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Inhibition of Cell Cycle Progression by Rapamycin Induces T Cell Clonal Anergy Even in the Presence of Costimulation

Jonathan D. Powell, Cara G. Lerner, and Ronald H. Schwartz

Costimulation (signal 2) has been proposed to inhibit the induction of T cell clonal anergy by either directly antagonizing negative signals arising from TCR engagement (signal 1) or by synergizing with signal 1 to produce IL-2, which in turn leads to proliferation and dilution of negative regulatory factors. To better define the cellular events that lead to the induction of anergy, we used the immunosuppressive agent rapamycin, which blocks T cell proliferation in late G1 phase but does not affect costimulation-dependent IL-2 production. Our data demonstrate that full T cell activation (signal 1 plus 2) in the presence of rapamycin results in profound T cell anergy, despite the fact that these cells produce copious amounts of IL-2. Similar to conventional anergy (induction by signal 1 alone), the rapamycin-induced anergic cells show a decrease in mitogen-activated protein kinase activation, and these cells can be rescued by culture in IL-2. Interestingly, the rapamycin-induced anergic cells display a more profound block in IL-3 and IFN-γ production upon rechallenge. Finally, in contrast to rapamycin, full T cell activation in the presence of hydroxyurea (which inhibits the cell cycle in early S phase) did not result in anergy. These data suggest that it is neither the direct effect of costimulation nor the subsequent T cell proliferation that prevents anergy induction, but rather the biochemical events that occur upon progression through the cell cycle from G1 into S phase.

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costimulation directly inhibits anergy induction by preventing the enhancement of Nil-2a function following TCR occupancy.

In the present work we sought to better define the events responsible for anergy induction by using the immunosuppressive agent rapamycin. Rapamycin is a macrolide antibiotic with a similar biochemical structure to cyclosporin A (CSA)\(^2\) and FK506 (17). Indeed, the intracellular target for rapamycin is FK506 binding protein (FK506BP). However, unlike CSA and FK506, rapamycin does not inhibit IL-2 production (18). Rather, it inhibits the ability of lymphocytes to proliferate in response to IL-2. It is thought that the rapamycin-FK506BP complex does this by binding to and inhibiting the mammalian target of rapamycin (mTOR), a serine/threonine kinase that is believed to be a critical step in the pathway leading to the down-regulation of the cell cycle inhibitor Kip-1 following IL-2R engagement (17). As a result, the cell fails to progress from G1 to S phase and does not proliferate. Therefore, we incubated the CD4\(^+\) Th1 clone A.E7 overnight with or without costimulation in the presence or absence of rapamycin.

Our data show that even when given full signal 1 and signal 2, A.E7 cells are rendered anergic if cultured in the presence of rapamycin. Furthermore, the rapamycin-induced anergy appears to be more complete than conventional anergy in that there is also a marked decrease in IFN-\(\gamma\) and IL-3 production upon restimulation. On the other hand, A.E7 T cells stimulated with signal 1 plus 2 in the presence of hydroxyurea, an agent which arrests proliferation in S phase, are not rendered anergic. These data suggest that it is neither direct costimulation nor proliferation per se that prevents anergy induction, but rather the biochemical events that occur upon progression through the cell cycle from G1 to S phase.

