Rapamycin-resistant Proliferation of CD8+ T Cells Correlates with p27kip1 Down-regulation and bcl-xL Induction, and Is Prevented by an Inhibitor of Phosphoinositide 3-Kinase Activity*

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Rapamycin inhibits the proliferation of many mammalian cell types, including lymphocytes, making the compound useful as an immunosuppressant. Rapamycin has also been a useful tool for studying signaling mechanisms regulating cellular proliferation. However, the effects of rapamycin remain poorly understood, and the precise mechanisms of clinical action remain elusive. Previously, we found that, depending on the strength of the signal delivered to the T cell via both the T cell receptor and the costimulatory molecule CD28, CD8+ T cells are capable of rapamycin-resistant proliferation. Here, we have further elucidated the mechanism of rapamycin-resistant proliferation of human CD8+ T cells. Under conditions where rapamycin inhibited proliferation, p27kip1 down-regulation was prevented, whereas under conditions resulting in rapamycin-resistant proliferation, p27kip1 was down-regulated. Further, T cell receptor/CD28-dependent induction of bcl-xL expression was not inhibited by rapamycin, which correlated with both rapamycin-resistant proliferation and increased cell survival. Moreover, an inhibitor of phosphoinositide 3-kinase activity was able to eliminate rapamycin-resistant proliferation of freshly isolated CD8+ human cells, strongly suggesting that phosphoinositide 3-kinase activity is required for the rapamycin-resistant proliferation of CD8+ T cells. The selective immunosuppressive effect of rapamycin in human CD8+ T cell populations could be predictive of a selective effect allowing cytotoxic responses during microbial infections where there are strong strengths of signals associated with high affinity T cell receptors and strong costimulatory second signals. In contrast, the weaker autoimmune and perhaps allogeneic responses can be selectively inhibited by the actions of rapamycin.

Rapamycin is an immunosuppressant that functions by inhibiting entry into the cell cycle. This inhibition of proliferation by rapamycin occurs at a later stage of cellular activation than the inhibition by cyclosporin A and FK506, which inhibit calcineurin or calcineurin-dependent transcriptional activation of lymphokine genes (Ref. 1; reviewed in Ref. 2). It is thought that rapamycin primarily inhibits growth factor signaling rather than growth factor synthesis (3). The potent immunosuppressive activity of rapamycin has led to its wide clinical use in solid organ transplantation (reviewed in Ref. 4). More recent studies demonstrated successful islet cell transplantation in patients with type I diabetes treated with low dose rapamycin in combination with humanized anti-IL-2 receptor monoclonal antibody (mAb) and FK506, which prevented autoimmune destruction of islet cells (5). However, the clinical mechanism of action of rapamycin in suppressing immune responses is not well defined.

Although it is clear that entry into the cell cycle is generally inhibited by rapamycin, we recently made the surprising observation that increasing the strength of signal delivered to the T cell via both the T cell receptor (TcR) and the costimulatory molecule CD28 allowed subpopulations of CD8+ T cells to proliferate in the presence of rapamycin (6). In fact, CD8+ T cells isolated and stimulated ex vivo often exhibited increases in 3Hthymidine uptake following exposure to rapamycin in vitro. The rapamycin-resistant proliferation was not dependent on p70s6k activation, and the mobility shift that corresponds to phosphorylation of p70s6k was inhibited even under conditions resulting in rapamycin-resistant proliferation. However, the antigen-driven, rapamycin-resistant proliferation of a T cell clone could be converted to rapamycin-sensitive in the presence of neutralizing anti-IL-2 antibodies.

These observations in both antigen-reactive CD8+ T cell clones and CD8+ T cells isolated ex vivo provide a novel model for studying the intracellular mechanism of action of rapamycin, about which a great deal is known. The inhibition of the mammalian target of rapamycin (mTOR) by the FKBP12/rapamycin complex halts cell cycle progression at the G1-S transition. mTOR activity appears to be upstream of several pathways involved in regulating progression through the cell cycle, including p70s6k (7), cyclin/cdk activity (8, 9), and 4EBP (PHAS) (Refs. 10–12; reviewed in Ref. 13). Both p70s6k and 4EBP are involved in regulating translation via different mechanisms. p70s6k phosphorylates the 40S ribosomal subunit, and thereby controls synthesis of a subset of proteins, whereas 4EBP is the suppressor of eukaryotic initiation factor 4E and thereby regulates many proteins involved in the cell cycle.

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1 The abbreviations used are: IL, interleukin; mAb, monoclonal antibody; FCS, fetal calf serum; TcR, T cell receptor; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; CFSE, carboxyfluorescein diacetate, succinimidyl ester; FITC, fluorescein isothiocyanate.

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Phosphorylation of p70S6K by mTOR is thought to promote its activity, and phosphorylation of 4EBP reduces its affinity for 4E, releasing its blockade of translation. mTOR has been shown to phosphorylate specific residues on both 4EBP and p70S6K (12).

An emerging concept in the regulation of immune responses relates to the strength of signal delivered to the T cell. This occurs with formation of an immune synapse between the antigen-presenting cell and the T cell and depends on both the antigen-specific interaction and critical contributions of co-receptors (14, 15). Thus, the differential effects of rapamycin on CD8+ T cell entry into the cell cycle, as related to the integrated strength of signal delivered through the immune synapse, are likely to have an important influence on the cellular mechanism of action of the drug.

