38); anti-CD28: 9.3, IgG2a (Dr. C. June, Naval Research Institute, Bethesda, MD) (39); anti-ICAM-1: RR1.1, IgG1 (40) and anti-LFA-1a: TS1/22, IgG1 (41) (Dr. T. Springer, Center for Blood Research, Boston, MA); and anti-CD16: 3G8, IgG1 (used as ascites) (Dr. J. Ritz, Dana Farber Cancer Institute). Anti-CD28 Fab fragments were generated in our laboratory from the 9.3 mAb, by papain digestion and purification on a protein A column, according to the manufacturer's instructions (Pierce, Rockford, IL). Human CTLA4-Ig was prepared as previously described (30). Control fusion protein (control Ig), was identically constructed, except using a recombinant human–mouse chimeric mAb in which the H and

L chain variable domains were derived from a murine anti-HIV envelope mAb, and the remainder of the chimeric mAb was derived from the identical human IgG1 sequences used to construct CTLA4-Ig. The chimeric control mAb was purified on an immobilized protein A column and did not react with either human or murine lymphocytes as measured by flow cytometry.

NIH 3T3 Cell Transfection. The MHC class II–negative, B7-negative murine fibroblast cell line NIH 3T3, was transfected by electroporation with either DRα and DR7β chain (t-DR7), B7 (t-B7), ICAM-1 (t-ICAM-1), DRα, DR7β chain and B7 (t-DR7/B7) or DRα, DR7β chain and ICAM-1 (t-DR7/ICAM-1) genes. The preparation of t-DR7, t-B7, and t-DR7/B7 transfectants has been previously described (30). t-mock cells were constructed by transfecting the SV2-Neo-Sp65 plasmid only. For ICAM-1 transfection, a plasmid encoding human ICAM-1 in the pH3M vector ([27], kindly provided by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA) was used. For each transfection, 20 μg of SfiI linearized ICAM-1 plasmid and 5 μg of ClaI linearized pPGK-Hygro were electroporated into NIH 3T3 cells or into t-DR7 cells. After selection in media containing 400 μg/ml Hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) for t-ICAM-1 or 200 μg/ml G418 (GIBCO BRL, Gaithersburg, MD) and 400 μg/ml Hygromycin B for t-DR7/ICAM-1, cells were sorted for ICAM-1 and DR expression by indirect immunofluorescence, cultured for 4 wk, resorted, and cloned by limiting dilution. Transfectants expressing similar levels of DR and/or ICAM-1 were selected for use. The expression of ICAM-1 was assessed using the RR1.1 mAb. The expression of MHC class II and B7 were assessed using the mAbs 9-49 and 133, respectively. These cells are referred to as artificial alloantigen-presenting cells (allo-APCs) throughout the text. Before use, allo-APCs were treated with 10 μg/ml mitomycin-C (mito-C) (Sigma Chemical Co., St. Louis, MO), at 37°C for 4 h, washed extensively, and resuspended in complete culture medium. The required number of cells was added to the appropriate wells in 96-well flat bottomed microtiter plates (Nunc, Roskilde, Denmark) and incubated overnight at 37°C in 5%, before the addition of the responder T cells.

Primary Mixed Lymphocyte Reaction (MLR). To determine the appropriate numbers of NIH 3T3 transfected cells bearing the allogeneic class II molecule (t-DR7) required to induce a submitogenic signal for T cell activation, a dose–response curve was performed using 10^5 CD4$^+$ T cells/well as responders, and progressively, increasing numbers of mito-C–treated t-DR7 cells as stimulators. The dose of 5 × 10^4 cells/well was the highest number of t-DR7 that did not induce allogeneic T cell proliferation. All primary MLRs were performed using 5 × 10^4 stimulators/well. For experiments with Cyclosporin A (CsA) (Sandoz Pharmaceuticals, East Hanover, NJ) used at a final concentration of 10^{-6} M, T cells were incubated with CsA for 10 min at 4°C before the initiation of culture. When indicated, stimulator NIH 3T3 cells were incubated for 30 min at 4°C with either 10 μg/ml anti-MHC class II, anti-B7, or anti-ICAM-1 mAbs, or 10 μg/ml CTLA4-Ig or control-Ig before addition to the experimental plates. Similarly, responder CD4$^+$ T cells were incubated with 15 μg/ml anti-CD28 Fab or 10 μg/ml anti-LFA-1, for 30 min at 4°C. To assess the kinetics of primary response, eight identical plates for each condition were set up and harvested daily from day 1 to 8 after the initiation of culture. For experiments with mixed populations of t-DR7 and t-ICAM-1 or t-B7 cells as stimulators, equal numbers of the two different types of transfectants were mixed and the indicated numbers of cells were used as stimulators.

Secondary Stimulation. After the appropriate time for maximal response in a primary MLR (7 d of culture with t-DR7 or t-DR7/

B7 cells and 5 d of culture with t-DR7/ICAM-1 cells), viable alloreactive T cell blasts were isolated by Percoll gradient in the 40–50% fraction, washed extensively, and cultured in media for 24 h. Each T cell population was subsequently rechallenged with (a) each one of the primary stimulators; (b) an irrelevant stimulator cell population not expressing HLA-DR7 antigen (PBMC from a DR1 homozygous individual) (DR1-PBMC); (c) an EBV-transformed B7$^+$, lymphoblastoid B cell line from a DR7 homozygous individual (DR7-LBL); (d) 100 U/ml recombinant human IL-2 (Collaborative Biomedical Products, Bedford, MA); or (e) a mitogenic combination of 1 ng/ml PMA and 100 ng/ml calcium ionophore Ionomycin (IM) (both from Sigma Chemical Co.). Samples were cultured in triplicate at 37°C for the appropriate time period (1–8 d with allo-APCs DR1-PBMC or DR7-LBL and for 3 d with IL-2 or PMA and IM) in 5% CO_2. To determine the length of primary exposure to alloantigen that is necessary for the development of specific tolerance, T cells were cultured from 12 h–7 d with artificial allo-APCs in a primary MLR, and the above described isolation of blasts and restimulation procedure was performed.

