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Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation

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ABSTRACT **The maximal T-cell response to its antigen requires presentation of the antigen by a major histocompatibility complex class II molecule as well as the delivery of one or more costimulatory signals provided by the antigen-presenting cell (APC). Although a number of candidate molecules have been identified that are capable of delivering a costimulatory signal, increasing evidence suggests that one such critical pathway involves the interaction of the T-cell surface antigen CD28 with its ligand B7, expressed on APCs. In view of the number of potential costimulatory molecules that might be expressed on the cell surface of APCs, artificial APCs were constructed by stable transfection of NIH 3T3 cells with HLA-DR7, B7, or both. Here, we show that in a human antigen-specific model system, when tetanus toxoid peptide antigen is presented by cells cotransfected with HLA-DR7 and B7, optimal T-cell proliferation and interleukin 2 production result. In contrast, antigen presentation, in the absence of B7 costimulation, results in T-cell clonal anergy. These results demonstrate that it is possible to induce antigen-specific clonal tolerance in human T cells that have been previously sensitized to antigen. The artificial antigen-presenting system provides a useful model for the investigation of the biochemical events involved in the generation of tolerance and for the study of signals necessary to overcome tolerance.**

The maximal T-cell response to its antigen requires presentation of the antigen by a major histocompatibility complex (MHC) class II molecule and the delivery of one or more costimulatory signals provided by heterotypic adhesion between receptor ligand pairs on the T cell and the antigen-presenting cell (APC) (1–3). Signaling through the T-cell receptor (TCR) complex alone can result in a down-regulatory signal for human T-cell growth (4, 5). *In vitro* systems in the mouse have demonstrated that in the absence of a costimulatory signal, occupancy of the TCR by peptide fragments in the context of MHC class II is capable of inducing a long-lasting antigen-specific unresponsiveness, termed anergy (1, 3, 6).

Increasing *in vitro* evidence in murine and human systems suggests that one such critical costimulatory pathway involves the interaction of the T-cell surface antigen CD28 with its ligand B7 on the APC (7–10). CD28 is constitutively expressed on 95% of resting $CD4^+$ and 50% of $CD8^+$ human T lymphocytes (11, 12). After T-cell activation, CTLA4, a second higher-affinity ligand for B7 (13), is expressed and CD28 expression increases. B7 expression is limited to cells capable of presenting antigen, including activated B cells (14), activated monocytes (15), and dendritic cells (16). After T-cell activation, ligation of CD28 by anti-CD28 monoclonal antibody (mAb) or by B7 induces maximal proliferation and interleukin 2 (IL-2) secretion (7, 8, 17, 18). Two recent studies in murine systems suggest that the inhibition of the CD28–B7 pathway appears to be central to the induction of peripheral T-cell tolerance. (*i*) Anti-CD28 mAb could block the induction of anergy in murine T-cell clones (18). (*ii*) Blocking the CD28/CTLA4–B7 pathway by the *in vivo* administration of the recombinant (r) CTLA4–immunoglobulin (Ig) fusion protein prolonged xenogeneic pancreatic-islet-cell graft survival in mice and resulted in the induction of long-term antigen-specific tolerance (19).

In the present report, we demonstrate in an antigen-specific model that antigen presentation by MHC class II molecules in the absence of B7 costimulation results in human T-cell clonal anergy. In contrast, when the antigen is presented by MHC class II molecules on cells that also express B7, optimal T-cell proliferation and cytokine production are induced. These *in vitro* experiments provide a foundation to investigate the clinical utility of the inhibition of this pathway in the induction of tolerance in humans.

MATERIALS AND METHODS

Human Antigen-Specific T-Cell Clones. Tetanus toxoid (TT)-specific T cells from TT-primed HLA-$DR7^+$ individuals were cloned by limiting dilution in 96-well round-bottomed plates (Nunc) in the presence of irradiated autologous peripheral blood mononuclear cells (PBMCs) and antigen. Growing clones were further expanded and restimulated every 2 weeks with irradiated PBMCs (HLA-$DR7^+$) and antigen. The TT peptide antigen specificity of the T-cell clones was established in a proliferation assay with syngeneic adherent PBMCs pulsed with various concentrations of TT peptide.