Here, we examined the biochemical relationship between the strength of signal delivered through the immune synapse and the proliferation of CD8+ T cells in the presence of rapamycin. We found that p27kip1 down-regulation, unlike p70S6K phosphorylation, correlated with rapamycin-resistant proliferation of both a human CD8+ T cell clone and freshly isolated human CD8+ T cells. Further, CD3/CD28-dependent induction of bcl-xL expression was not inhibited by rapamycin treatment, and this correlated with [3H]thymidine incorporation. Finally, using a panel of inhibitors of specific signaling pathways, we discovered that a PI3K inhibitor was able to eliminate rapamycin-resistant proliferation. Under these conditions, p70S6K down-regulation was blocked and bcl-xL expression was not induced. Therefore, rapamycin-resistant proliferation of CD8+ human peripheral blood T cells requires PI3K activity and is accompanied by both p27kip1 down-regulation and bcl-xL induction. Thus, the down-regulation of p27kip1 in association with bcl-xL induction correlates with the escape of human CD8+ T cells from rapamycin inhibition, and suggests novel methods of immune monitoring in patients undergoing rapamycin therapy.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Lymphocytes were isolated from the whole blood of normal donors after platelet phoresis by centrifugation through Ficol-Paque (Amersham Biosciences, Uppsala, Sweden) and plastic adherence. CD8+ T cells were positively selected following incubation with anti-human CD8 microbeads using the Dynabead magnetic cell separation system (Dynal Inc., Lake Success, NY). Purified CD8+ T cells were rested overnight at 37 °C before use. CD8+ T cells were cultured at 37 °C with 5% CO2 in RPMI 1640 (Biowhittaker, Walkersville, MD) with 10% heat-inactivated fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, and 2 mM l-glutamine (all from Biowhittaker) (termed complete RPMI). CD8+ human T cell clones reactive to human T-cell lymphotropic virus type I peptide Tax11–19 were generated as described, they have been extensively characterized functionally elsewhere (6, 16). For use in experiments, T cell clones were thawed, restimulated with phytohemagglutinin plus irradiated mononuclear cells, and expanded in RPMI 1640 containing 10% heat-inactivated pooled human serum (Omega Scientific, Tarzana, CA), 10% T-stim (Collaborative Biomedicine Products, Bedford, MA), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, and 2 mM l-glutamine (all from Biowhittaker).

Proliferation Assay—Cells were pretreated with rapamycin (Calbiochem-Novabiochem Corp., San Diego, CA) or ethanol (vehicle control) for 30 min to 2 h before they were used in proliferation assays. Plates were coated with anti-CD3 (1 μg/ml) murine anti-human CD3 mAb OKT3 (ATCC), or with anti-CD28 (5 μg/ml murine anti-human CD28 mAb 3D10), as indicated, at 37 °C for at least 2 h and then washed five times with two volumes of PBS to remove free antibody prior to use. T cells were plated in 96-well round-bottom plates (Costar, Cambridge, MA) at 104/well (in triplicate) in a final volume of 200 μl and cultured for 3–5 days as indicated. Where indicated, LYT298 (42 μM) was included in culture use. (Calbiochem-Novabiochem). Proliferation was assessed by the incorporation of [3H]thymidine (PerkinElmer Life Sciences), after harvest (Tomtec, Orange, CT) following an 18-h pulse. Incorporation was measured using a liquid scintillation counter (Wallace, Gaithersburg, MD).

Western Blots—Human purified CD8+ T cells or CD8− T cell clones were cultured in the presence or absence of rapamycin for the time period indicated, in 24-well plates coated with either anti-CD3 or anti-CD3 plus anti-CD28. Following incubation, cells were washed twice with cold RPMI containing 1 mM sodium orthovanadate (Sigma) and either frozen in liquid nitrogen or processed immediately. Cells were lysed for 15 min on ice in cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Detergent extracts were clarified by centrifugation at 14,000 × g for 10 min at 4 °C. The resulting supernatants were harvested and separated by electrophoresis through SDS-polyacrylamide gels (SDS-PAGE) (Protogel, National Diagnostics, Atlanta, GA). Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA), and immunoblotted with anti-ε-Myc, anti-p27kip1, anti-Bcl-xL, anti-p70S6K (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin D2 or anti-cyclin D3 (Neomarkers, Freemont, CA), as indicated, and detected by enhanced chemiluminescence (ECL) (Amersham Biosciences) according to the instructions from the manufacturer. Where indicated, blots were stripped (Chemicon International, Inc., Temecula, CA), reblocked, and reprobed with antibody.

Carboxyfluorescein Diacetate, Succinimidyl Ester (CFSE) Labeling—Cells were labeled with CFSE following the protocol of Lyons (17). Briefly, cells were washed and resuspended in PBS at a concentration of 2 × 106/ml. An equal volume of 0.25 μM CFSE (Molecular Probes, Eugene, OR) was added, and cells were incubated for 5 min at room temperature with intermittent mixing. After 5 min, an equal volume of heat-inactivated FCS was added and the cells were incubated for an additional 1 min at room temperature, spun down, and washed three times with complete RPMI. Cells were plated at a concentration of no more than 1 × 106/ml and stimulated as indicated. After stimulation, cells were harvested, spun down, and resuspended in fluorescence-activated cell sorting medium (PBS containing 2% heat-inactivated FCS). Samples were run on a Becton Dickinson FACScan, and data were analyzed using CellQuest.

Annexin V Staining—Human purified CD8+ T cells were cultured in the presence or absence of rapamycin in 48-well plates coated with anti-CD3 plus anti-CD28. Cells were harvested, washed with PBS, and labeled with annexin V-FITC, propidium iodide, or both using an Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) according to instructions from the manufacturer. Briefly, cells were incubated for 15 min in the dark at room temperature in 100 μl of binding buffer containing the appropriate labeling reagent, followed by the addition of 400 μl of binding buffer. Samples were run on a FACScan, and data were analyzed using CellQuest.

