Brief Definitive Report

The Combination of Anti-B7 Monoclonal Antibody and Cyclosporin A Induces Alloantigen-specific Anergy during a Primary Mixed Lymphocyte Reaction

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Summary

Interaction of CD28/CTLA-4 on T cells with B7 on antigen-presenting cells constitutes an important costimulatory signal for T cells and is responsible for cyclosporin A–resistant interleukin 2 (IL-2) gene expression and potentially also for prevention of anergy induction after T cell receptor triggering. In this paper, we demonstrate that addition of a monoclonal antibody to B7, which blocks B7–CD28/CTLA-4 interaction, and of cyclosporin A together, but not separately, to a primary mixed lymphocyte reaction of freshly isolated human T cells towards a human B cell line, induces nonresponsiveness of alloantigen-specific cytotoxic T lymphocyte precursors, whereas reactivity to a third party stimulator is intact. Nonresponsiveness could be reversed by culture in IL-2, indicating that anergy, and not clonal deletion, is responsible for this phenomenon. Our finding opens important perspectives for the development of new therapeutic strategies in transplantation.

Optimal therapy to prevent graft rejection and GVHD should rely on the induction of alloantigen-specific anergy or elimination of donor-reactive and host-reactive T cells, respectively, without the need for long-term immunosuppression. T cell anergy is currently thought to be the result of an intracellular signaling event after TCR/MHC–peptide interaction in the absence of one or more particular costimulatory signals (1–4). Among the multiple costimulatory signals identified so far (5), the best-known candidate to determine whether TCR triggering leads to full T cell activation or to T cell anergy, is the interaction of CD28 on the T cells with B7 on APC (6, 7). Costimulation through CD28 induces tyrosine phorphorylation of a number of specific substrates (8), and greatly enhances T cell activation (9–12). This effect involves stabilization of mRNA for several cytokines, including IL-2 (10). A CD28-responsive element has been demonstrated in the enhancer of the IL-2 gene, indicating that there is also regulation at the transcriptional level (13, 14).

B7, the natural ligand for CD28 (15), is a monomeric transmembrane glycoprotein with a molecular mass of 45–65 kD and is, like CD28, a member of the Ig superfamily. B7 also binds to the CTLA-4 molecule (15), another member of the Ig superfamily restricted to activated T cells. The B7 molecule is expressed on activated B cells and activated monocytes, and constitutively on dendritic cells in both lymphoid and nonlymphoid tissues (16). Very recently, the B7 molecule was shown to be involved in T cell activation in vivo. A soluble fusion protein of human CTLA-4 and the Ig G1 Fc region (CTLA-4-Ig), which strongly binds to both mouse and human B7, was able to prevent rejection of human pancreatic islets after transplantation in mice (17). CTLA-4-Ig could also enhance the survival of cardiac allografts transplanted into fully MHC-mismatched rats (18). Moreover, blocking the B7–CD28/CTLA-4 interaction induces a specific hyporesponsiveness in human T cells after activation with alloantigens (19).

Of importance with respect to transplant rejection, T cell coactivation mediated by CD28 has been reported to be calcium independent (20) and to be relatively resistant to inhibition with cyclosporin A (CsA)[1] (11, 12). T cell activation thus involves a number of CsA-sensitive mechanisms, including the Ca^{2+}-associated TCR/CD3 complex–mediated intracellular signal transduction (21), besides a CsA-resistant costimulatory signal from B7 (11, 12). We therefore hypothesized that the combination of CsA and mAb B7-24, which blocks B7–CD28/CTLA-4 interaction (22), would more efficiently block T cell activation. In a recent study (23), we could indeed demonstrate that this combination strongly inhibits

[1] *Abbreviation used in this paper:* CsA, cyclosporin A.

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alloantigen-induced T cell activation in an MLR (23). In the present study, we questioned whether the presence of CsA and a mAb against the B7 molecule during a primary MLR between freshly isolated human T cells and a B7-expressing EBV-transformed human B cell line as stimulator, could induce nonresponsiveness or anergy of human CTL precursors. T cell nonresponsiveness was studied at the cytotoxic effector level, which was selected as being most relevant for transplant rejection and most difficult to induce (24, 25). Our findings may be of considerable importance for the development of new therapeutic strategies aimed at prevention of transplant rejection and treatment of CsA- and corticosteroid-resistant GVHD.