Thymidine Incorporation. Thymidine incorporation was used as an index of mitogenic activity. During the last 18 h of the appropriate culture period, cells were incubated with 1 μCi (37 kBq) of [methyl-^{3}H]thymidine (Du Pont, Boston, MA). The cells were harvested onto filters and the radioactivity on the dried filters was measured in a beta plate liquid scintillation counter (Pharmacia, Piscataway, NJ).

Cell Cycle Analysis. CD4$^+$ T cells were stimulated in a primary MLR, as described above, with t-DR7, t-DR7/B7, t-DR7/ICAM-1 allo-APCs, or t-DR7 and IL-2. After the completion of the appropriate time for maximal response, viable alloreactive T cells were recovered as described above and 10^6 cells were resuspended in 500 μl of propidium iodide solution (propidium iodide 50 μg/ml [Sigma Chemical Co.], 0.1% NP-40, and 0.1% Na citrate). After incubation for 30 min at 4°C, DNA analysis was performed by flow cytometer (Coulter Electronics). As controls, T cells were cultured in media for 5 and 7 d and DNA preparation was performed.

IL-2 and IL-4 Assays. Supernatants from primary and secondary stimulations were collected at 24 and 48 h after the initiation of the culture, as indicated, and IL-2 and IL-4 concentrations were assayed in duplicate by ELISA (R & D Systems, Minneapolis, MN).

Results

B7 and ICAM-1 Provide Distinct Costimulatory Signals for Proliferation of Alloreactive CD4$^+$ T Cells.

Considering the number of candidate costimulatory molecules that might be expressed on the cell surface of normal human allo-APCs, transfectants expressing HLA-DR7, B7, and ICAM-1 either alone or in combination, were prepared in order to functionally dissect the B7:CD28/CTLA4 and ICAM-1:LFA-1 costimulatory pathways. These artificial allo-APCs were constructed by stably transfecting (a) HLA-DRα and -DR7β chain genes (t-DR7); (b) B7 gene (t-B7); (c) ICAM-1 gene (t-ICAM-1); (d) the combination of HLA-DRα and -DR7β chains and B7 genes (t-DR7/B7); or (e) the combination of HLA-DRα and -DR7β chains and ICAM-1 genes (t-DR7/ICAM-1) into NIH 3T3 cells, as described in Materials and Methods. The expression or coexpression of these genes was documented by FACS® analysis as depicted in Fig. 1.

To determine the relative contributions of B7 and ICAM-1
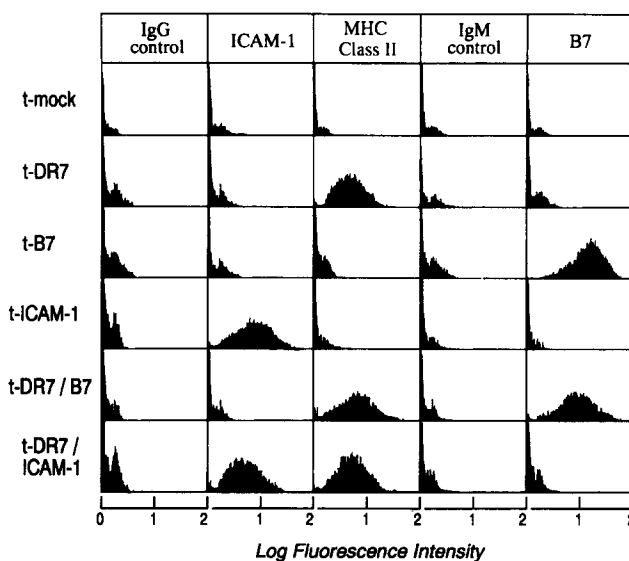
Figure 1. Surface expression of human MHC class II, B7, and ICAM-1 of transfected NIH 3T3 cells. Cells were stained with 9–49, 133, or RR1.1 mAb followed by FITC-labeled goat anti–mouse Ig and analyzed by flow cytometry.

to alloantigen-induced proliferation, CD4$^+$ T cells from HLA-DR7- and DR1-negative individuals were cocultured with mito-C–treated allo-APCs in a primary MLR for 1–8 d as described in Materials and Methods and proliferation was assessed. When increasing numbers of stimulator t-DR7 cells were added to constant numbers of responder T cells, proliferation was first observed at a stimulator/responder ratio of >1:2 (Fig. 2 a). Peak t-DR7 induced proliferation occurred at 7 d of culture at a stimulator/responder ratio of 1:1 and declined thereafter. Increasing numbers of t-B7 or t-ICAM-1, up to stimulator/responder ratio of 1:1 did not induce significant proliferation of T cells (Fig. 2, b and c) compared to controls cultured with autologous T cells or mito- C–treated t-mock transfectants (data not shown). At concentrations at which t-DR7 cells did not induce proliferation (stimulator/responder ratio 1:5), t-DR7/B7 cells induced significant proliferation, demonstrating that B7 provided a costimulatory signal to submitogenically alloactivated T cells (Fig. 2 b). Peak proliferation by t-DR7/B7 cells occurred at a stimulator/responder ratio of 1:2 and was nearly threefold of that observed with t-DR7 cells alone. Equal numbers of t-DR7/ICAM-1 cells provided a similar magnitude of costimulation as t-DR7/B7 cells (Fig. 2 c). Although the maximal costimulatory signals delivered by t-DR7/B7 or t-DR7/ICAM-1 were nearly identical, the kinetics of the response differed as B7 costimulation peaked at 7 d whereas ICAM-1 costimulation peaked at 5 d of culture (data not shown).