RESULTS

CD8+ T Lymphocytes Undergo Cell Division despite the Presence of Rapamycin—Proliferation of CD8+ T cells and a subset of CD8+ T cell clones induced by higher concentrations of plate-bound anti-CD3 mAb plus anti-CD28 mAb (or a high concentration of anti-CD3 alone for clones) was resistant to inhibition by rapamycin (6). Given that CD8+ T cell clonal expansion ex vivo as measured by [3H]thymidine incorporation after co-cross-linked anti-CD3 (10 or 50 μg/ml) and anti-CD28 (1 μg/ml) was partially blocked by rapamycin, and that only a subset of the clones tested exhibited rapamycin-resistant proliferation, it was possible that only a subset of circulating T cells were resistant to the effects of rapamycin. To validate the results of the thymidine incorporation assay, we used a second method to confirm rapamycin-resistant proliferation of the CD8+ T cells. T cells were labeled with CFSE. CFSE is incorporated into the cell, where it irreversibly couples to both intracellular and cell surface proteins. As the labeled cell divides, the CFSE is evenly divided among the daughter cells such that each generation has half the fluorescent intensity of the parent; this can be readily assessed using flow cytometry (17). Using this technique, we compared the proliferating population of CD8+ T cells in the presence and absence of rapamycin. CD8+ T cells were labeled with CFSE and cultured for 5 days in uncoated or anti-CD3/anti-CD28-coated plates in the presence or absence of 1 μM rapamycin (Fig. 1A). On day 5, cells were harvested and analyzed by flow cytometry. Unlabeled
Mechanism of Rapamycin-resistant CD8+ T Cell Proliferation

Fig. 1. CD8+ T cells can divide in the presence of rapamycin. A, human CD8+ T cells were washed and labeled with a final concentration of 0.125 μM CFSE (diluted in PBS) for 5 min at room temperature with intermittent mixing. An equal volume of PCS was then added, and cells were incubated an additional 1 min at room temperature and washed three times with complete RPMI. Cells were plated in uncoated wells or wells coated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml), in the absence or presence of 1 μM rapamycin. Five days later, cells were harvested, washed, and analyzed on a Becton Dickinson FACScan. Data from a representative experiment are shown. B, the results of five separate experiments were compiled. Cells were grouped into three categories based on the number of divisions (0, 1, or 2 or more) they had undergone at the time of analysis. Numbers were plotted as the mean percentage of total number of gated cells in each category ± standard deviation.

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Rapamycin-resistant Proliferation Correlates with p27kip1 Down-regulation in Response to Stimulation—Several pathways, and specific proteins in these pathways, involved in progression through the cell cycle that are induced in response to stimulation are affected by rapamycin. By example, rapamycin has been shown to inhibit the induction of c-Myc expression in response to stimulation in cells that are sensitive to inhibition by rapamycin (18). Conversely, rapamycin resistance in tumor cell lines has been shown to correlate with the inability of the drug to inhibit the induction of c-Myc (18). Given this, we examined the induction of c-Myc expression in freshly isolated CD8+ human T cells after stimulation under rapamycin-resistant conditions. Freshly isolated human CD8+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of rapamycin (Fig. 2A). Although c-Myc was absent in unstimulated cells, it was induced upon stimulation with anti-CD3 and anti-CD28, and this expression was not prevented by treatment with rapamycin (Fig. 2A). Thus, in freshly isolated human CD8+ T cells, rapamycin did not inhibit c-Myc induction under conditions leading to rapamycin-resistant proliferation.

p27kip1 is a negative regulator of the cell cycle, which is normally down-regulated in response to cytokines or growth factors, permitting cell cycle progression (19). Thus, a diminution of p27kip1 levels is seen following proliferative stimulation; conversely, non-proliferating cells have high basal levels of p27kip1. IL-2 is capable of providing such a signal, and exposure to IL-2 can promote the down-regulation of p27kip1, thereby enabling cell division (9). Rapamycin has been shown to prevent the down-regulation of p27kip1 (9, 20), and rapamycin resistance in murine cells was associated with the loss of the cyclin-dependent kinase inhibitor p27kip1 (21). Given these results, we examined the expression of p27kip1 in human CD8+ T cell clones in response to stimulation in the presence or absence of rapamycin. The human CD8+ T cell clone TP7 was pretreated with either vehicle control or 1 μM rapamycin and then exposed to plate-bound anti-CD3. Base-line expression of p27kip1 is evident in the unstimulated samples at both the 15- and 24-h time points (Fig. 2B). However, after these cells were stimulated with plate-bound anti-CD3 for either 15 or 24 h, p27kip1 levels diminished (Fig. 2B). Notably, treatment with rapamycin did not change the level of down-regulation relative to untreated, stimulated T cells. Correlation of the entry into the cell cycle with p27kip1 expression was verified by the results of a thymidine incorporation assay set up simultaneously (Fig. 2C). Proliferation induced by 1 or 5 μg/ml plate-bound anti-CD3 mAb was not inhibited by the presence of rapamycin at concentrations as high as 500 nM (Fig. 2C).