Materials and Methods

Cells and Cell Lines. Peripheral blood T cells were isolated from healthy volunteers as described (12). The resulting population is devoid of B cells, NK cells, and monocytes. The cells were cultured in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% iron-supplemented bovine calf serum (complete medium). Two EBV-transformed B cell lines were used as stimulator cells for the MLR: ARC, obtained from the American Type Culture Collection (ATCC [Rockville, MD]) (HLA-DR w8,w8), and MM, produced at Innogenetics (HLA-DR 1,2). The P815 cell line (ATCC) is a DBA/2-derived NK-resistant mouse mastocytoma cell line that expresses FcγRII and FcγRIII.

Monoclonal Antibodies and Other Reagents. mAb B7-24 (IgG2a, κ) was obtained from a fusion with splenocytes from a mouse immunized with Sf9 insect cells expressing the human B7 molecule (22, 26) and was used as purified Ab. Anti-CD3 mAb OKT3 (IgG2a, κ) was used as purified Ab isolated from the supernatant of the OKT3 clone obtained from the ATCC. CsA (purchased from Sandoz Pharmaceuticals, Basel, Switzerland) was dissolved in RPMI 1640 (stored at 4°C). rIL-2, produced by Hoffmann-La Roche (Nutley, NJ), was obtained through the Biological Response Modified Program (National Cancer Institute, Frederick, MD). A second rIL-2 preparation was purchased from Boehringer-Mannheim (Mannheim, Germany). rIL-4 was produced at Innogenetics.

Mixed Lymphocyte Reactions. For the primary MLR, 10^6 T cells were cultured with 0.25×10^6 ARC cells (irradiated with 2,000 rad), for 5–7 d in 24-well flat-bottom tissue culture plates in 1 ml of medium. mAb B7-24 and/or CsA were added from the beginning of the culture period. After 5–7 d, the cells were harvested and resuspended in medium for direct analysis of cytotoxic capacity or for restimulation in a secondary MLR. In the latter case, the cells were first rested in medium alone during 1–2 d in the absence of the stimulator cells. The T cells were then restimulated in a secondary MLR with irradiated ARC cells or MM cells at a T cell/stimulator cell ratio of 4:1 without CsA or anti-B7. 4 d later, the cells were harvested and resuspended in medium for analysis of cytotoxic capacity or for further culture. If a tertiary MLR was performed, the cells were again restimulated with ARC cells or MM cells (T cell/stimulator cell ratio, 4:1) for 4–5 d without CsA or anti-B7, and then tested for their cytotoxic capacity. IL-2 in the culture supernatants was assayed with a bioassay as previously reported (27).

Cytotoxicity Assay. Cytotoxic activity was measured in a 4-h target cell lysis assay using murine P815 cells or ARC cells as targets. In the case of the P815 target cells, the CTL were bridged nonspecifically to the target cells using the anti-CD3 mAb OKT3 at 2 μg/ml (12). This anti-CD3–redirected cytotoxicity assay thus evaluates all T cell cytotoxic activity, irrespective of antigen specificity. When the ARC cells were used as target cells, only the alloantigen-specific CTL supposedly participated in the killing process. One million target cells were labeled with 200 μCi of [^{51}Cr]sodium chromate (Amersham International, Amersham, Bucks, UK). The CTL assays were performed in 96-well V-bottom microtiter plates using 5,000 ^{51}Cr-labeled target cells with 5×10^4 T cells (E/T ratio = 10, unless otherwise indicated) in a total volume of 200 μl/well. Four wells were filled with 5×10^3 target cells in 200 μl medium alone, and four wells with 5×10^3 target cells in 100 μl medium and 100 μl saponin for evaluation of spontaneous and maximal release, respectively. The percentage of anti-CD3–dependent ^{51}Cr release of P815 cells was calculated as described (12). Percent lysis of ARC cells was calculated as follows: 100 × [(total release by the T cells − spontaneous release)/(maximal release by saponin − spontaneous release)].