Although t-DR7/B7 and t-DR7/ICAM-1 appear to equally enhance alloreactive T cell proliferation, this can be due to either transmission of a costimulatory signal or simply to enhanced adhesion. To address this issue, T cells were cultured with: (a) increasing numbers of equal mixed populations of t-DR7 and t-B7; (b) increasing numbers of equal
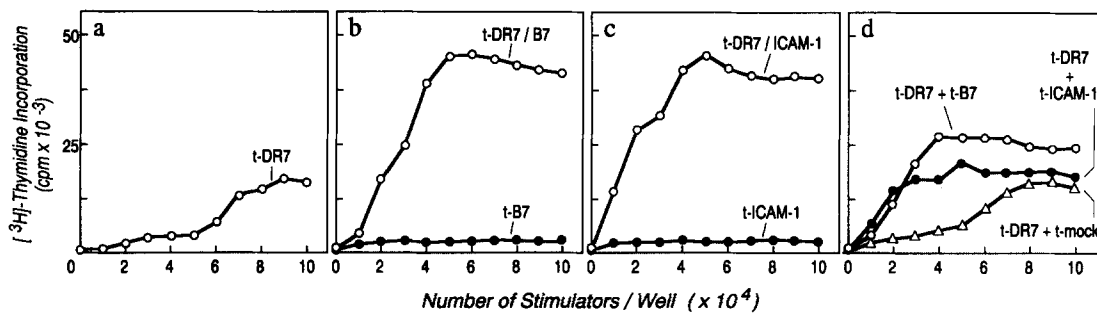
Figure 2. Dose–response of CD4+ T cells to alloantigen, presented on allo-APCs with or without B7 or ICAM-1 costmulation. 10^5 T cells were cultured with increasing numbers of each type of NIH 3T3 transfectants as stimulators, and thymidine incorporation was assessed for the last 18 h of a 7-d culture with t-DR7, t-B7, or t-DR7/B7 and of a 5-d culture with t-ICAM-1 or t-DR7/ICAM-1. In experiments in which mixed populations of transfectants were used as stimulators (d), cell numbers were selected so that DR7 molecules and costimulatory molecules per well were equal to those present on the corresponding double transfectants t-DR7/B7 and t-DR7/ICAM-1; the indicated numbers represent those of each cell type. This represents one of three experiments with mixed populations of t-DR7 and t-mock as stimulators, and five additional experiments under all other indicated conditions.

mixed populations of t-DR7 and t-ICAM-1; and (c) submitogenic concentrations of PMA and t-ICAM-1 or t-B7 cells. As seen in Fig. 2 d, t-DR7 and t-B7 or t-DR7 and t-ICAM-1 allo-APCs provided costimulatory signals of comparable magnitude, beginning at stimulator/responder ratios of 1:5. Although the maximal response induced by these mixed populations was not markedly augmented compared to that induced by t-DR7 alone, it occurred at a low stimulator/responder ratio, at which t-DR7 did not induce T cell response. However, in all experiments, proliferative response induced by mixed populations of transfectants was significantly less than that

induced by either t-DR7/B7 or t-DR7/ICAM-1. No significant additive stimulatory effect was observed by mixed populations of t-DR7 and t-mock. Mito-C–treated t-B7 or t-ICAM-1 allo-APCs in the presence of submitogenic concentrations of PMA, equally costimulated T cell proliferation (Table 1). No costimulation was induced by t-mock cells (data not shown). Although both ICAM-1 and B7 are involved in adhesion between APCs and T cells, these results are consistent with the hypothesis that they also deliver costimulatory signals independent of their adhesive function.

In addition, indication that B7 and ICAM-1 costimulate

Table 1. *Effect of CsA on B7 and ICAM-1 Costimulation of CD4+ T Cell Proliferation and IL-2 Accumulation*

| Culture	Conditions	Thymidine incorporation		IL-2	
		Media	CsA	Media	CsA
		cpm		*pg/ml*	
CD4+T cells		452	359	20	20
	+ PMA	4,787	4,148	<20	<20
	+ PMA + t-B7	84,888	76,150	580	320
	+ PMA + t-ICAM-1	73,130	7,555	<20	<20
	+ PMA + IM	63,134	12,871	1,100	50
	+ PMA + IM + t-B7	127,927	122,583	2,000	1,800
	+ PMA + IM + t-ICAM-1	160,030	37,293	1,200	60
	+ t-DR7/B7	37,354	33,761	1,100	800
	+ t-DR7 + t-B7	22,321	20,786	250	230
	+ t-DR7/ICAM-1	20,548	3,587	<20	<20
	+ t-DR7 + t-ICAM-1	16,654	2,966	<20	<20

CD4+ T cells were cultured in the presence of the indicated stimuli. When activation was provided by PMA or PMA + IM and costimulation by either B7 or ICAM-1, IL-2 accumulation was assessed at 24 h and proliferation at 3 d of culture. When activation was provided by alloantigen, IL-2 accumulation and proliferation were assessed as described in the legend to Fig. 3. Values represent means of triplicate cultures (SEM <15%).

through distinct pathways is derived from their differential sensitivity to CsA. CD4$^+$ T cells were activated with either PMA, PMA and IM, or t-DR7 allo-APCs, and costimulation was provided by either B7 or ICAM-1. As seen in Table 1, costimulation by B7 is CsA resistant, whereas costimulation by ICAM-1 is CsA sensitive.

B7 but Not ICAM-1 Induces Detectable IL-2 Production by Alloreactive T Lymphocytes. Since both B7 and ICAM-1 appear to provide equally potent costimulation for alloreactive T cell proliferation, we next sought to determine whether they also induced IL-2 secretion. For these experiments, to minimize any possible contribution from additional ligands that might be present on the NIH-3T3 cells, the numbers of stimulators were selected so that t-DR7 cells alone, would not induce proliferation (stimulator/responder ratio 1:2) and therefore provide a submitogenic first signal. To confirm the specificity of the activation and costimulatory signal provided by the artificial allo-APCs, mAbs and fusion proteins directed against TCR:MHC class II, B7:CD28/CTLA4, and ICAM-1: LFA-1 pathways were added at the inception of these cultures. At 2 d, IL-2 and IL-4 concentration in the culture supernatants was assessed by ELISA and proliferation was assessed at 5 d for t-DR7/ICAM-1 and at 7 d for t-DR7/B7-induced response. Here, t-mock, t-DR7, and t-ICAM-1 cells did not induce proliferation or detectable IL-2 or IL-4 production (Fig. 3, *a* and *b*). Fig. 3 *a* examines the costimulatory effect of the ICAM-1:LFA-1 pathway. t-DR7/ICAM-1 cells delivered a significant proliferative signal that was inhibited by >95% with either anti-class II, anti-ICAM-1, or anti-LFA-1 but not with isotype-matched control mAbs; no IL-2 or IL-4 accumulation was detected. t-DR7/B7 cells induced a proliferative response that was comparable to that induced by t-DR7/ICAM-1 but unlike t-DR7/ICAM-1 it was accompanied by the presence of significant amounts of IL-2. No IL-4 accumulation was detected (Fig. 3 *b*). Response was inhibited by anti-MHC class II mAb, and the specificity of B7 to induce IL-2 is demonstrated by the complete abrogation of proliferation and IL-2 accumulation by blocking the B7:CD28/CTLA4 costimulatory pathway with either anti-B7 or anti-CD28 Fab mAb, or CTLA4-Ig (high affinity receptor for B7). No inhibitory effect was observed for isotype-identical control mAb or control-Ig. Specificity for B7 and ICAM-1–mediated costimulation was confirmed since anti-B7, anti-CD28 Fab mAbs, or CTLA4-Ig did not affect costimulation induced by t-DR7/ICAM-1, and anti-ICAM-1 or anti-LFA-1 mAbs did not affect the costimulation induced by t-DR7/B7 (data not shown).