Antigen. TT peptide corresponding to the sequence aa 830–844 (QYIKANSKFIGITE) (20) was synthesized on an Applied Biosystems model 430A peptide synthesizer and purified by reversed-phase HPLC. The peptide was used at 1 μg/ml, the concentration shown to produce optimal proliferation.

cDNA Clones and Transfection. NIH 3T3 cells, a MHC class II-negative B7-negative murine fibroblast cell line, were transfected by electroporation with DR7 (t-DR7), B7 (t-B7), or both DR7 and B7 (t-DR7/B7). For each transfection 20 μg of *Pvu* I-linearized plasmid containing the B7-pLEN construct (8) (a kind gift of California Biotechnology, Mountain View) and/or 20 μg each of the DRα and DRB1*0701 β-chain cDNAs (21) were transfected with 5 μg of *Pvu* I-linearized SV2-Neo-Sp65 plasmid (22). The expression of MHC class II and B7 molecules was assessed using mAbs 9-49 and anti-B7, respectively. After selection in medium containing G418 at 200 μg/ml, cells were sorted for B7 and DR expression by indirect immunofluorescence, cultured for 4 weeks, resorted, and cloned by limiting dilution. Transfectants expressing

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Abbreviations: TCR, T-cell receptor; mAb, monoclonal antibody; IL-2, interleukin 2; APC, antigen-presenting cell; TT, tetanus toxoid; MHC, major histocompatibility complex; h, human; Ig, immunoglobulin; r, recombinant; PMA, phorbol 12-myristate 13-acetate; IM, ionomycin.

similar levels of DR and B7 were selected for use. Full-length HLA-DR α-chain and HLA-DRB1*0701 β-chain cDNA clones in the pcD expression vector were a gift from R. Karr (University of Iowa, Iowa City).

Expression and Purification of Human CTLA4–Ig (hCTLA4–Ig). The coding sequence for the extracellular portion of hCTLA4 was joined to the heavy chain constant region C_H1–hinge–C_H2–C_H3 domains of human genomic IgG1 gene (23) by PCR using a strategy similar to that reported (13). The hCTLA4–Ig genetic fusion was cloned into expression vector pNRDSH (Repligen, Cambridge, MA) containing the neomycin-resistance gene for selection in G418 and the *DHFR* gene for amplification by methotrexate under control of the modified cytomegalovirus promoter (24). Chinese hamster ovary (CHO) cells were transfected by electroporation and cultured in α-modified minimum essential medium (α-MEM, GIBCO) with G418 (500 μg/ml). hCTLA4–Ig was purified by binding to immobilized protein A (IPA-300, Repligen), eluted, and dialyzed overnight against 0.5× phosphate-buffered saline at 4°C. hCTLA4-Ig was analyzed by SDS/PAGE and visualized using Coommassie blue staining and on Western blots using anti-hCTLA4 antisera R1438 (25). Purified hCTLA4–Ig existed primarily in the dimeric form and was detected by mAbs that recognize the native form. hCTLA4–Ig bound to its ligand, B7, as shown by its ability to immunoprecipitate hB7Ig and to stain CHO cells expressing human B7 via flow cytometry.

Proliferation Assay. Transfected murine fibroblasts (NIH 3T3) were treated with mitomycin C at 5 μg/ml for 2 h and extensively washed. The treated cells were then plated in flat-bottom 96-well plates at 2×10^4 cells per well and incubated with various concentrations of TT peptide in an overnight pulse and then washed extensively. After several washes, 5×10^4 cloned T cells were added to the cultures. Where indicated, purified anti-B7 mAb 133 (IgM) (26) or hCTLA4–Ig was added to 10 μg/ml. During the last 12 h of a 72-h culture, cells were incubated with 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine (ICN Flow, Costa Mesa, CA) and proliferation was assessed by thymidine incorporation.