Materials and Methods

Cell culture and anergy induction

A.E7 is a CD4\(^+\) Th1 clone specific for the pigeon cytochrome c (PCC) peptide 81–104. It was grown and maintained as previously described (19). Briefly, cells were stimulated for 48 h with whole PCC and irradiated (3000 rad) B10.A splenocytes as a source of APCs. The cells were next expanded 20:1 in 10 U/ml of rIL-2. After a minimum of 12 days in culture, when the IL-2 was consumed and the cells were rested, the live cells were isolated from a Ficoll gradient and utilized. Anergy induction was achieved by overnight incubation of 20–40 \(\times\) 10\(^4\) A.E7 cells in a 775 tissue culture flask (Costar, Cambridge, MA) that had previously been coated with anti-TCR (Ab H57-597) (20) at a concentration of 10 \(\mu\)g/ml. The cultures were also supplemented with ascitic fluid containing the anti-CD28 mAb 37.51 (21) (a kind gift from Dr. James Allison) at a final dilution of 1:5000. CSA (Calbiochem, Cambridge, MA) was added to some cultures at a final concentration of 100 or 1000 nM, while rapamycin (a generous gift of Dr. S. Sehgal, Wyeth-Ayerst, Princeton, NJ) was added to other cultures at the same concentrations. Both the cyclosporin and the rapamycin were dissolved in ethanol. Preliminary studies revealed that adding an equivalent volume of ethanol (vehicle alone) did not affect cell cultures (data not shown). Hydroxyurea (Sigma, St. Louis, MO) was added to some cultures at a concentration of 2 mM. None of the drugs used caused a decreased viability as determined by trypan blue exclusion during the overnight incubation period. After the overnight incubation, the cells were removed from the flask, washed, and recultured in fresh medium for 5–14 days before rechallenge. At this time, the cells were reisolated and assayed for their ability to proliferate or produce IL-2.

Proliferation assay

Anergized or control clones were assessed for their ability to proliferate to PCC by adding 2 \(\times\) 10\(^4\) A.E7 cells to 5 \(\times\) 10\(^4\) B10.A irradiated splenocytes (3000 rad) and increasing doses of PCC in 96-well plates in triplicate. After 48 h, the cells were pulsed with \([\text{\textsuperscript{3}}H]\)thymidine and harvested 16 h later, and thymidine incorporation was determined using a betaplate reader.

### Results

**Rapamycin inhibits proliferation to IL-2 but not IL-2 production by A.E7 cells**

The strategy behind using rapamycin was that it would enable us to stimulate A.E7 cells with signal 1 plus 2, allow for the production and secretion of IL-2 as well as engagement of the IL-2R, but prevent IL-2-induced proliferation. Previously, Dumont et al. demonstrated that rapamycin inhibited proliferation but not IL-2 production by murine T cells (18). Therefore, initial experiments were performed to confirm that rapamycin was able to inhibit IL-2-induced proliferation in the A.E7 clone and not affect IL-2 production. In these experiments, similar doses of CSA were used for comparison. Fig. 1A formally demonstrates that CSA does not inhibit proliferation of the A.E7 cells in response to exogenous IL-2 (50 U/ml), while rapamycin inhibits proliferation in a dose-dependent fashion. The ID\(_{50}\) for rapamycin was ~4 nM. On the other hand, we see the exact opposite effect for IL-2 production. A.E7 cells were cultured with plate-bound anti-TCR and soluble anti-CD28 in 24-well plates overnight, and supernatant fluids were collected and assayed by ELISA for IL-2 production. As seen in Fig. 1B, CSA inhibited IL-2 production completely at a concentration of 100 nM. On the other hand, for the same concentrations of rapamycin tested, IL-2 production was similar to that of the control cells. This overnight stimulation protocol is precisely the same as that we used to induce anergy. Of note, when the clones were stimulated with anti-TCR and anti-CD28 overnight and then pulsed for an additional 12 h with \([\text{\textsuperscript{3}}H]\)thymidine, proliferation resulted in the uptake of 24,500 cpm, while in the presence of 1,000 nM CSA or rapamycin there was only 242 and 267 cpm, respectively. In this case, the CSA inhibited proliferation by inhibiting IL-2 production, while the rapamycin inhibited proliferation by endogenously produced IL-2.

**Rapamycin induces anergy even in the presence of costimulation**

To induce anergy, A.E7 cells are normally incubated with plate-bound anti-TCR overnight (induction phase). The cells are then harvested, washed, and rested in fresh medium. After a minimum

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\(^2\)Abbreviations used in this paper: CSA, cyclosporin A; TCR, T-cell receptor; IL-2, interleukin 2; mTOR, mammalian target of rapamycin; MAP, mitogen-activated protein; TCC, chicken T-cell lymphoma; PCC, pigeon cytochrome c; ERK, extracellular regulatory kinase.
of 5 days, the cells are rechallenged with either APCs plus Ag or anti-TCR plus anti-CD28. In the following experiments, anti-CD28 with or without CSA or rapamycin was added to the induction phase. Of note is the fact that the rapamycin and CSA are only present during the 16-h overnight incubation and not during the resting or rechallenge phase. After the overnight culture to induce anergy, the cells are washed and the drugs are removed.