Results and Discussion

We analyzed the effect of CsA and of blocking B7–CD28/CTLA-4 interaction with mAb B7-24 during a primary MLR of freshly isolated human T cells against EBV-transformed human B cell lines (ARC or MM) on subsequent restimulation with the same or different stimulator cells. Alloantigen-specific and total anti-CD3–redirected cytotoxic activity were measured as a read-out system for CTL activation. T cells primed with ARC displayed cytotoxic activity after a primary, and more so, after a secondary MLR (Fig. 1). As also demonstrated in Fig. 1, the combination of B7-24 mAb and CsA, but not each of them separately, blocked the generation of CTL during the primary MLR, and, more importantly, prevented subsequent CTL generation in a secondary MLR performed with the same stimulator cells but in the absence of these blocking agents. In other words, T cells primed in an MLR in the presence of both anti-B7 and CsA, are nonresponsive upon restimulation with the same alloantigen. Tan et al. (19) demonstrated that CTLA-4-Ig alone induces alloantigen-specific hyporesponsiveness in human T cell cultures as measured by proliferation. In our experiments using a CTL evaluation for induction of nonresponsiveness, the mAb B7-24 alone did not consistently enter the cells in a nonresponsive state, although we also sometimes found a state of hyporesponsiveness after a primary MLR with anti-B7 alone (data not shown). However, the combination of CsA and mAb B7-24 added during the primary MLR consistently prevented alloantigen-induced CTL generation in a secondary MLR (Fig. 2). Varying the CsA concentration from 200 to 1,600 ng/ml or the anti-B7 concentration from 0.1 to 10 μg/ml resulted in an identical state of nonresponsiveness (data not shown). Also, after a second restimulation with ARC (tertiary MLR), the cells made nonresponsive during the primary MLR with CsA and mAb B7-24 displayed no cytotoxic activity (Fig. 2), indicating the persistence of nonresponsiveness. CsA with anti-CD58 (LFA-3) mAb or with anti-CD54 (intercellular adhesion molecule 1 [ICAM-1]) mAb could not induce this state of nonresponsiveness (data not shown).

To exclude cell death as a cause of nonresponsiveness in the secondary MLR, T cells were first rendered nonrespon-