Induction of T-Cell Anergy. T-cell clones (10^6 cells per well) were cultured for 48 h in a 24-well plate containing 4×10^5 mitomycin C-treated TT-peptide-treated (1 μg/ml) NIH 3T3 transfectants. The NIH 3T3 cells were separated from the T-cell clones by Percoll density-gradient centrifugation. The T cells were washed several times and added to new wells containing medium without rIL-2 for 72 h. Cells were restimulated in 96-well microtiter plates (10^6 cells per ml) with TT-peptide-treated (1 μg/ml) t-DR7, t-DR7/B7, or t-DR7/B7 (4×10^5 cells per ml) and hCTLA4–Ig at 10 μg/ml (final concentration). In parallel cultures, T-cell clones were stimulated with rIL-2 (100 units/ml) or with phorbol 12-myristate 13-acetate (PMA; 1 ng/ml) and ionomycin (IM; 100 ng/ml). Culture supernatants were harvested 24 h after the initiation of the culture. IL-2 concentrations were assayed by ELISA (Quantikine, R&D System, Minneapolis). Proliferation was measured as described above.

Quantification of Cell Death. T-cell clones (10^6 cells per ml) were cultured in 24-well plates (Nunc) in the presence of rIL-2 (100 units/ml), IM (250 ng/ml), or with TT-peptide-treated t-DR7 or t-DR7/B7. After 16 h, cells were collected and stained with saturating concentrations of fluorescein-labeled anti-CD4 mAb and the DNA intercalating dye propidium iodide at 5 μg/ml. Red and green fluorescence was then analyzed on a FACScan flow cytometer.

RESULTS

B7 Mediates Costimulation in TT-Specific T-Cell Clones. In view of the number of potential costimulatory molecules that might be expressed on the cell surface of normal human APCs, transfectants expressing HLA-DR and/or B7 were prepared to functionally isolate the B7–CD28 costimulatory pathway. These artificial APCs were constructed by stably transfecting (*i*) HLA-DR α-chain and -DR7 β-chain genes (t-DR7), (*ii*) the B7 gene (t-B7), or (*iii*) a combination of HLA-DR α-chain and -DR7 β-chain and B7 genes (t-DR7/B7) into NIH 3T3 cells. The expression of these genes is depicted in Fig. 1.

TT-peptide-specific T-cell clones were prepared from HLA-DR7-positive individuals. All T-cell clones expressed CD2, CD3, TCRαβ, CD4, CD25, CD29, CD45R0, CD69, CD71, and MHC class II. CD28 expression was weak on resting T-cell clones but increased after activation. The T-cell clones did not express B7. To determine whether TT peptide could be presented by artificial APCs, mitomycin C-treated transfectants were incubated with increasing doses of TT peptide and cultured overnight. After extensive washing to remove free peptide, artificial APCs were then cocultured with antigen-specific T-cell clones for 60 h, and proliferation was assessed. Fig. 2 *A* and *B* depicts representative results of two of three T-cell clones studied. As shown in Fig. 2*A*, t-B7 did not induce proliferation of T-cell clone 6A7 at any dose of TT peptide tested. In contrast, when T-cell clones were cultured with TT-peptide-treated t-DR7/B7, a marked proliferative response was seen, at levels similar to those observed using TT-treated "natural" APCs (data not shown). The t-DR7 was also capable of presenting TT peptide and induced low-level proliferation at doses of TT equal to or greater than 0.1 μg/ml. Proliferation induced by TT-treated t-DR7 did not significantly increase with peptide between 1 and 10 μg/ml, and in all experiments maximal proliferation was between 12 and 20% of that observed using t-DR7/B7. To demonstrate that the augmented proliferative response induced by the TT-peptide-treated t-DR7/B7 was mediated by B7 costimulation, anti-B7 mAb or soluble chimeric hCTLA4–Ig fusion protein was added to the cultures. Fig. 2*B* shows the proliferative responses of clone 5A2 and demonstrates that either anti-B7 mAb or hCTLA4–Ig fusion protein totally abrogated the augmented proliferative response. Isotype-matched control mAb added to the culture had no effect (data not shown).

Supernatants from the above cultures were collected at 24 h and IL-2 secretion was assayed. As shown in Fig. 2*C*, TT-treated t-DR7/B7 cells induced IL-2 secretion in an antigen-dose-dependent fashion. Secretion of detectable levels of IL-2 was completely blocked by the addition of anti-B7 mAb to the cultures. There was no detectable IL-2 secretion in supernatants when T-cell clones were exposed to antigen-treated t-DR7, even at the higher TT peptide concentrations that induced minimal proliferation.