A.E7 cells stimulated in the presence of signal 1 alone proliferate less effectively upon rechallenge with APCs and Ag than cells that received signal 1 plus 2 during the induction phase; this is conventional anergy (Fig. 2A). As has been previously shown, the presence of CSA during the induction phase inhibits the development of the anergic state (5). In other experiments, we have determined that the enhanced proliferation of the CSA-treated cells when compared with the signal 1 plus 2-treated cells is actually the same level of proliferation as nonmanipulated cells (data not shown). In contrast, the presence of rapamycin during the induction phase with signal 1 alone does not interfere with anergy induction, but rather results in strong anergy (Fig. 2B). In fact, in this particular experiment, the presence of rapamycin appears to enhance conventional anergy. We have noted this enhancement in a number of experiments, though not all, (see Fig. 3 for example),

FIGURE 1. A, Rapamycin but not CSA inhibits proliferation of A.E7 cells to exogenous IL-2. A.E7 cells were incubated with 50 U/ml of IL-2 in the presence of increasing doses of either CSA or rapamycin. After 48 h, [3H]thymidine was added to each well and the cells were harvested and assayed for proliferation after an additional 16 h of culture. Comparable data were obtained in three other experiments. B, CSA but not rapamycin inhibits IL-2 production. A.E7 cells were incubated overnight with plate-bound anti-TCR and soluble anti-CD28 in the presence of increasing doses of either CSA or rapamycin. Supernatant fluids were harvested and assayed for IL-2 by ELISA.

FIGURE 2. A, CSA inhibits anergy induction. A.E7 cells were incubated overnight with plate-bound anti-TCR alone (signal 1) or plate-bound anti-TCR plus soluble anti-CD28 (signal 1 plus 2) in the presence or absence of CSA. The cells were harvested, washed, and rested for 7 days in fresh medium. The cells were then tested for their ability to proliferate to Ag and APCs. Note, the CSA is only present during the induction phase of anergy and not during the rest period or rechallenge phase. In this and subsequent figures, the legend refers to the conditions of the induction phase. B, Rapamycin augments anergy induction even in the presence of costimulation. In the same experiment as shown in A, A.E7 cells were incubated overnight with plate-bound anti-TCR alone or plate-bound anti-TCR plus soluble anti-CD28 in the presence or absence of rapamycin. The signal 1 and signal 1 plus 2 groups are the same as in A to facilitate easy comparison. Once again, the rapamycin was only present during the induction of anergy and not during the rest or rechallenge phases. C, Rapamycin-induced anergic cells proliferate to exogenous IL-2. The cells that were anergized in A and B were tested for their ability to proliferate to 50 U/ml of exogenous IL-2. A.E7 cells were cultured for 48 h and pulsed with [3H]thymidine for an additional 16 h and then harvested and assayed for incorporation of radioactivity into DNA. Proliferation of cells in the absence of IL-2 was <1000 cpm for all conditions except signal 1 plus 2, which was 2400 cpm (not shown). Comparable data were obtained in three other experiments.
particularly if the signal 1-induced anergy is not optimal. Rapamycin in the absence of anti-TCR had no effect on the cell cultures (data not shown). Most important, though, is the observation that the presence of rapamycin promotes anergy induction even in the presence of full costimulation. Thus, in the presence of rapamycin, costimulation does not inhibit the induction of anergy. Finally, a characteristic of anergic T cell clones is that they are able to proliferate to exogenous IL-2, because they constitutively express low levels of the high-affinity IL-2R. As seen in Fig. 2, the cells anergized in the presence of rapamycin proliferated to exogenous IL-2 as well as the conventionally anergized cells and slightly less effectively than the cells that were given signal 1 plus 2 without rapamycin. Also, as expected, the cells that were initially incubated with CSA proliferated to the exogenous IL-2. In addition to their ability to respond to exogenous IL-2, the rapamycin-induced anergic cells were able to up-regulate CD25 and CD69 in response to TCR stimulation as well as normal T cell clones and their conventionally anergized counterparts (data not shown). The TCR level was also comparable (data not shown).