It was important to confirm these results in freshly isolated human CD8+ peripheral blood T cells. As with the T cell clone, robust base-line expression of p27kip1 was evident at both 24- and 48-h time points (Fig. 2D). Stimulation with anti-CD3 alone for 24 h resulted in a slight diminution of p27kip1 expression; this diminution was more obvious when cells were stimulated with both anti-CD3 and anti-CD28. At both time points, the expression of p27kip1 was dramatically decreased after CD3/CD28 stimulation as compared with expression in unstimulated cells. In both instances, this down-regulation was not affected by the addition of 1 μM rapamycin. However, the difference in the levels of p27kip1 in the unstimulated versus the stimulated cells was maximal at 48 h (Fig. 2D); therefore, further experiments examining p27kip1 expression were carried out at 48-h time points. The down-regulation of p27kip1 correlated well with the proliferation results from the same experiment (Fig. 2E). Consistent with our previous results (6), [3H]thymidine incorporation was induced by stimulation with anti-CD3 alone (Fig. 2E and inset); this proliferation was readily inhibited by either a minimal dose of 1 nM or a maximal...
A dose of 1 μM rapamycin (Fig. 2E, inset). However, the proliferation induced by the combination of anti-CD3 and anti-CD28 was resistant to both 1 nM and 1 μM rapamycin. Therefore, because the effects were the same, we used 100 nM, 0.5 μM, or 1 μM rapamycin throughout this report. Thus, we found that, in contrast to p70^65S^ activation, levels of p27\(^{kip1}\) correlated with the functional proliferative phenotype.

Expression of Cyclin D Can Be Induced in CD8\(^+\) T Cells in the Presence of Rapamycin—Another level at which progression through cell cycle is regulated is the timing of expression of the cyclins, the regulatory component of the cyclin-dependent kinases (reviewed in Refs. 22 and 23). The D-type cyclins appear to be involved early on in the response to incoming mitogenic signals. There are three cyclin D family proteins, cyclins D1, D2, and D3. The effect of rapamycin on cyclin D1 is somewhat controversial; some have shown expression of cyclin D1 to be unaffected by rapamycin (24, 25), whereas others have found growth factor-induced expression of cyclin D1 to be unaffected by rapamycin (26). Regardless, because T cells do not express cyclin D1 (27), the induction of the cyclins D2 and D3 is a possible target of the anti-proliferative effects of rapamycin (3, 28). Given these previous data and our p27\(^{kip1}\) data described herein, we undertook a kinetic study of the effects of rapamycin on specific proteins involved in the progression through cell cycle in CD8\(^+\) T cells.
Mechanism of Rapamycin-resistant CD8+ T Cell Proliferation

**Fig. 3.** The kinetics of cyclin D expression in the presence of rapamycin. A, freshly isolated CD8+ T cells (4.5 × 10^6) were treated with ethanol (−) or with rapamycin (+) and left unstimulated (−) or stimulated (+) with plate-bound anti-CD3 (1 μg/ml) + anti-CD28 (5 μg/ml) for the time period indicated. Following the incubation, cells were lysed and proteins were separated on 10% SDS-PAGE. Proteins were transferred to PVDF, and the membrane was cut into two halves. The top half was immunoblotted with anti-c-Myc (top panel), whereas the bottom was probed with anti-cyclin D2 (middle panel), and the proteins were visualized by ECL. The bottom half of the membrane was then stripped and reprobed with anti-cyclin D3 (bottom panel), and the proteins were visualized by ECL. B, the number of experiments (on the y axis) in which rapamycin inhibited cyclin D2 expression (open bars) is plotted against the number of experiments in which rapamycin did not inhibit cyclin D2 expression (filled bars) at various time points (on the x axis).

The time periods indicated. Following stimulation, cells were collected and lysed and proteins separated by 10% SDS-PAGE. Proteins were transferred to PVDF, which was separated into two halves; the top and bottom halves were immunoblotted with anti-c-Myc and anti-cyclin D2, respectively (Fig. 3A, top and middle panels). Stimulation with anti-CD3 and anti-CD28 resulted in the detection of cyclin D2 protein, which peaked between 7 and 16 h (data not shown and Fig. 3A). D2 protein expression was still evident at 24 h but was often undetectable by 48 h. c-Myc expression followed a similar time course, with expression evident by 7.5 h (data not shown and Fig. 2A), continued expression at 16 and 24 h and no expression by 48 h (Fig. 3A). The expression of these proteins was unaffected by the presence of rapamycin. In contrast, when the bottom half of the membrane was reprobed with anti-cyclin D3, there was a dramatic shift in the time course of expression upon exposure to rapamycin (Fig. 3A, bottom panel). In stimulated T cells, cyclin D3 expression was evident at 16 h, maximal at 24 h, and undetectable by 48 h. When the cells were treated with rapamycin, the kinetics of expression appeared to be shifted, with expression first detectable at 24 h that was maximal by 48 h and gone by 72 h. There was variability in these experiments with respect to cyclin D2 expression at early time points; a graphic summary of these results is shown in Fig. 3B. That is, at earlier time points (8 h or less), cyclin D2 expression was variably inhibited by rapamycin; at 24 h, rapamycin failed to inhibit cyclin D2 expression in most experiments, whereas by 48 h or later, rapamycin did not inhibit the expression of cyclin D2 when it was still evident. Cyclin D3 expression was consistently inhibited (though not abrogated) by rapamycin at the early time points examined. Thus, although rapamycin may delay the expression of cyclin D2 and D3 in human CD8+ T cells, it does not block the induction of cyclin D expression, a finding consistent with our proliferation results.