Absence of B7 Costimulation Induces T-Cell Clonal Anergy. To determine whether TT antigen presentation in the absence of B7 costimulation could induce T-cell clonal anergy, T-cell

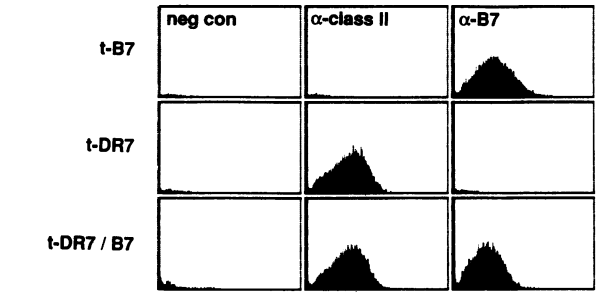

FIG. 1. Cell surface expression of B7 and MHC class II molecules on NIH 3T3 cells transfected with B7 (t-B7), HLA-DR7 (t-DR7), or both (t-DR7/B7) as assessed by flow cytometry. neg con, negative control.

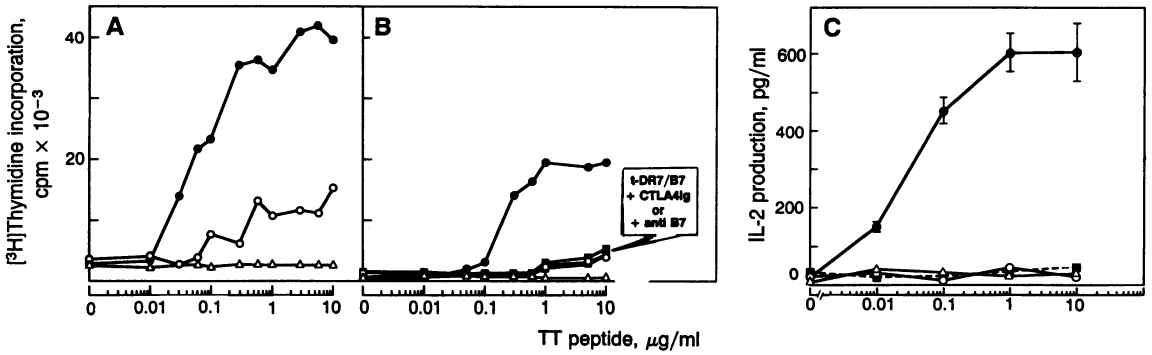

FIG. 2. Dose–response relationship of T-cell clones to TT peptide antigen presented by artificial APCs t-DR7/B7 (●), t-DR7 (○), or t-B7 (△). APCs were treated with mitomycin C, treated with TT peptide antigen, washed, and incubated with the T-cell clone in the presence or absence of anti-B7 mAb (10 μg/ml) (□) or hCTLA4–Ig fusion protein (10 μg/ml) (■). [^{3}H]Thymidine incorporation was measured for the last 12 h of a 72-h incubation. (*A*) Clone 6A7. (*B*) Clone 5A2. (*C*) Culture supernatants from the T-cell cultures in *A* were collected at 24 h and IL-2 was measured by ELISA. Dashed line, tDR7/B7 plus anti-B7 mAb.

clones were cultured with TT-peptide-treated (1 μg/ml) transfectants t-DR, t-DR/B7, or with t-DR/B7 in the presence of CTLA4–Ig (10 μg/ml). After 48 h, T-cell clones were separated from the transfectants, washed, and allowed to rest for 72 h in medium to remove TT peptide and permit reexpression of the TCR complex. After this interval, CD3 expression returned to levels observed prior to stimulation (data not shown). The T-cell clones were then restimulated with TT-peptide-treated t-DR7 or t-DR7/B7 or cultured with rIL-2 or mitogenic doses of PMA and IM.