Cyclosporin inhibits rapamycin-induced anergy
As seen in Fig. 2, CSA inhibits anergy induction while the presence of rapamycin results in anergy induction even in the presence of signal 2. Thus, the question arises as to what happens when the two drugs are both present during the induction phase. Fig. 3 shows once again that the presence of rapamycin during the induction phase promotes anergy even in the presence of costimulation. On the other hand, when CSA is also added to this culture, anergy induction is abrogated. CSA’s ability to inhibit anergy dominates over the ability of rapamycin to induce anergy. This is presumably because CSA blocks anergy induction proximally by inhibiting the production of negative regulatory factors. The results are consistent with the idea that rapamycin works distally, possibly by inhibiting proliferation and/or the inactivation of the negative regulatory factors.

Hierarchy of anergy induction
Cells that were stimulated during the induction phase with both signal 1 and signal 2 appeared to be less responsive than nonmanipulated resting cells. These cells appeared to be partially anergized. To test this directly, we compared the proliferative response of cells that had been stimulated overnight with signal 1 plus 2, rested, and rechallenged, to cells that were “mock” stimulated, rested, and rechallenged. As seen in Fig. 4A, cells that were stimulated with signal 1 plus 2 during the induction phase proliferated less effectively upon rechallenge than cells that were not stimulated at all (mock stimulation). This observation is consistent with the results of Beverly et al., who showed that anergy could be induced in the presence of signal 1 plus 2 if APCs and the medium were removed and the cells washed free of IL-2 (10). In our system, after 16 h of signal 1 plus 2, the stimulating Abs and medium are also removed. It might be that despite the high levels of IL-2 produced during the 16 h with signal 1 plus 2, there is not sufficient time to induce the cells to proliferate or completely inactivate the
negative regulatory factors. In addition, under such culture conditions, the cells are continuously being stimulated by signal 1 and thus presumably continuously replenishing negative factors. By adding rapamycin to this culture, the anergy induced is more complete and rivals that of the cells anergized conventionally with signal 1 alone. This is consistent with the idea that rapamycin completely blocks the ability of IL-2 to prevent anergy during the initial stimulation phase. As is the case for conventional anergy, the partial anergy induced by signal 1 plus 2 and the profound anergy induced by signal 1 plus 2 plus rapamycin is not secondary to down-regulation of the TCR as determined by flow cytometry (data not shown).

A hallmark of anergic cells is their inability to produce IL-2 upon rechallenge (2). Thus, we wanted to confirm that the lack of proliferation upon rechallenge of the rapamycin-induced anergic cells also correlated with a decrease in their ability to produce IL-2. Cells were anergized, rested, and then assayed for their ability to produce IL-2 upon rechallenge with plate-bound anti-TCR and soluble anti-CD28. As seen in Fig. 4B, there was a >220-fold decrease in the IL-2 produced by the conventionally anergized cells. Likewise, the cells anergized in the presence of signal 1 plus 2 plus rapamycin demonstrated a >370-fold reduction in IL-2 production. In addition, consistent with their decreased proliferative ability upon rechallenge, the cells that were stimulated with signal 1 plus 2 during the induction phase also showed a decrease in IL-2 production when compared with the nonmanipulated cells (19-fold), though their response was still >20-fold that of the fully anergized cells.