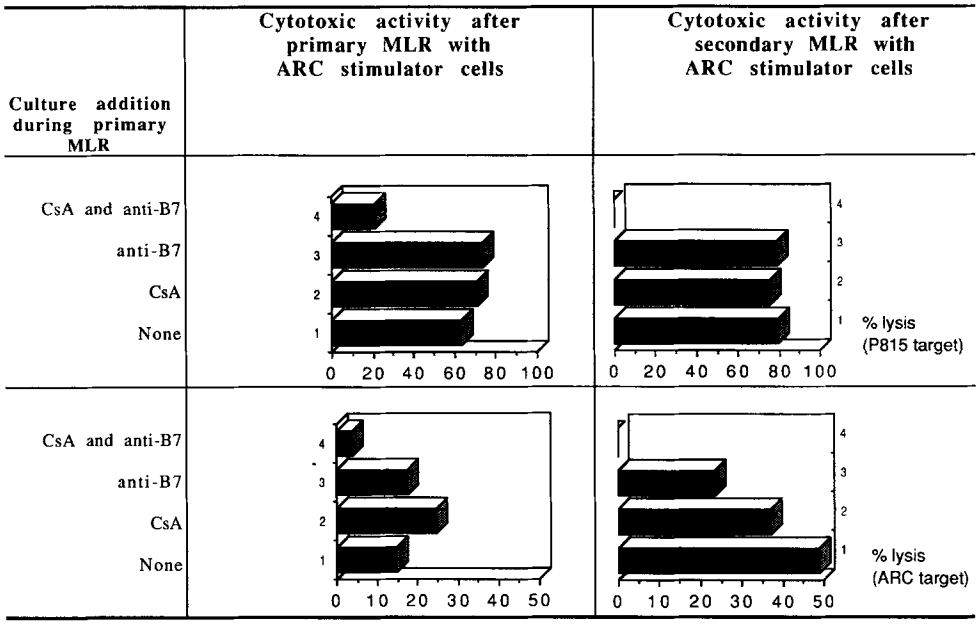

Figure 1. Synergy between mAb B7-24 and CsA in blocking the primary MLR and in inducing persistent alloantigen nonresponsiveness of CTL precursors. Purified human T cells were stimulated with irradiated ARC cells in a 7-d primary MLR. CsA (400 ng/ml) and/or mAb B7-24 (10 μg/ml) was added during the primary MLR as indicated. T cells were then rested for 2 d and restimulated with ARC cells in a 4-d secondary MLR, in the absence of CsA or anti-B7. CTL activity after primary and after secondary MLR was measured in an anti-CD3–redirected cytotoxicity assay against P815 mastocytoma cells (*top*) and in an alloantigen-specific cytotoxicity assay against the original stimulator cells (*bottom*) and is expressed as percent lysis of the target cells. Results of one representative experiment out of 10.

sive to ARC as before, and were then restimulated polyclonally with immobilized anti-CD3 and IL-2. Anti-CD3–redirected cytotoxicity was used as a read-out system for polyclonal CTL generation. As can be seen in Fig. 3 *a*, when the cells were cultured in the primary MLR with ARC, CsA, and anti-B7, they could still be stimulated polyclonally to the same extent as the control cells primed with ARC alone. Induction of nonresponsiveness could be the result of either anergy, or of clonal deletion. In the first case, it should be possible to overcome the anergic state with IL-2 (2, 6), whereas IL-2 would have no effect after clonal deletion. T cells that were either primed with ARC or rendered nonresponsive to ARC, were restimulated with ARC or with IL-2. It is shown in Fig. 3 *b* that T cells cultured in the primary MLR with ARC, CsA, and anti-B7 could not be restimulated with ARC. However, after culture in IL-2, these cells displayed again a strong cytotoxic activity towards ARC. It can be concluded that nonresponsiveness in this in vitro model is due to anergy induction.