The results obtained in these secondary responses are shown in Fig. 3. T-cell clones that had been stimulated initially with TT-peptide-treated t-DR7 demonstrated no significant proliferative response to rechallenge with TT peptide presented by either t-DR7 cells or t-DR7/B7 cells. These unresponsive T-cell clones were viable and could proliferate in response to rIL-2 or to mitogenic stimulation with PMA and IM. In contrast, T-cell clones that had been exposed to TT-peptide-treated t-DR7/B7 cells during the primary stimulation could respond to TT-peptide-treated t-DR7 or t-DR7/B7 cells. In all experiments, secondary restimulation was greater when the TT peptide was presented by the t-DR7/B7 rather than by t-DR7. T-cell proliferation induced by antigen presentation by t-DR7/B7 appeared maximal as the addition of exogenous IL-2 or mitogenic stimulation with PMA and IM did not result in increased proliferation. The induction of anergy was antigen-dependent. T-cell clones that were cultured with t-DR7 that had not been treated with TT peptide proliferated in response to TT-peptide-treated t-DR7 or t-DR7/B7 in a dose-dependent manner.

To confirm that the signal preventing the induction of anergy was mediated through the CD28/CTLA4–B7 pathway, hCTLA4–Ig (10 μg/ml) was added to the primary cultures containing TT-peptide-treated tDR7/B7 to block B7-mediated costimulation. As can also be seen in Fig. 3, blocking B7 costimulation resulted in T-cell clonal anergy. Again these cells were capable of proliferating in response to exogenous rIL-2 and the mitogens PMA and IM.

Since B7 costimulation was necessary for the dose-dependent increase in IL-2 secretion in the primary stimulation, IL-2 concentration was measured in the supernatants of the rechallenged cells. T-cell clones stimulated initially with TT-peptide-treated t-DR7 secreted no measurable IL-2 in response to subsequent rechallenge with TT peptide

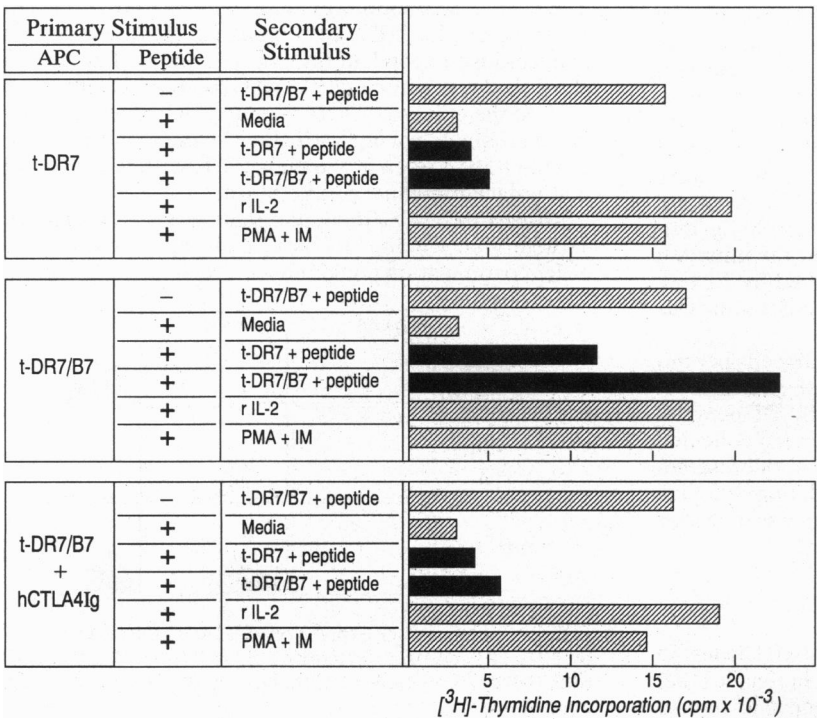

FIG. 3. T cells are tolerized by antigen presentation in the absence of B7 costimulation. T-cell clones were cultured in the primary stimulus—t-DR7, t-DR7/B7, or t-DR7/B7 plus CTLA4–Ig that had been treated with TT peptide (+) or medium (−). These cells were then allowed to rest in medium for 48 h and cultured in a secondary stimulus as shown. [^{3}H]Thymidine incorporation was measured for the last 12 h of a 72-h incubation. Shading of bars is for emphasis only.