**Fig. 4.** Stimulation of CD8+ T cells in the presence of rapamycin increases the number of live cells. A, freshly isolated CD8+ T cells (2 × 10^6) were pretreated with ethanol or with rapamycin (lower right panel), left unstimulated (panels on left) or stimulated (panels on right) for 3 days with plate-bound anti-CD3 (1 μg/ml) + anti-CD28 (5 μg/ml). Cells were harvested, stained with annexin V-FITC and propidium iodide, and analyzed using a FACScan. The upper left panel shows unstained cells. B, numbers are shown as percentage of unstimulated control. The mean plus standard deviation of five separate experiments is shown, with a statistically significant difference between the rapamycin-treated and untreated groups.

Rapamycin Exposure Increases the Number of Live CD8+ T Cells upon Stimulation with Anti-CD3 and Anti-CD28—We observed that the proliferation of rapamycin-treated CD8+ T cells (as measured by either thymidine incorporation or CFSE dilution) in response to a strong signal is equal to or greater than that of similarly stimulated control cells (Fig. 2, C and E). One possible explanation is that rapamycin prevents activation-induced cell death, resulting in a greater number of living cells better able to respond to the stimulation as measured in our assays. To test this hypothesis, we assessed the number of live versus apoptotic versus dead cells after stimulation in the presence and absence of rapamycin. Annexin V binds to exposed phosphatidylserines on the cell membrane; this exposure is the result of membrane disruption characteristic of and preceding apoptosis. Propidium iodide is excluded from live cells with intact cell membranes and readily taken up by cells with compromised membrane integrity. Thus, cells that are negative for both propidium iodide and annexin staining are living cells, annexin-positive cells are thought to be apoptotic, and propidium iodide-positive cells are dead. Cells that are double positive could be either dead or dying; it is impossible to make this distinction with these dyes. CD8+ T cells were labeled with annexin V-FITC and propidium iodide. The number of live, dead, and dying cells was determined using flow cytometry (Fig. 4A). The lower left quadrant shows cells that stained with neither annexin nor FITC; these are live cells. After 18–48 h of culture, 78–87% of the stimulated cells were
alive, with no obvious difference between rapamycin-treated and untreated cells (data not shown). However, after 3 days of culture, only 66% of the cells remained alive (Fig. 4A, lower left panel, lower left quadrant). The number of live cells was further reduced to 42% after stimulation with anti-CD3 and anti-CD28 (Fig. 4A, upper right panel, lower left quadrant). However, the percentage of live cells was dramatically increased to 64% by the presence of rapamycin (Fig. 4A, lower right panel, lower left quadrant). The percentage of live cells after stimulation (∓ rapamycin) was plotted as percentage of unstimulated, live cells (Fig. 4B). The mean and standard deviations of five separate experiments are shown, revealing a statistically significant difference between the control and the rapamycin-treated group (p < 0.05). Thus, stimulation with anti-CD3/anti-CD28 in the presence of rapamycin resulted in a greater number of live cells than did the same stimulation in the absence of rapamycin.

*bcl-xL* Induction in Response to Anti-CD3 and Anti-CD28 Stimulation Was Not Prevented by Rapamycin—The signal for rapamycin-resistant proliferation of freshly isolated human CD8+ peripheral blood T cells was provided by the simultaneous engagement of CD3 and CD28; other co-receptors failed to provide this signal for rapamycin-resistant proliferation (6). The expression of the cell survival factor bcl-xL is induced upon simultaneous engagement of CD3 and CD28; other co-receptors failed to provide this signal for rapamycin-resistant proliferation (6). The expression of bcl-xL, under conditions leading to rapamycin-resistant proliferation, CD8+ human peripheral blood T cells were left unstimulated, or were stimulated with plate-bound anti-CD3 alone or in combination with plate-bound anti-CD28, in the presence or absence of 1 μM rapamycin. After 48 h, cells were collected and lysed and proteins separated by 12% SDS-PAGE. Proteins were transferred to PVDF and immunoblotted with anti-bcl-xL antibody (Fig. 5A, top panel). Unstimulated cells expressed no detectable bcl-xL protein; induction with anti-CD3 stimulation was minimal. Simultaneous stimulation with both anti-CD3 and anti-CD28 resulted in a robust increase in the expression of bcl-xL protein. Again, the minimal expression of bcl-xL induced by stimulation with plate-bound anti-CD3 alone was prevented by the presence of rapamycin, whereas the addition of CD28 stimulation resulted in the induction of bcl-xL expression that correlated with both rapamycin-resistant proliferation and increased cell survival.

This membrane was stripped and reprobed with anti-p27\kip1 antibody (Fig. 5A, bottom panel). p27\kip1 expression was inversely correlated with the bcl-xL expression. As in Fig. 2, resting, unstimulated cells demonstrated high basal levels of p27\kip1 protein. p27\kip1 was nearly abrogated by stimulation with anti-CD3 and anti-CD28; this was not prevented by the exposure to rapamycin. We found that bcl-xL expression was not inhibited by rapamycin; thus, the induction of bcl-xL expression correlated with rapamycin-resistant proliferation. Further, the induction of bcl-xL expression and the stimulation of p27\kip1 down-regulation were inversely correlated; this is most clearly illustrated by the graphic depiction of densitometric analysis done on the gel (shown in Fig. 5B). Both the induction of bcl-xL expression and the stimulation of p27\kip1 down-regulation by anti-CD28 plus anti-CD3 were unchanged by exposure to rapamycin.