**IL-2 rescues cells from rapamycin-induced anergy**

The anergic state of T cell clones is reversed by culturing the cells in exogenous IL-2 and allowing them to proliferate. We wanted to determine whether the anergic state induced in the presence of rapamycin was also reversible. Anergy was induced in the presence and absence of costimulation and the presence and absence of rapamycin. The cells were harvested and split into either fresh medium or fresh medium containing 50 U/ml of IL-2. The cells were cultured for 12–14 days and then harvested. As seen in Fig. 5A, induction with signal 1 or signal 1 plus 2 plus rapamycin results in hypoproliferation in response to rechallenge with APCs and Ag when compared with the cells that were induced with signal 1 plus 2. However, culturing these cells in IL-2 during the rest period reverses the anergy. In addition, we see that culturing cells in IL-2 that were initially stimulated with signal 1 plus 2 rescues them from their state of partial anergy. As shown in Fig. 5B, the clones were tested for their ability to produce IL-2 after being rested in the presence or absence of exogenous IL-2. Note the hierarchy of IL-2 production in the control groups in this experiment: the nonmanipulated cells produced the greatest amount of IL-2, followed by the cells stimulated with signal 1 plus 2 then the cells stimulated with signal 1 alone or 1 plus 2 plus rapamycin. Thus, 14 days after their exposure to signal 1 plus 2 and rapamycin the clones remain anergic. In contrast, when these cells were stimulated with IL-2, following removal of rapamycin, the anergic state was reversed. In terms of IL-2 production, the reversal was not as complete as that seen for the reversal of proliferation (Fig. 5A). In this experiment, there was a 5.4-fold increase in IL-2 production after culture of anergic cells in IL-2, while in three other experiments we have seen between a 5- and 12-fold increase. The failure to completely reverse anergy and achieve maximum IL-2 production upon rechallenge might relate to the deeper state of anergy induced with rapamycin (Fig. 2B and Fig. 7 to be discussed below).

![FIGURE 5. Culturing cells in IL-2 rescues them from anergy.](http://www.jimmunol.org/)
while the bottom panel is the same blot stripped and reblotted with anti-ERK. The upper and lower arrows indicate ERK 1 and 2, respectively. Extracts were derived from cells anergized under various conditions and then either left unstimulated or stimulated with plate-bound anti-TCR for 10 min. The upper panel was blotted with anti-phospho-ERK, while the bottom panel is the same blot stripped and reblotted with anti-ERK. The upper and lower panels indicate ERK 1 and 2, respectively.

Extracts from nonanergic A.E7 cells are denoted by “Mock,” conventionally anergized cells are denoted as “1 alone,” partially anergized cells are denoted as “1 + 2,” and rapamycin-anergized cells are denoted as “1 + 2 + R.”

FIGURE 6. Rapamycin-induced anergy results in a block in the MAP kinase pathway. Extracts were derived from cells anergized under various conditions and then either left unstimulated or stimulated with plate-bound anti-TCR for 10 min. The upper panel was blotted with anti-phospho-ERK, while the bottom panel is the same blot stripped and reblotted with anti-ERK. The upper and lower panels indicate ERK 1 and 2, respectively. Extracts from nonanergic A.E7 cells are denoted by “Mock,” conventionally anergized cells are denoted as “1 alone,” partially anergized cells are denoted as “1 + 2,” and rapamycin-anergized cells are denoted as “1 + 2 + R.”

FIGURE 7. Rapamycin-induced anergy results in decreased IFN-γ and IL-3 production. Cells were anergized, rested in fresh medium, and stimulated with plate-bound anti-TCR and anti-CD28 ascites. Supernatant fluids were assayed by ELISA for IFN-γ (A) and IL-3 production (B). Cytokine production was <2.5 pg/ml of IL-3 and undetectable for IFN-γ for all unstimulated cultures (only unstimulated “Mock” culture is shown). Comparable results were observed in three experiments.