To study the alloantigen specificity of the anergy induc-

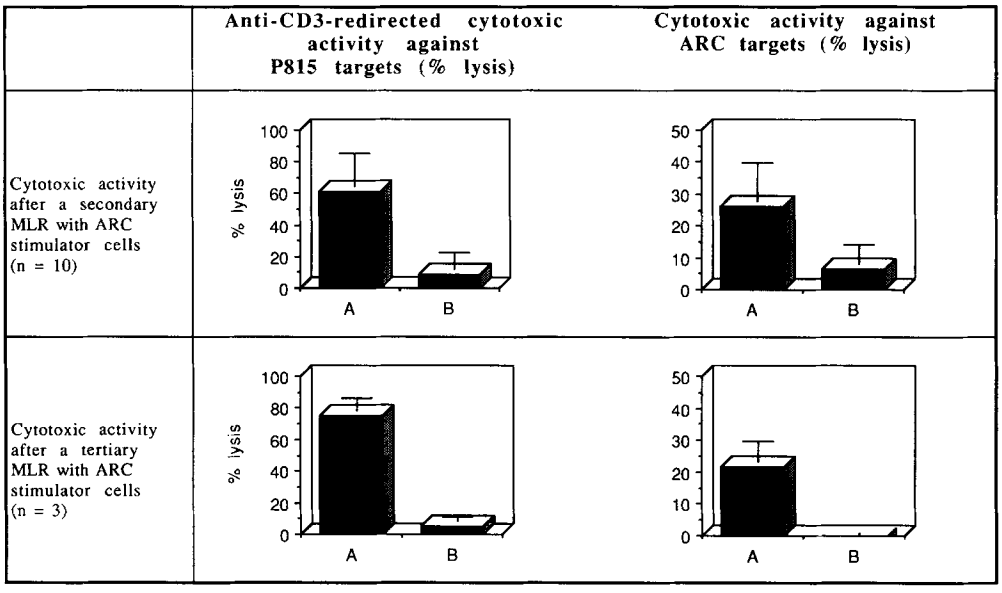

Figure 2. The combination of CsA and anti-B7 during a primary MLR induces persistent alloantigen nonresponsiveness upon restimulation in secondary and tertiary MLR. T cells were stimulated during a primary MLR, using ARC as stimulator cells, without or with mAb B7-24 (10 μg/ml) and CsA (400 ng/ml) and were then restimulated with ARC alone. Addition of anti-B7 and CsA to the primary MLR significantly reduced the cytotoxic activity after secondary MLR (n = 10, *top*) or tertiary MLR (n = 3, *bottom*), measured with the anti-CD3–redirected cytotoxic assay against P815 (*left*, Student's t test of paired samples: $p < 0.0001$) or with an antigen-specific cytotoxic assay against the original ARC stimulator cells (*right*, Student's t test of paired samples: $p < 0.005$). (*A*) T cells cultured in the primary MLR with ARC; (*B*) T cells cultured in the primary MLR with ARC, anti-B7, and CsA.

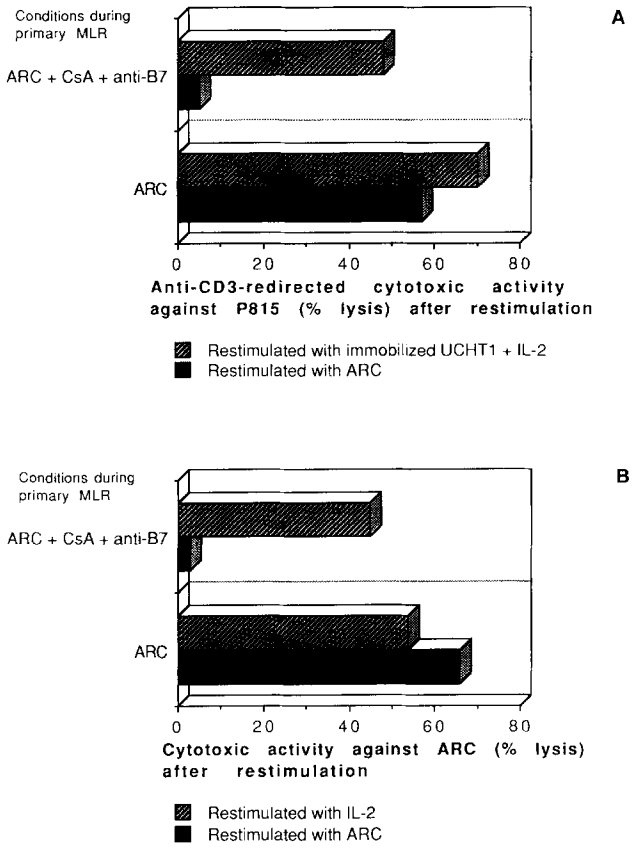

Figure 3. Restimulation with immobilized anti-CD3 (*A*) or with IL-2 (*B*) can reverse anergy. (*A*) T cells were first stimulated during 7 d with irradiated ARC cells without or with CsA (400 ng/ml) and mAb B7-24 (10 μg/ml). Thereafter, the cells were restimulated with ARC cells or with immobilized anti-CD3 (*UCHT1*) + IL-2 (10 U/ml, Boehringer Mannheim) for 4 d. Immobilization of UCHT1 was done by preincubation of the culture plates with purified UCHT1 (5 μg/ml) for 4 h at 37°C (12). CTL activity was measured in an anti-CD3-redirected cytotoxic assay against P815 mastocytoma cells (E/T = 20). The data are representative of two experiments. (*B*) T cells were primed with irradiated ARC or rendered nonresponsive against ARC during a primary MLR with the combination of CsA (400 ng/ml) and mAb B7-24 (10 μg/ml). Thereafter, cells were restimulated with ARC cells or IL-2 (10 U/ml). Antigen-specific CTL activity was measured after 4 d (E/T = 20). The data are representative of three experiments. No LAK activity against ARC was found in control experiments with unprimed T cells cultured in IL-2 (10 U/ml) alone.

tion, ARC-primed and nonresponsive T cells were restimulated once or twice with ARC cells (HLA-DR w8,w8) or third party MM (HLA-DR 1,2) cells. The T cells treated with CsA and B7-24 mAb in a primary MLR towards ARC, displayed cytotoxic activity when restimulated with MM cells, indicating that only the ARC-specific T cells were nonresponsive (Table 1).