The PI3K Inhibitor LY294002 Abrogates Rapamycin-resistant Proliferation of CD8+ T Cells—To determine which intracellular signaling pathways were required for the rapamycin-resistant CD8+ T cell proliferation, we employed a panel of inhibitors of specific signaling pathways (data not shown). Reversal of the rapamycin-resistant proliferation with a specific inhibitor would implicate the targeted pathway in rapamycin-resistant proliferation. CD8+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of rapamycin and in the presence or absence of LY294002, a specific inhibitor of PI3K activity. Proliferation after 4 days was assessed by [3H]thymidine incorporation (Fig. 6A). The combination of anti-CD3 and anti-CD28 induced robust proliferation, which was unaffected by the addition of either rapamycin or LY294002. However, in the presence of both drugs, proliferation was dramatically inhibited (Fig. 6A).

The requirement for PI3K activity was confirmed with a CFSE dilution assay. CD8+ T cells were labeled with CFSE, and then cultured for 5 days in the presence or absence of rapamycin, and LY294002. Unstimulated cells showed a uniform peak whereas untreated, stimulated cells underwent multiple cell divisions, as indicated by diminishing fluorescence. The addition of rapamycin did not change the number of cell divisions observed with stimulation; the addition of LY294002 alone resulted in less cell division. When both drugs were used in tandem, there was a complete absence of cell division in this assay, consistent with the results of the [3H]thymidine assay (Fig. 6A).

Next, we examined the down-regulation of p27\kip1 and the induction of bcl-xL in the presence of the PI3K inhibitor. CD8+ T cells were left unstimulated (Fig. 6C, lanes 1–4), stimulated with anti-CD3 alone (Fig. 6C, lanes 5–7 and 9), or stimulated with anti-CD3 and anti-CD28 (Fig. 6C, lanes 8 and 10–12) for 48 h. During this time course, some cells were exposed to rapamycin alone (Fig. 6C, lanes 2, 6, and 10), LY294002 alone (Fig. 6C, lanes 3, 7, and 11) or both rapamycin and LY294002 (Fig. 6C, lanes 4, 9, and 12). Following the 48-h incubation, cells were lysed, and proteins were separated on 12% SDS-PAGE. Proteins were transferred to PVDF and immunoblotted with anti-p27\kip1 (Fig. 6C, upper panel). As in Figs. 2 and 4, basal p27\kip1 expression was abrogated by stimulation with
Inhibition of PI3K activity abrogates rapamycin-resistant proliferation, p70^{S6K} down-regulation, and bcl-x<sub>L</sub> induction.

A, 96-well, round-bottom plates were coated with 1 μg/ml anti-CD3 + 5 μg/ml anti-CD28. Human peripheral blood CD8<sup>+</sup> T cells were plated ± rapamycin, ± LY294002. Proliferation was assessed at 4 days by the incorporation of [3H]thymidine. The average of triplicate wells from a representative experiment is shown.

B, human CD8<sup>+</sup> T cells were washed and labeled with a final concentration of 0.125 μM CFSE (as in Fig. 1). Cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml), in the absence or presence of rapamycin = LY294002. Five days later, cells were harvested, washed, and analyzed on a Becton Dickinson FACScan. C, CD8<sup>+</sup> T cells (2 × 10<sup>5</sup>) were pretreated with ethanol (lanes 1, 5, and 8), rapamycin (lanes 2, 4, 6, 9, 10, and 12), or LY294002 (lanes 3, 4, 7, 9, 11, and 12). They were left unstimulated or stimulated for 48 h with plate-bound anti-CD3 (1 μg/ml) (lanes 5-7 and 9), or with anti-CD3 (1 μg/ml) + anti-CD28 (5 μg/ml) (lanes 8 and 10-12). Lysates were prepared and p27<sup>kip1</sup> detected as in Fig. 2 (upper panel). The blot was stripped and reprobed with anti-bcl-x<sub>L</sub>, antibody, and proteins were visualized by ECL (lower panel). D, the gel shown in C was subjected to densitometric analysis using NIH Image. Numbers represent densitometric units obtained after background subtraction and are plotted as bcl-x<sub>L</sub> values versus p27<sup>kip1</sup> values. E, CD8<sup>+</sup> T cells (5.4 × 10<sup>5</sup>) were treated with ethanol, rapamycin, and/or LY294002 and left unstimulated or stimulated for 7.5 h with plate-bound anti-CD3 (1 μg/ml) + anti-CD28 (5 μg/ml), as indicated. Lysates were prepared, and c-Myc was detected as in Fig. 2 (upper panel). Blot was stripped and reprobed with anti-bcl-x<sub>L</sub> antibody, and proteins were visualized by ECL (lower panel).

**DISCUSSION**

Rapamycin is widely used as an immunosuppressive agent in the treatment of transplant rejection and is undergoing trials in patients with autoimmune disorders. Upon binding to its intracellular binding protein (FKBP12), the activity of mTOR is blocked. Rapamycin is regarded as a global inhibitor of cell cycle progression, although patients do not experience a higher incidence of infections that would suggest a global state of immunosuppression (clinical trials reviewed in Ref. 4). We
recently demonstrated that rapamycin does or does not suppress T cell proliferation depending upon the strength of the signal delivered to the T cell, providing an in vitro correlate with the observed in vivo actions of the drug. Here, we examined the biochemical basis for this observation.

The strength of signal delivered through the immune synapse formed by engagement of the T cell receptor with major histocompatibility complex-peptide complex along with associated costimulatory coreceptor interactions determines the functional outcome of the activating signal. Lower strengths of signals associated with recognition of self antigen without second costimulatory signals results in either anergy or entry into the cell cycle with secretion of cytokines that generally down-regulate immune responses such as IL-4 and transforming growth factor-β (30). Pathologic autoimmune responses appear to involve stronger stimulatory signals that are still quantitatively lower than the signaling events associated with response to microbial pathogens. Thus, immunosuppressants that selectively inhibit cell cycle entry depending on the strength of signal may provide a means of more specific inhibition of immune responses. The in vitro assay we describe here for CD8+ T cells allows examination of immune responses in relationship to the integrated T cell strength of signal.