presented by t-DR7 cells or t-DR7/B7 cells (Table 1). However, these T-cell clones secreted IL-2 in response to mitogenic stimulation with PMA and IM. In contrast, T-cell clones that had been stimulated initially with TT-peptide-treated t-DR7/B7 secreted IL-2 in response to rechallenge with TT peptide presented by t-DR7/B7 cells. However, the T-cell clones that had been stimulated initially with TT-peptide-treated t-DR7/B7 did not secrete IL-2 when restimulated with TT peptide and t-DR7 in the absence of secondary restimulation with B7. This demonstrates that the IL-2 secretion after restimulation was not the result of costimulation with B7 during the primary stimulation. T-cell clones initially stimulated by TT-peptide-treated t-DR7/B7 in the presence of hCTLA4-Ig did not secrete IL-2 in response to secondary stimulation, a pattern that is identical to the pattern seen with the T-cell clones initially stimulated with t-DR7.

TT Clones Are Viable After Primary Stimulation with t-DR7 but Rapidly Undergo Apoptosis After Removal of rIL-2. To determine T-cell viability after a tolerogenic signal, T-cell clones were cultured for 72 h with a high dose of IM, TT-peptide-treated t-DR7, or TT-peptide-treated t-DR7/B7. Cell death, as assessed by propidium iodide uptake, was not seen when clonal anergy was induced with the TT-peptide-treated t-DR7 (Fig. 4*A*) or when proliferation was induced with TT-peptide-treated t-DR7/B7 although up to 60% of the T cells died when cultured with a high dose of IM (data not shown). Since T-cell clones that had been rendered tolerant could still proliferate maximally in response to the addition of exogenous rIL-2, we attempted to determine whether these rIL-2-treated tolerized cells were now competent to respond to subsequent rechallenge with peptide presented by t-DR7/B7. After incubation of the tolerized T-cell clones with rIL-2 for 72 h, cells were allowed to rest for 48 h in medium alone. At the completion of this period, no viable cells could be isolated and propidium iodide uptake was observed in >95% of the cells (Fig. 4*B*). DNA extracted from these cells had undergone fragmentation in a pattern characteristic of apoptotic cell death (Fig. 4*C*). In contrast, cells that had been initially stimulated with TT-peptide-treated t-DR7/B7, restimulated with IL-2, and then allowed to rest in medium alone for 48 h had >50% viability, and DNA extracted from these cells had not undergone fragmentation (Fig. 4*C*). These cells were capable of proliferating in response to a third challenge using TT-treated t-DR7/B7 (data not shown). These results show that tolerized cells are much more sensitive to IL-2 deprivation. Because of their rapid cell death in the absence of IL-2, it has not been possible to demonstrate the role of IL-2 in overcoming tolerance in this *in vitro* cell model.

Table 1. IL-2 production after rechallenge of tolerized vs. activated T cells

Primary stimulus		IL-2, production after secondary stimulus, pg/ml		
APC	Peptide	t-DR7 + peptide	t-DR7/B7 + peptide	PMA + IM
Clone 6A7				
t-DR7	−	<20	750	550
	+	<20	<20	600
t-DR7/B7	−	<20	900	375
	+	<20	875	400
t-DR7/B7 + CTLA4-Ig	+	<20	<20	350
Clone 5A2				
t-DR7	−	<20	950	800
	+	<20	<20	800
t-DR7/B7	−	<20	1050	900
	+	<20	900	750
t-DR7/B7 + CTLA4-Ig	+	<20	<20	850

Secondary stimuli were t-DR7 plus TT peptide, t-DR7/B7 plus TT peptide, and PMA plus IM. Culture supernatants were collected 24 h after rechallenge and IL-2 was measured by ELISA.

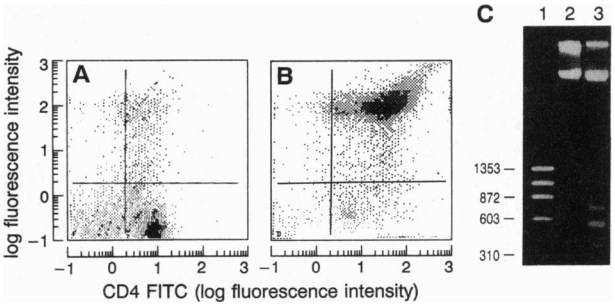

FIG. 4. Cell viability of T-cell clones after tolerization with TT-peptide-treated t-DR7 (*A*) or after culture of tolerized clones with rIL-2 and then culture in medium alone for 48 h (*B*). (*C*) Agarose gel DNA electrophoresis. Lanes: 1, molecular mass markers in bp; 2, DNA extracted from stimulated (TT-peptide-treated t-DR7/B7); 3, DNA extracted from tolerized (TT-peptide-treated t-DR7) T cells cultured with rIL-2 for 72 h and then cultured in medium alone for 48 h. FITC, fluorescein isothiocyanate.