At the completion of primary MLR with t-DR7 cells as stimulators, the fraction of T cells in each phase of the cell cycle was not significantly different compared to the controls cultured with media alone (Table 2). Coculture with t-DR7 and IL-2 or t-DR7/ICAM-1 and more strikingly with t-DR7/B7, induced T cells to enter the cell cycle and progress to S phase.

B7 but not ICAM-1 Costimulation Prevents the Induction of Alloantigen-specific Tolerance. We next sought to determine whether alloantigen presentation in the absence of costimulation could induce alloantigen-specific tolerance and, more
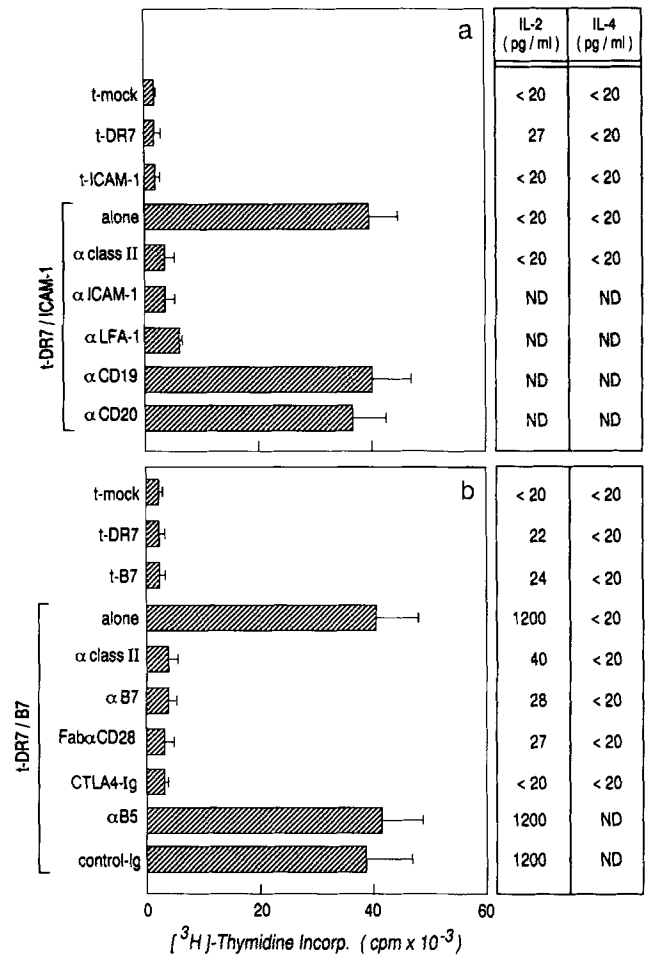
Figure 3. B7 and ICAM-1 mediate distinct and specific costimulatory signals in primary MLR. Freshly isolated CD4$^+$ T cells were cocultured with allo-APCs in media or in the presence of the indicated mAbs. Anti-CD20 is an isotype control for anti-class II and anti-CD19 is the control for anti-ICAM-1 and anti-LFA-1 mAbs; anti-B5 is the control for anti-B7 mAb and control-Ig is the control for CTLA4-Ig. Proliferation was assessed at 7 d of culture with t-mock, t-DR7, t-B7, t-DR7/B7 and at 5 d of culture with t-mock, t-DR7, t-ICAM-1, and t-DR7/ICAM-1. (*a*) Nine experiments; (*b*) seven experiments. IL-2 and IL-4 concentrations shown were assessed in supernatants of 48 h of culture by ELISA. The concentration of 20 pg/ml was the lowest detection limit of the assay.

importantly, whether either B7 or ICAM-1 could prevent the induction of tolerance. To this end, we undertook a three-step culture: (*a*) primary sensitization of T cells to allo-APCs; (*b*) reculture of these cells in media; and finally (*c*) rechallenge in a secondary culture with either allo-APCs, third-party alloantigen, exogenous IL-2, or mitogens. Specifically, T cells were cultured with either t-DR7, t-DR7/B7, or t-DR7/ICAM-1. Primary MLR culture was continued until the completion of maximal response (7 d with t-DR7 and t-DR7/B7, and 5 d with t-DR7/ICAM-1 allo-APCs). After primary stimulation, viable alloreactive blasts from each culture were isolated, washed, and recultured in media for 24 h to reexpress TCR complex (data not shown). These cells were then restimulated with (*a*) t-DR7, t-DR7/B7, or t-DR7/ICAM-1;

Table 2. *Effect of Alloantigen and B7 or ICAM-1 Costimulation on Cell Cycle Progression of Alloreactive CD4+ T Cells*

Culture conditions of CD4+ T cells	Percent cells in each cell cycle phase		
	G0/G1	S	G2
Media	94.3	4.4	1.9
t-DR7	94.6	5.4	2.0
t-DR7 + IL-2	75.2	24.8	2.0
t-DR7/B7	52.6	39.5	1.9
t-DR7/ICAM-1	78.0	28.4	1.8

CD4+ T cells were cultured in a primary MLR with the indicated stimuli and resuspended in propidium iodide solution. 10^6 T cells were analyzed for each sample.