Rapamycin-induced anergy results in a more profound block in IL-3 and IFN-γ production

Despite the profound block in IL-2 production by anergic cells, they still produce other cytokines upon rechallenge, albeit in decreased amounts. Typically, the anergic clones in our laboratory display a 50% decrease in IFN-γ production and a 10-fold decrease in IL-3 production under optimal activation conditions (10). To examine the production of these two cytokines by the rapamycin-induced anergic cells, supernatant fluids from 16-h stimulated cells were assayed by ELISA for IFN-γ and IL-3 levels. These are the same supernatant fluids from Fig. 5B that demonstrated 240 U/ml IL-2 for the stimulated cells vs 8 U/ml and 7 U/ml for the signal 1 and rapamycin-induced anergic cells, respectively. As can be seen in Fig. 7A, there is a ~50% decrease in IFN production as measured in the signal 1-induced anergic supernatants, when compared with the nonanergic supernatant fluid. On the other hand, there is a >10-fold reduction in IFN production in the supernatant fluid derived from the rapamycin-induced anergic cells. Likewise, as seen in Fig. 7B, there is a typical sixfold reduction in IL-3 production between the supernatant fluids derived from the conventionally anergized cells and the nonanergic cells, while the supernatant fluids derived from the rapamycin-induced anergic cells showed nearly a 25-fold difference. Thus, it appears as if the cells anergized in the presence of costimulation and rapamycin have a more profound block in the production of these other cytokines than the decrease seen in cells that are anergized by stimulation with signal 1 alone. This observation was also made for cells treated with signal 1 and rapamycin in the absence of costimulation, suggesting that costimulation is not necessary for the deeper state of rapamycin-induced anergy (data not shown). Furthermore, as is the case for signal 1-induced anergy, the hierarchy of inhibition (IL-2 > IL-3 > IFN-γ) is also maintained in the rapamycin-induced anergy. Interestingly, this hierarchy of inhibition is also observed for the signal 1 plus 2-induced partial anergy. As seen in Fig. 7, A and B, there is essentially no inhibition of IFN-γ in the supernatant fluid from the cells incubated initially with signal 1 plus 2, while there is a 2.5-fold decrease in the production of IL-3 and a 5-fold decrease in the production of IL-2 (Fig. 5B).

Cell cycle progression from G1 to S phase prevents the induction of anergy

In as much as we have been able to demonstrate the induction of T cell clonal anergy for cells cultured in the presence of signal 1 plus 2 and rapamycin, the data thus far support the Jenkins and Beverly hypothesis that TCR engagement in the absence of proliferation leads to anergy induction. Because rapamycin blocks IL-2-induced proliferation at the level of mTOR, it was possible that it was not proliferation and subsequent dilution of negative regulatory factors that prevented anergy induction, but rather the biochemical events that are the result of progression through the cell cycle. If indeed such were the case, then we would predict that by incubating the clones with signal 1 plus 2 and hydroxyurea, which blocks proliferation in early S phase, anergy would not be induced. Fig. 8A demonstrates that increasing concentrations of hydroxyurea inhibit IL-2-induced T cell proliferation as potently as rapamycin (see Fig. 1A). In addition, the clones still produce IL-2 in the
these data are consistent with a model in which CSA blocks the
ble for preventing anergy lies between G1 and S phase. Overall,
acts proximally to hydroxyurea and that the mechanism responsi-
anergy (Fig. 8B). CSA (Fig. 3), hydroxyurea does not inhibit rapamycin-induced
the presence of costimulation. Furthermore, in sharp contrast to
cause anergy in negative controls for their butyrate-induced anergy
of Gilbert and Weigle, who also found that hydroxyurea did not
do not become anergic. These findings are consistent with the data
up-regulation of “anergic factors” and rapamycin blocks their deg-
radiation/inactivation, while hydroxyurea, despite its ability to
block proliferation, neither blocks the up-regulation nor down-reg-
ulation of the factors that promote anergy.