The mechanism of action for rapamycin is well studied in single cell organisms. Upon binding to its intracellular binding protein (FKBP12), the activity of mTOR is altered (reviewed in Refs. 31 and 32). TOR has been shown to act as a “nutrient sensor” in yeast, serving to evaluate environmental conditions based on cues such as growth factors and nutrients (33). The response of TOR to favorable growth conditions is the promotion of progression through the cell cycle at many levels. The response to unfavorable environmental conditions is the converse: halting of cell cycle progression by the inhibition of, or failure to activate, downstream events required for cell cycle progression such as translation of mRNAs. Thus, it is thought that rapamycin recapitulates a growth factor-deprived state. Downstream factors affected by the presence of rapamycin include p70(S6K), cyclin-dependent kinases, cyclins, and 4EBP (PHAS). p70(S6K) is one of the most well studied downstream targets of rapamycin inhibition, as exposure to rapamycin inhibits p70(S6K) phosphorylation. However, we previously reported the uncoupling of p70(S6K) activation and proliferation in human CD8+ T cells in response to a strong TcR stimulus in combination with specific co-receptor stimuli in the presence of rapamycin. Under these conditions, rapamycin inhibited p70(S6K) phosphorylation, yet the cells were able to proliferate, as measured by [3H]thymidine incorporation, in the presence of rapamycin.

In the present study we were first able to demonstrate, using CFSE to assess proliferation, that the [3H]thymidine incorporation by CD8+ T cells in the presence of rapamycin represents actual cell division. CFSE labeling of cells permits the direct assessment of cell division by flow cytometry, as the fluorescence intensity of cells decreases with each subsequent cell division. The results we obtained using this method were in agreement with our previous results, demonstrating that strong stimulation through the TcR in combination with CD28 costimulatory signals resulted in rapamycin-resistant cell division. The number of cells that had undergone one or at least two divisions did not differ significantly after stimulation in the presence or absence of rapamycin. However, these results still do not exclude the possibility that a subpopulation of CD8+ T cells was responsible for the rapamycin-resistant proliferation.

The progression of normal cells through the cell cycle in response to growth factors is strictly regulated by a family of proteins known as cell cycle inhibitors, because dysregulated cellular proliferation and growth has deleterious effects. p27(kip1), a member of the Cip family of cell cycle inhibitors, is expressed at detectable levels in resting cells, and functions by coupling with cdk2 and cyclin E to prevent their activity, thus inhibiting subsequent cell cycle progression. We previously observed that proliferation of CD8+ T cells could be resistant to rapamycin treatment, and that the antigen-specific rapamycin resistance could be converted to rapamycin sensitivity in the presence of neutralizing anti-IL-2 mAb. These data suggested that rapamycin-resistant, antigen-driven proliferation was IL-2-dependent. Here we demonstrated that the down-regulation of p27(kip1) correlated with the proliferation of the CD8+ T cells, even in the presence of rapamycin. This investigation was enabled by our in vitro model, in which we could dissociate treatment of cells with rapamycin cells from inhibition of T cell proliferation.

In previous studies, rapamycin inhibited the IL-2-induced down-regulation of p27(kip1) (9), and in other systems rapamycin induced the expression of p27(kip1) (20). These previous reports differed from ours in several substantial ways. Nourse et al. (9) examined the CD4+ cell line D10, cultured T cells, or primary T cells stimulated with IL-2 or with anti-CD3 plus plate-bound anti-CD2. Kawamata et al. (20) used a malignantly transformed human T-cell lymphotrophic virus type I-infected CD4+ T cell line. Notably, both of these studies differed from ours in that proliferation or progression through the cell cycle was inhibited by rapamycin when p27(kip1) was not down-regulated. In contrast, in our experiments, under conditions in which rapamycin did not inhibit proliferation of IL-2-dependent T cells clones or CD8+ T cells isolated ex vivo, rapamycin did not prevent the stimulation-dependent p27(kip1) down-regulation. Importantly, under conditions in which rapamycin readily inhibited proliferation, rapamycin also inhibited p27(kip1) down-regulation (Fig. 2). Thus, the down-regulation of p27(kip1), unlike the phosphorylation of p70(S6K), correlates with proliferation, irrespective of the presence of rapamycin.

p27(kip1) down-regulation has been well studied, yet the mechanistic details remain controversial (reviewed in Ref. 23). Cell type specificity may contribute to difficulty in extrapolating results from one system to another. It has been clearly demonstrated that p27(kip1) down-regulation can occur via proteasome-dependent pathways (34, 35). In our studies, exposure to the proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) inhibited the stimulation-dependent down-regulation of p27(kip1) in CD8+ T cells, in both the presence and absence of rapamycin (data not shown). However, exposure to this drug also increased the percentage of dead cells, clouding interpretation of these studies. Regardless, this suggests that the mechanism of p27(kip1) down-regulation in CD8+ human T cells in response to stimulation through CD3 and CD28 is the same in the presence and absence of rapamycin.