The anergy induction obtained by alloantigen stimulation in the presence of mAb B7-24 and CsA has to be considered as an active process for several reasons. First, a minimum of 5 d culture with stimulator cells, followed by 1 or 2 d culture in medium alone, were needed to yield an anergic state (data not shown). Moreover, the activation marker HLA-DR was expressed on a proportion of the cells stimulated with alloantigen in the presence of mAb B7-24 and CsA (24 and 33% HLA-DR positive cells in two experiments), compared to 2 and 4% in the starting population, and 51 and 31% after stimulation with alloantigen alone. The cells cultured with mAb B7-24 and CsA during alloantigen stimulation did, however, not express CD25 (IL-2 receptors) (data not shown).

IL-2 has been shown to prevent the anergic state of T cells, anergized by antigen stimulation in the absence of costimulatory signals (19). Fig. 4 demonstrates that alloantigen-specific anergy induction in human T cells by CsA and mAb B7-24 could also be prevented by adding IL-2 during the primary MLR. This IL-2 effect could be blocked by a combination of mAb anti-Tac and mikβ1 against the p55 and p70 chain of the IL-2 receptor (data not shown). These data suggest the importance of the IL-2 production defect for induction

Table 1. *Alloantigen Specificity of Nonresponsiveness Demonstrated by Restimulation with the Priming Cells or Third Party Stimulator Cells*

Stimulator cells		Percent lysis of P815 cells* by	
Secondary MLR‡	Tertiary MLR‡	ARC primed cells§	ARC nonresponsive cells§
ARC	-	40	6
MM	-	ND	38
ARC	ARC	83	8
MM	ARC	ND	53
ARC	MM	84	16
MM	MM	ND	54

* After the secondary or tertiary MLR, the cells were harvested and an anti-CD3-redirected cytotoxic assay was performed with an effector/target ratio of 10:1 against anti-CD3-coated P815.

‡ After the primary MLR with ARC stimulator cells, and after a 1-d period of culture in medium alone, the cells were restimulated with ARC or with MM (T cell/stimulator cell ratio, 4:1). After 5 d of secondary MLR, the remaining cells were harvested and cultured in medium alone for 1 d. A tertiary MLR was performed with ARC or MM as indicated (T cell/stimulator cell ratio, 4:1).

§ Alloantigen-primed or nonresponsive cells were generated in a 5-d primary MLR, depending on whether T cells were stimulated with ARC in the absence (primed cells) or presence (nonresponsive cells) of anti-B7 (10 μg/ml) and CsA (400 ng/ml).

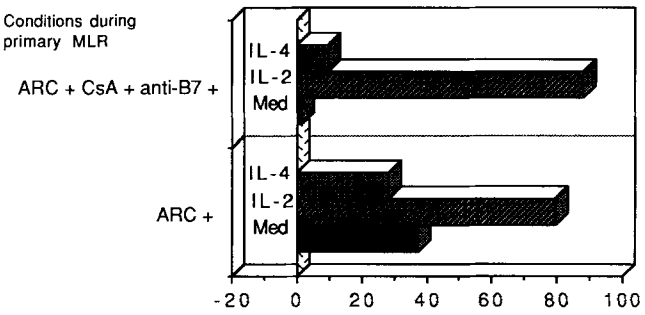

Figure 4. IL-2 prevents the induction of anergy with mAb B7-24 and CsA in a primary MLR. Purified human T cells were stimulated with irradiated ARC cells in a primary MLR (5 d) without or with CsA (400 ng/ml) and mAb B7-24 (10 μg/ml). IL-2 (10 U/ml, Hoffmann-La Roche) or IL-4 (200 U/ml) were added during the primary MLR as indicated. After the secondary MLR (4 d), CTL activity was measured in an anti-CD3-redirected cytotoxic assay against P815 mastocytoma cells.

of the anergic state. In contrast, adding IL-4 to the primary MLR in the presence of mAb B7-24 and CsA had no effect (Fig. 4).