DISCUSSION

Artificial APCs, transfected with human HLA-DR7, B7, or both, permitted us to determine the relative contributions of these molecules to the generation of immunity and tolerance. HLA-DR, peptide antigen, and B7 were necessary to induce maximal proliferation and IL-2 secretion by TT-specific T-cell clones. In contrast, presentation of HLA-DR and peptide antigen in the absence of or by specific blocking of B7 costimulation resulted in the inability of clones to proliferate or secrete detectable levels of IL-2 after rechallenge with TT-peptide-treated dual transfectants. T-cell clones tolerized by inhibition of B7 costimulation were viable and could proliferate in response to an activation signal (PMA and IM) or exogenous IL-2 alone. Although these tolerized T-cell clones could proliferate in response to exogenous IL-2, they did not regain responsiveness to antigen presented by HLA-DR7/B7 transfectants but promptly underwent apoptosis after IL-2 deprivation. These results demonstrate that B7 costimulation is sufficient to induce antigen-specific T-cell clonal proliferation and that engagement of the TCR in the absence of B7 costimulation is sufficient to induce antigen-specific clonal T-cell anergy. Antigen presentation and B7 costimulation induced maximal cellular proliferation and IL-2 secretion. Very high, probably nonphysiologic, doses of peptide antigen presented by HLA-DR alone can induce T-cell clonal proliferation that was only 20% of that observed with B7 costimulation and no detectable IL-2 was secreted.

These data demonstrate that it is possible to induce antigen-specific clonal tolerance in human T cells that have been previously sensitized to antigen. It was recently demonstrated that costimulation with murine anti-CD28 mAb could inhibit induction of antigen-specific clonal tolerance in the mouse (18). *In vivo* animal experiments have also demonstrated that CTLA4-Ig infusion could block human pancreatic islet rejection in mice and that long-term donor-specific tolerance could be induced (19). CTLA4-Ig similarly delayed rejection of cardiac allografts in a rat model (27). All the above studies suggested that the CD28/CTLA4-B7 pathway appears to be critical for the induction of antigen-specific tolerance. Our studies do not address the potential contribution(s) of other costimulatory signals to the generation of antigen-specific immunity nor do they address whether these potential alternative costimulatory signals can inhibit the generation of tolerance in humans. It was demonstrated

recently in a nonclonal alloantigen-driven human system that inhibition of the CD28/CTLA4–B7 costimulatory pathway resulted in alloantigen-specific hyporesponsiveness, but not anergy, to rechallenge with donor cells but not third-party allogeneic cells (28).

Although tolerized cells are viable and proliferate in response to exogenous IL-2, it is not known whether IL-2-treated tolerized cells will remain unresponsive to subsequent antigen challenge. This is a critical issue since the feasibility of utilizing the CD28/CTLA4–B7 pathway to induce tolerance for transplantation will require maintenance of the anergic state *in vivo* where exogenous IL-2 will likely be present in the microenvironment. In our studies, IL-2-induced proliferation of tolerized cells did not restore responsiveness to subsequent antigen rechallenge but, in contrast, resulted in apoptosis of tolerized cells upon deprivation of IL-2. The choice between long-term T-cell inactivation and T-cell death may, therefore, be determined by the level of IL-2 present in the microenvironment.

The development of techniques to tolerize T lymphocytes *in vitro* will likely facilitate the development of *ex vivo* strategies to induce antigen-specific tolerance. The system that we have developed, employing T-cell clones, peptide antigen, and artificial APCs, provides useful tools for the investigation of the biochemical events involved in the generation of tolerance as well as the discovery of those signals that might overcome tolerance.

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