(*b*) a stimulator cell population not expressing DR7 antigen (PBMC from a DR1 homozygous individual) (DR1-PBMC); (*c*) recombinant human IL-2; or (*d*) a mitogenic combination of PMA and IM. Proliferation was assessed at 3 d of culture with IL-2 or PMA and IM and at 1–8 d of culture with each one of the other stimuli. As controls, T cells were exposed in the primary culture to either t-mock, t-B7, or t-ICAM-1. T cells recovered from these control primary cultures, demonstrated a proliferative response on rechallenge with either t-DR7, t-DR7/B7, or t-DR7/ICAM-1 identical to that induced by these transfectants in a primary stimulation (data not shown).

When T cells were primarily stimulated with t-DR7 or t-DR7/ICAM-1 allo-APCs, they were tolerized and no significant proliferation was observed at restimulation with either t-DR7, t-DR7/B7, or t-DR7/ICAM-1 cells (Fig. 4 *a*). This proliferative response was identical to that observed in the controls of the secondary stimulation, in which T cells from each primary culture were rechallenged with either au-
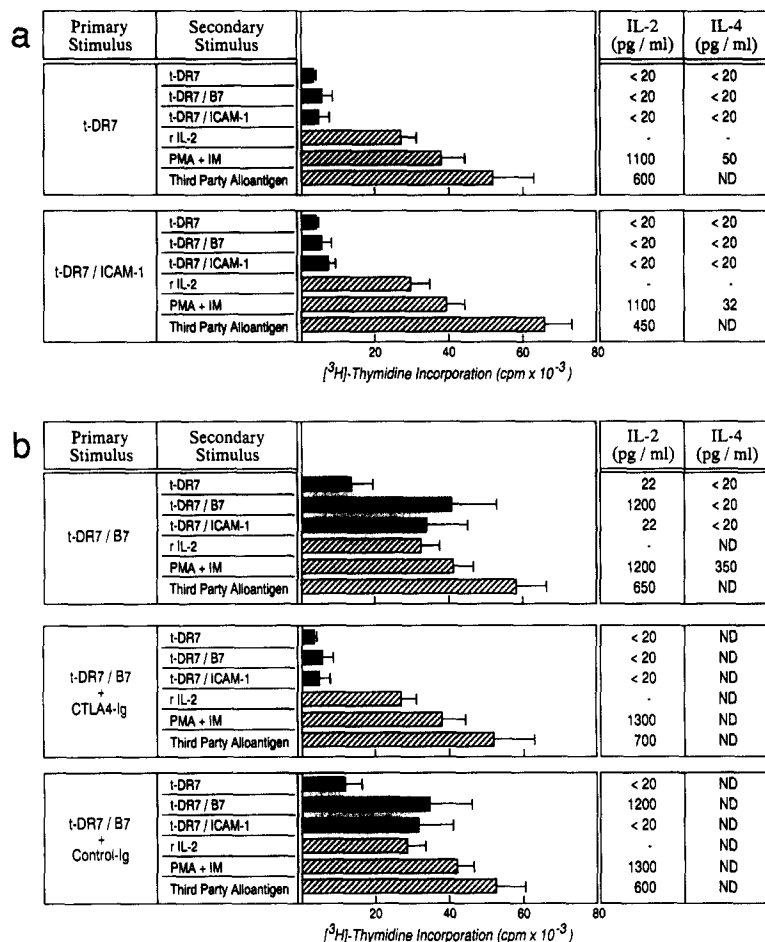

Figure 4. B7 but not ICAM-1 costimulation can prevent the induction of tolerance. CD4+ T cell were primarily stimulated with t-DR7, t-DR7/B7, or t-DR7/ICAM-1. Viable blasts were rested in media for 24 h and cultured with a secondary stimulus, as shown. Thymidine incorporation was measured for the last 18 h of a 3-d culture with either t-DR7, t-DR7/B7, t-DR7/ICAM-1, IL-2, or PMA + IM and of a 7-d culture with third-party alloantigen as secondary stimuli. Results represent 18 experiments with t-DR7 or t-DR7/B7 and seven experiments with t-DR7/ICAM-1 stimulators in primary culture. IL-2 and IL-4 concentrations were assessed in supernatants of 48 h of secondary culture with third-party alloantigen and of 24 h of secondary culture with each one of the other stimuli.

tologous cells or t-mock, t-B7, or t-ICAM-1 (data not shown). Although the tolerized cells did not respond to any of the above allo-APCs during the secondary challenge, there was significant proliferation in response to either IL-2, mitogenic combination of PMA and IM, or third-party alloantigen.

In contrast, when T cells were primarily stimulated with t-DR7/B7 allo-APCs, they responded to all types of allo-APCs in a secondary culture and the proliferative response peaked at day 3 (Fig. 4 *b, top*), characteristic of a secondary response. Interestingly, proliferation consistently was highest with t-DR7/B7 allo-APCs, followed by t-DR7/ICAM-1, and to a lesser extent with t-DR7. The response to t-DR7 occurred at a cell concentration that did not induce proliferation in the primary MLR, suggesting that this represented a secondary proliferative response. Significant proliferation was also induced by IL-2, PMA, and IM or third-party alloantigen. When the primary stimulation with t-DR7/B7 allo-APCs was performed in the presence of CTLA4-Ig to block the B7:CD28/CTLA4 pathway, secondary responses demonstrated virtually identical results to those seen with T cells primarily stimulated with t-DR7 allo-APCs, i.e., the T cells were tolerized (Fig. 4 *b, middle*). No effect was observed when control-Ig was added in the primary culture (Fig. 4, *bottom*). This observation confirms that the B7 costimulatory signal is sufficient for prevention of the induction of tolerance to alloantigen.