We subsequently measured IL-2 production in a primary MLR with a bioassay as described (27). IL-2 production in the presence of mAb B7-24 decreased to 28% of the control MLR (being 75 U/ml). CsA alone lowered the IL-2 production to 21% of the control level. However, using both CsA and mAb B7-24, the residual IL-2 production was only 2% of the control level. Thus, anti-B7 and CsA together almost completely block IL-2 production in the primary MLR, and this might be responsible for anergy induction, as the latter could be prevented by addition of IL-2 during the induction phase. The failure of anti-B7 alone to induce anergy can most likely be explained by a persistent IL-2 production, induced by TCR triggering in combination with signaling from other accessory molecules such as LFA-3 or ICAM-1 on APC (5). These additional costimulatory signals could then probably be blocked by CsA. CsA can prevent T cell activation only when the activation is dependent on elevations in cytosolic Ca^{2+} (21). The CD2–LFA-3 ligation induces a phospholipase-Cγ1 tyrosine phosphorylation, regulates CD3 signaling, and induces an increased concentration of free cytoplasmic Ca^{2+} (28). It has been demonstrated that this CD2-mediated pathway of activation is highly CsA sensitive (29). Although the mechanism of costimulation through LFA-1 remains unclear, evidence for signaling through LFA-1 comes from studies in which crosslinking of T cell LFA-1 with mAbs was found to induce increased intracellular Ca^{2+} concentrations (30), which may also suggest a CsA-sensitive intracellular signal transduction. The B7–CD28/CTLA-4 interaction indeed seems to be the only signal that confers CsA-resistant IL-2 production (11, 12).

Our findings that CsA contributes to the induction of a state of anergy, are in apparent contradiction with earlier work by Jenkins et al. (1–3) who found that CsA blocked the induction of nonresponsiveness in mouse T cell clones stimulated with antigen and chemically modified APC, and suggested that a calcium signal, mediated via the TCR, was essential for induction of anergy. Several differences between the model of Jenkins and our model are obvious. We used freshly isolated human T cells and anergy was studied at the cytotoxic effector level. Fresh virgin T cells versus memory cells (T cell clones) might have totally different requirements for anergy induction, and anergy induction models have already been shown to differentially affect IL-2 versus IL-4 production (19) or proliferation versus CTL induction (24, 25).

The mechanism of how blocking of TCR signal transduction by CsA can contribute to anergy induction remains an intriguing problem. It cannot be excluded that a calcium-independent limb of the TCR signaling pathway, unaffected by CsA, is required for induction of anergy. As CsA affects late steps of intracellular signal transduction, changes in early signaling events after TCR triggering might be responsible for hyporesponsiveness after TCR triggering in the presence of anti-B7 and CsA. Alterations in tyrosine phosphorylation signaling events with reduced amounts of the protein tyrosine kinase $p56^{lck}$ and constitutively elevated levels of the protein tyrosine kinase $p56^{fyn}$ have already been described using a model of anergy induction with immobilized anti-CD3 mAb (31). Further studies are required to address these issues.

In conclusion, CTL precursors in vitro become anergic when signals derived both from CsA-resistant B7–CD28/CTLA-4 interaction and from CsA-sensitive pathways are blocked during alloantigen stimulation. This approach may have major implications for organ and bone marrow transplantations: prevention of graft rejection and of GVHD. However, further studies in animal models are required to substantiate this approach before trials in humans can be started.

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Note Added in Proof: ARC cells weakly express B70 (a second ligand for CD28, M. Azuma, et al., *Nature [Lond.]* 1993:366;76–79) as determined by staining with mAb IT2 (a kind gift from M. Azuma, Department of Immunology, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-Ku, Tokyo 113, Japan) and by PCR.

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