The progression of normal cells through the cell cycle is also regulated by the timing of expression of the cyclins, the regulatory component of the cyclin-dependent kinases (reviewed in Refs. 19 and 23). However, the stimulation-dependent induction of cyclin D2 and cyclin D3 in human T cells has been shown to be unaffected by rapamycin, even when growth factor-induced progression through the cell cycle was inhibited by rapamycin (28). It has therefore been proposed that rapamycin affects cell cycle progression in lymphoid cells after the emergence of cyclin D2 and D3, although the effects may differ in other cell types (Ref. 28; reviewed in Ref. 3). In our studies, we observed that rapamycin, although it may delay the expression of cyclin D2 and D3 in human CD8+ T cells, does not block the expression of cyclin D, a finding consistent with both our proliferation results and previously published results (28). An-
other interpretation of these data that cannot be excluded at present is that a subset of the CD8+ T cells exposed to rapamycin are impervious to its effects and that, because these represent only a portion of the total pool of cells, the effects of rapamycin on the expression of cyclin D2 and D3 in this subset are difficult to study. Experiments to address this possibility are under way, sorting the cells that have divided in the presence of rapamycin to assess their expression of the cyclin D proteins.

Cell survival is another highly regulated process, controlled in part by expression of the bcl-2 family members. The induction of anti-apoptotic bcl-2 family members, in response to specific signals delivered through surface receptors such as TcR and CD28 for T cells, or growth factors (notably IL-2), results in protection from cell death. The mechanism of this protection from death afforded by bcl expression is thought to be the preservation of mitochondrial integrity in death promoting situations (36, 37). Thus, cell survival is often a balance between pro- and anti-apoptotic factors elicited upon stimulation. Here, we verified the induction of the expression of a bcl-2 family member, bcl-xL, in response to stimulation through the TcR in combination with stimulation through CD28. We further observed that this induction was not inhibited by the presence of rapamycin under stimulatory conditions that allowed proliferation and cell survival. Our results are consistent with those of Miyazaki et al. (38), who found that, although the IL-2-dependent induction of bcl-2 mRNA in an IL-2R-expressing pro-B cell line was inhibited by rapamycin, the expression of bcl-xL under the same conditions was not. More recently, Calastretti et al. (39) found that, in human folicular B cell lymphoma lines, the amount of bcl-2 protein was increased by exposure to rapamycin. However, neither of these studies examined the expression of bcl-xL in primary T cells. In our system, the rapamycin-resistant induction of bcl-xL expression in CD8+ T cells is a direct response to the signals delivered specifically through CD28 in conjunction with CD3, and correlates with proliferation. Our observation that bcl-xL expression was not induced when rapamycin inhibited proliferation is consistent with this. Thus, it appears that both bcl-xL induction and p27kip1 down-regulation correlate with the effects of rapamycin on proliferation of human CD8+ T cells; it remains to be seen whether the same holds true for CD4+ T cells.

In our experiments, not only did rapamycin fail to inhibit the proliferation of CD8+ T cells after CD3/CD28 co-cross-linking, or of the T cells clones following CD3 cross-linking, but the incorporation of [3H]thymidine was not prevented by rapamycin, as we and others have shown (6, 7, 47). The induction of c-Myc expression was inhibited by LY294002, consistent with the involvement of the PI3K pathway in the induction of c-Myc expression in response to stimulation (48–50). Taken together, our results suggest that the induction of bcl-xL expression and the concurrent down-regulation of p27kip1 protein are more accurate measures of inhibition of CD8+ T cell proliferation by rapamycin than is phosphorylation of p70s6k or the induction of c-Myc expression. These appear to correlate more consistently with inhibition of proliferation by rapamycin. Thus, direct ex vivo monitoring of bcl-xL and p27kip1 in T cells during rapamycin therapy may provide new insights into the mechanism of immunosuppression in humans undergoing drug treatment. Moreover, further efforts to characterize the effects of rapamycin on specific proteins involved in the progression through the cell cycle in CD8+ T cells, in particular those inhibited by p27kip1 (cyclin E and cyclin A), will be of interest.

The strength of signal delivered to T cells determines the functional outcome of the activating signal. Because of thymic selection against T cells that recognize self-antigens, autoreactive T cells have lower affinity T cell receptors as compared with T cells that recognize foreign microbial antigens. Moreover, responses to infectious agents are associated with strong co-stimulatory signals. We postulate that the selective immunosuppressive effect of rapamycin in human CD8+ T cell populations could be predictive of a selective effect allowing cytotoxic responses during microbial infections where there are strong strengths of signals associated with high affinity T cell receptors and strong costimulatory second signals. In contrast, the weaker autoimmune and perhaps allogeneic responses can be selectively inhibited by the actions of rapamycin.

REFERENCES

1. Fruman, D. A., Klee, C. B., Bierer, B. E., and Burakoff, S. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3686–3690
2. Hemenway, C. S., and Heitman, J. (1999) Cell Biochem. Biophys. 30, 115–151
3. Dumont, F. J., and Su, Q. (1996) Life Sci. 58, 373–385
4. Saunders, R. N., Metcalfe, M. S., and Nicholson, M. L. (2001) Kidney Int. 59, 3–16
5. Shapiro, A. M. J., Lakey, J. R. T., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M., and Rajsitch, R. V. (2000) N. Engl. J. Med. 343, 230–238
6. Slavik, J. M., Lim, D. G., Burakoff, S. J., and Hafler, D. A. (2001) J. Immunol. 166, 3201–3209
7. Calvo, C. W., Crews, C. W., Vlk, T. A., and Bierer, B. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7571–7575
8. Morice, W., Wiederrecht, G., Brunn, G., Siekerka, J., and Abraham, R. (1993) J. Biol. Chem. 268, 22737–22745
9. Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994) Nature 372,
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