To determine whether secondary proliferative responses were driven by IL-2 or potentially by IL-4, supernatants were harvested at 48 h of rechallenge with third-party alloantigen and at 24 h of rechallenge with each one of the other stimuli, and IL-2 and IL-4 concentration was assessed by ELISA. Only t-DR7/B7 allo-APCs induced both detectable amounts of IL-2 and increased proliferation. Both alloactivated and tolerized T cells, however, produced significant amounts of IL-2 in response to mitogenic combination of PMA and IM or third-party alloantigen (Fig. 4). In contrast, IL-4 was detectable only after secondary stimulation with PMA and IM. It is of note that primary sensitization in the presence of B7 costimulation, primed the cells for IL-4 secretion in restimulation with PMA and IM (Fig. 4 *b, top*).
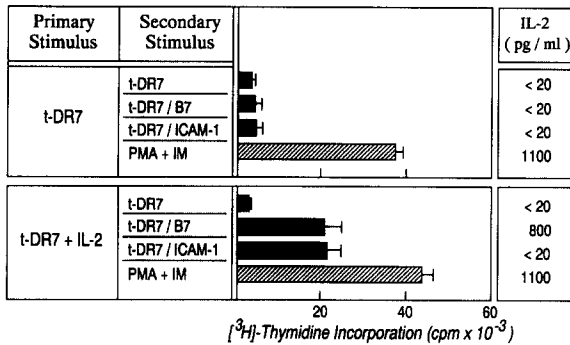
Since T cells primarily sensitized with either t-DR7 or t-DR7/ICAM-1 did not proliferate or secrete IL-2 on rechallenge with t-DR7/B7 allo-APCs, they were rechallenged with a lymphoblastoid cell line (DR7-LBL) or with PBMC (DR1-PBMC), that express alloantigens and presumably other costimulatory molecules not expressed on the surface of the transfectants. T cells tolerized to DR7 using t-DR7 or t-DR7/ICAM-1 during primary stimulation, demonstrated low levels of proliferation after rechallenge with DR7-LBL, which peaked at day 7 of culture (Fig. 5, *top* and *bottom*). In contrast, T cells primarily sensitized with t-DR7/B7, demonstrated a dramatic secondary response on rechallenge with DR7-LBL, which peaked at day 3 of culture (Fig. 5, *middle*). All populations of T cells, however, responded identically to rechallenge with third-party HLA-DR alloantigen expressed on the DR1-PBMC, and proliferative response peaked at day 7 of culture (Figs. 4 and 5).
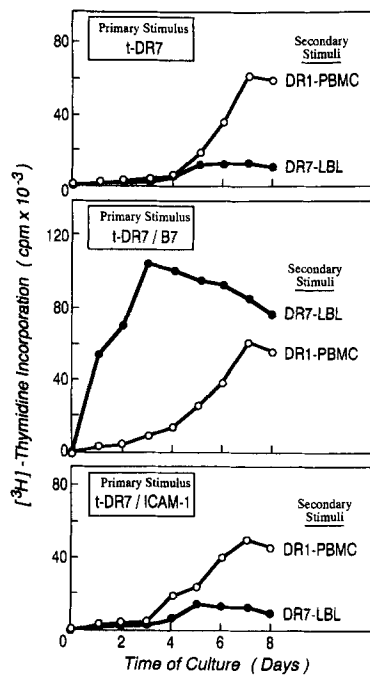
Figure 5. B7 costimulation in primary MLR primes CD4^{+} T cells for secondary alloantigen-specific response, but does not affect response to third-party alloantigen. CD4^{+} T cells were primarily stimulated with either t-DR7, t-DR7/B7, or t-DR7/ICAM-1. Viable blasts were cultured in media for 24 h and restimulated with an EBV-transformed cell line from a DR7 homozygous individual (*DR7-LBL*) or with PBMC from a DR1 homozygous individual (*DR1-PBMC*). At the indicated time points of culture, thymidine incorporation was assessed; one of seven experiments.

To determine the time interval of primary exposure to alloantigen required to induce alloantigen-specific tolerance, T cells were cultured with t-DR7, t-DR7/B7, or t-DR7/ICAM-1 allo-APCs for time intervals varying from 12 h to 7 d. After primary sensitization, viable alloreactive blasts were isolated and restimulated as described above. T cells exposed to alloantigen, presented by either t-DR7 or t-DR7/ICAM-1 stimulators, for periods of 24 h to 7 d, were rendered tolerant to the specific alloantigen. In contrast, T cells primarily sensitized for only 12 h were not tolerized and showed a primary response pattern at restimulation (data not shown).

IL-2 Prevents the Induction but Cannot Reverse the State of Alloantigen-specific Tolerance. Since B7 costimulation results in the production of multiple lymphokines including IL-2 (42–44), we were interested in determining whether the presence of IL-2 in the primary culture was the critical factor for preventing the induction of tolerance or whether, alternatively, B7 delivered a specific activating signal. T cells were cultured with primary tolerogenic signal (t-DR7) in the presence of media or 100 U/ml of IL-2. After primary culture, IL-2 was removed, cells were rested for 24 h, and then rechallenged with allo-APCs for 1–8 d or PMA and IM for 3 d. T cells tolerized with t-DR7, did not respond on rechallenge with t-DR7, t-DR7/B7, or t-DR7/ICAM-1 although they did proliferate in response to PMA and IM (Fig.

Primary Stimulus	Secondary Stimulus		IL-2 (pg/ml)
t-DR7	t-DR7		< 20
	t-DR7 / B7		< 20
	t-DR7 / ICAM-1		< 20
	PMA + IM		1100
t-DR7 + IL-2	t-DR7		< 20
	t-DR7 / B7		800
	t-DR7 / ICAM-1		< 20
	PMA + IM		1100

[³H]-Thymidine Incorporation (cpm x 10⁻³)

Figure 6. Primary stimulation with alloantigen in the presence of IL-2 can prevent the induction of tolerance. CD4⁺ T cells were primarily stimulated with t-DR7 with or without exogenous IL-2. Visible blasts were rested in media for 24 h and then cultured with a secondary stimulus, as shown. Results represent four experiments and show thymidine incorporation for the last 18 h of a 3-d secondary culture. IL-2 and IL-4 concentrations were measured in supernatants at 24 h of culture.
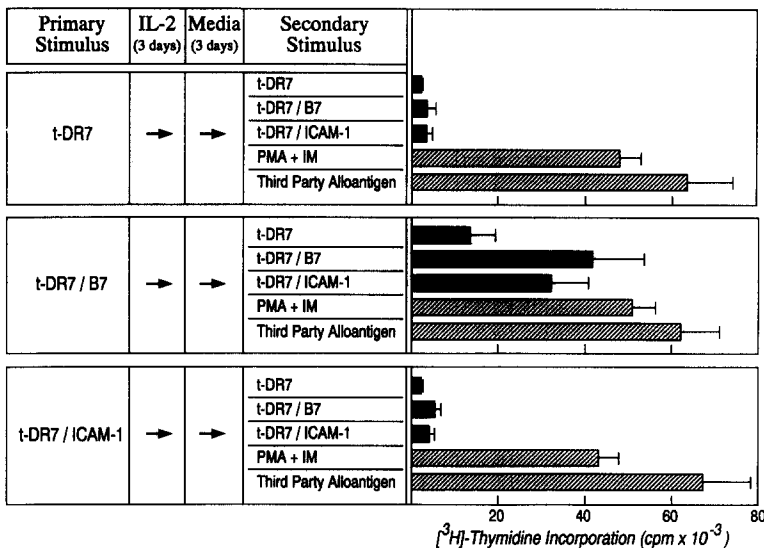
6, *top*). In contrast, T cells cultured with t-DR7 and IL-2 were not tolerized and could proliferate on rechallenge with t-DR7/B7 or t-DR7/ICAM-1. These T cells also secreted IL-2 in response to t-DR7/B7 (Fig. 6, *bottom*). The maximal secondary proliferative response after primary culture with t-DR7 and IL-2 was ~50% of that observed when cells had been primarily stimulated with t-DR7/B7 allo-APCs (Fig. 4 *b*, *top* and Fig. 6, *bottom*).

It has been observed previously that murine antigen-specific T cell clonal anergy can be reversed by prolonged culture of tolerized cells in the presence of exogenous IL-2 (45). In our model system, T cells tolerized to DR7 could briskly proliferate in the presence of IL-2 (Fig. 4 *a*, *top* and *bottom*). We therefore sought to determine whether short culture with IL-2 would reverse DR7-specific tolerance. Viable alloreactive blasts isolated from primary cultures with t-DR7, t-DR7/B7, or t-DR7/ICAM-1 allo-APCs, were cultured in the presence

of 100 U/ml IL-2 for 3 d, extensively washed, and cultured in media for 3 d. Cells were subsequently restimulated for 1–8 d with allo-APCs t-DR7, t-DR7/B7, and t-DR7/ICAM-1. T cells primarily tolerized with t-DR7 or t-DR7/ICAM-1 and cultured in IL-2 (Fig. 7, *top* and *bottom*), did not proliferate in rechallenge with t-DR7, t-DR7/B7, or t-DR7/ICAM-1. In contrast, cells primarily sensitized with t-DR7/B7 and recultured in IL-2 could respond in rechallenge with specific alloantigen and demonstrated maximal response at 3 d of culture (Fig. 7, *middle*). Rechallenge of all three populations with PMA and IM or third-party alloantigen resulted in significant proliferative response.

Discussion

Effective allogeneic transplantation in humans is limited by graft rejection and toxicity resulting from aggressive immunosuppression. These life-threatening complications might be prevented or ameliorated by inducing alloantigen-specific tolerance. One approach to achieve this objective is to identify and block all relevant costimulatory signals involved in the generation of the T cell immune response to alloantigen. Considering the number of potentially relevant molecules expressed on APCs, we developed a culture system to determine which of these molecules costimulate alloantigen-induced T cell proliferation and, more importantly, which can prevent the induction of human alloantigen–specific tolerance. To functionally isolate each of these costimulatory pathways, we prepared transfectants coexpressing HLA-DR and a candidate costimulatory molecule. Here, we demonstrate that transfectants coexpressing HLA-DR7 and either B7 or ICAM-1 provided equivalent costimulation for alloreactive CD4⁺ T cell proliferation. Although equivalent in their ability to costimulate alloreactive T cell proliferation, costimulation provided by B7 and ICAM-1 differed in two respects. First, B7 but not ICAM-1 induced significant production of IL-2. Second, B7 but not ICAM-1 prevented the induc-

Primary Stimulus	IL-2 (3 days)	Media (3 days)	Secondary Stimulus	
t-DR7	→	→	t-DR7	
			t-DR7 / B7	
			t-DR7 / ICAM-1	
			PMA + IM	
			Third Party Alloantigen	
t-DR7 / B7	→	→	t-DR7	
			t-DR7 / B7	
			t-DR7 / ICAM-1	
			PMA + IM	
			Third Party Alloantigen	
t-DR7 / ICAM-1	→	→	t-DR7	
			t-DR7 / B7	
			t-DR7 / ICAM-1	
			PMA + IM	
			Third Party Alloantigen	

[³H]-Thymidine Incorporation (cpm x 10⁻³)

Figure 7. IL-2 cannot reverse the state of established tolerance. CD4⁺ T cells were primarily stimulated with either t-DR7, t-DR7/B7, or t-DR7/ICAM-1 allo-APCs. Viable blasts were cultured with IL-2 for 3 d, in media without IL-2 for 3 d, and subsequently restimulated with a secondary stimulus as indicated. Results represent five experiments and show thymidine incorporation for the last 18 h of a 3-d secondary culture.

tion of alloantigen-specific tolerance. These results demonstrate that the delivery of a costimulatory signal that induces alloreactive T cells to proliferate is not sufficient to prevent the induction of tolerance. Moreover, these results demonstrate that this model system may permit us to determine which costimulatory molecules must be blocked in order to induce alloantigen-specific tolerance.

Several lines of evidence support the notion that B7- and ICAM-1–induced costimulation is not only due to improved adhesion between the APC and the T cell, but also to additional, distinct, and independently mediated costimulatory signals. First, presentation of the HLA-DR7 and B7 or ICAM-1 molecules on separate transfectants still results in costimulation, albeit in less proliferation than when alloantigen and costimulatory molecules are expressed on the same cell. Second, although B7 and ICAM-1 deliver equivalent proliferative costimulatory signals, only B7 results in significant, detectable IL-2 accumulation. Finally, only ICAM-1 costimulation is CsA sensitive whereas B7, in agreement with previous observations (46, 47), is not. Taken together, these data are consistent with the hypothesis that the proliferation induced by B7 and by ICAM-1 is the result of independently mediated costimulatory pathways.

Herein, we have shown absence of proliferation and IL-2 secretion in restimulation with the specific alloantigen, when alloreactive T cells were tolerized to DR7 after primary culture with t-DR7 or t-DR7/ICAM-1 cells. Although tolerized to specific alloantigen, alloreactive T cells can proliferate with IL-2 and proliferate and secrete IL-2 in response to mitogens or third-party alloantigen. Challenge of these tolerized cells with a HLA-DR7 homozygous lymphoblastoid line rather than with t-DR7/B7 transfectants resulted in modest proliferation. This response might be due to stimulation by additional alloantigens expressed on the lymphoblastoid cells or to other costimulatory molecules that can overcome tolerance.

Our results argue that inhibition of B7 costimulation will be essential but may not be sufficient to induce tolerance to alloantigen presented on donor grafts. These results are consistent with those reported by Tan et al. (48), who demonstrated that blockade of the B7:CD28/CTLA4 pathway with CTLA4-Ig resulted in hyporesponsiveness but not anergy of fully mismatched human PBMC. Although these investigators suggested that other costimulatory molecules might be responsible for the failure of tolerance induction, the expression of all potential alloantigens and candidate costimulatory molecules in both their primary and secondary MLR did not permit them to determine which costimulatory pathways were critical to the prevention of tolerance. Their results are less dramatic than those reported by Lenschow et al. (31), who demonstrated in an in vivo murine model that inhibition of the B7:CD28/CTLA4 pathway with CTLA4-Ig resulted in long-lasting tolerance to xenogeneic human islet cells. However, the inability of other costimulatory signals to prevent the induction of tolerance in this xenogeneic model, might be due to the previous observations that the B7:CD28 pathway is conserved between humans and mice but that other essen-

tial costimulatory molecules may not have this degree of phylogenetic conservation (9, 12). Therefore, our model provides an ideal system to functionally dissect surface molecules that potentially prevent the induction or reverse the state of tolerance. Transfection of other alloantigens (e.g., HLA-DP, HLA-DQ, etc.) as well as other costimulatory molecules will be necessary to conclusively determine which costimulatory pathways must be blocked in order to induce tolerance to alloantigen.

Since B7 costimulation upregulates IL-2 secretion, it was important to determine whether IL-2 alone could either prevent the induction or overcome the established state of tolerance. Our results confirm and extend previous observations (45, 48) which show that the presence of IL-2 during induction phase prevents the generation of antigen-specific tolerance. Since transfectants isolate the relative contributions of alloantigen and specific costimulation for IL-2 secretion, we can more accurately evaluate the role of costimulatory signals and IL-2 in preventing the induction of tolerance. Here, we have shown that alloantigen accompanied by either B7 costimulation or exogenous IL-2 is sufficient to prevent the induction of tolerance. Since IL-2 is one of multiple cytokines that are regulated by B7 (42–44), our data suggest that IL-2 is a critical consequence of B7 costimulation that prevents the induction of tolerance. This hypothesis is further supported by the observation that ICAM-1 does not lead to detectable accumulation of IL-2 and does not prevent the induction of tolerance. However, neither we nor others have identified the molecular basis of how IL-2 prevents the induction of tolerance.

Regardless of the mechanism, the ability of IL-2 to prevent the induction of tolerance has potential clinical implications for allogeneic transplantation. Our results suggest that the presence of any IL-2 in a microenvironment might permit the escape of alloreactive T cells from being rendered tolerant to a donor graft and thereby lead to only a partial or temporary state of tolerance. The observation that murine allogeneic cardiac graft survival was only transiently prolonged by CTLA4-Ig blockade of the B7:CD28/CTLA4 pathway (32) is consistent with this hypothesis and suggests that additional costimulatory blockade or immunosuppression may be necessary to induce a state of long-term tolerance. The second clinically relevant issue is whether IL-2 in the microenvironment can overcome the state of tolerance once it has been induced. This is obviously important to allogeneic transplantation, as tolerance could be potentially broken during an active immune response. Since our experiments were not done in a T cell clonal system, we cannot definitively resolve this issue. Our data, however, do not support the conclusion that IL-2 alone can break alloantigen-specific tolerance. Bulk cultures of alloreactive human CD4+ T cells tolerized to DR7 readily proliferate in the presence of IL-2 alone. In this nonclonal system, we do not know whether the proliferating T cells are alloantigen specific or alternatively activated, nonspecific T cell blasts. We have previously shown, however, that anergized human tetanus toxoid–specific T cell clones can proliferate to IL-2 alone (30). Moreover, our observation

that DR7-tolerized alloreactive T cell blasts can proliferate when stimulated with third-party class II alloantigen, is consistent with the assumption that the bulk CD4+ T cell blasts were contaminated by activated T cells that were not specific for the tolerizing alloantigen. Taken together, these results suggest that tolerized T cells can proliferate to IL-2 and that the observed proliferation of DR7-tolerized CD4+ T cell blasts in bulk, nonclonal culture is likely due to proliferation of both antigen-specific tolerized T cells and antigen-nonspecific activated T cell blasts. The capacity of tolerized T cells to proliferate to IL-2 alone suggests that the IL-2 receptor signal transduction pathway in tolerized T cells is intact.

In contrast to the reported results with mouse T cells (45), we have not been able to break tolerance with IL-2. After short-term culture with IL-2, rechallenge of DR7-tolerized cells with t-DR7/B7 does not result in proliferation. These results differ from those reported by Tan et al. (48) who

demonstrated in a nonclonal system that cells tolerized to alloantigen can proliferate in rechallenge with alloantigen and IL-2. Since those studies did not determine whether alloantigen-tolerized T cells could proliferate to IL-2 alone, they did not demonstrate that tolerance can be reversed. Moreover, they did not determine whether those IL-2/alloantigen stimulated cells could respond to rechallenge with alloantigen alone. Our results to date demonstrate that alloantigen-specific tolerance cannot be reversed by culture with IL-2 and are consistent with the hypothesis that IL-2 deprivation may induce apoptic cell death of previously tolerized T cells. We favor this possibility since we have previously shown that tolerized tetanus toxoid–specific T cell clones proliferate in response to IL-2 but undergo apoptosis when removed from the IL-2–rich environment (30). The answer, however, awaits the examination of this question in an alloantigen-specific T cell clonal system. If this result is confirmed, the concern that IL-2 may overcome tolerance will be a less serious clinical issue.

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