Discussion
T cell clonal anergy results from TCR engagement in the absence of
costimulation. Despite this observation, the precise role that
costimulation plays in preventing anergy has yet to be defined. Some
propose that CD28 signaling directly serves to prevent anergy (14, 15), while others argue that costimulation prevents an-
ergy inasmuch as it facilitates the production of IL-2 and subse-
quent T cell proliferation (10, 11). Taking advantage of the ability of
rapamycin to block IL-2-induced proliferation without inhibit-
ing costimulation-mediated IL-2 production, we sought to discrimi-
nate between these two models. Our data are consistent with the
idea that signaling through CD28 only prevents anergy under nor-
mal conditions by enhancing IL-2 production. On the other hand,
we have also presented evidence that it is not proliferation in and
of itself that inhibits anergy, but rather the biochemical events that
occur as a result of progression through the cell cycle from G1 into
S. Thus, although proliferation and dilution may contribute to the
prevention of anergy, we propose that anergy induction can be
prevented by the consequences of IL-2R engagement distal to the
serine/threonine kinase activity of mTOR.

Clearly, we have not formally ruled out the possibility that rapa-
mycin prevents the signaling of an as-yet unidentified CD28 “anti-
anergy” or signal 2t pathway (14). In fact, it has been shown that
rapamycin can inhibit CD28-mediated down-regulation of IL-2 and
up-regulation of CTLA4 (29, 30). However, at the very least, as
shown in Fig. 1B, in the presence of rapamycin, the CD28 medi-
ated signaling pathway responsible for the up-regulation of IL-2
production appears to be intact. Furthermore, our findings are con-
sistent with the observations of Gilbert and Weigle, who used the
histone deacetylase inhibitor n-butyrate to induce anergy (13).
They proposed that anergy is the result of TCR stimulation and
G1a sequestration. However, unlike rapamycin, histone deacetyl-
ase inhibitors also inhibit IL-2 production (13, 31), and, thus,
the ability of n-butyrate to induce anergy could also be consistent with the model of Jenkins and Beverly (10, 11). Finally, in terms of
whether or not regulation of the transcription factor Nil-2a con-
tributes to the antagonism of the anergic state, using electro-
phoretic mobility shift assays we could not demonstrate the down-
regulation of Nil-2a binding to the NRE in A.E7 cells stimulated
with anti-TCR and anti-CD28 Abs (data not shown). Thus, at this
time, the role, if any, that Nil-2a plays in anergy induction in the
A.E7 clone is not clear.

The anergic state induced in the presence of rapamycin shares
many similarities with conventionally anergized cells. They both
display a block in the MAP kinase pathway as determined by de-
creased ERK phosphorylation (27, 28) and they both were reversed
by adding exogenous IL-2. On the other hand, unlike convention-
ally anergized cells, the rapamycin-induced anergic cells appeared
to have a more profound block in the production of the cytokines
IL-3 and IFN-γ. It is not clear whether this is due to more complete
anergy, as a result of the ability of rapamycin to fully prevent the
G1 to S phase transition, or the ability of rapamycin to affect an
additional pathway. It is also of note that both IL-3 and IFN-γ
contain AP-1 sites in their promoters (32, 33). Because it has been
shown that there is a decrease in TRE-mediated transcription in
anergic cells, it might be that the differences seen in the production
of these cytokines in anergy is related to the relative contribution

**FIGURE 8.** A, Hydroxyurea inhibits IL-2-mediated T cell proliferation.
A,E7 T cells were incubated with 50 U/ml IL-2 and increasing concentra-
tions of hydroxyurea in a 96-well plate. The cultures were pulsed with
[3H]thymidine after 48 h and harvested at 64 h. In a separate culture, A,E7
cells were stimulated with plate-bound anti-TCR and anti-CD28 in the
presence of increasing concentrations of hydroxyurea. After 16 h, super-
natant fluid was harvested and IL-2 production was determined using
ELISA. B, The effect of hydroxyurea on anergy induction. A,E7 T cells
were stimulated overnight with either anti-TCR alone or anti-TCR plus
anti-CD28 in the presence or absence of hydroxyurea, rapamycin, or both.
The cells were washed and rested 7 days and then rechallenged with anti-
TCR and anti-CD28 for 16 h. Supernatant fluid was collected and assayed
for IL-2 production using the CTLL bioassay.
of these sites to TCR-induced activation. The consistency of the hierarchy of inhibition between IL-2, IL-3, and IFN-γ is striking and is observed under conditions of partial anergy (induced by 16 h of signal 1 plus 2 and removal of IL-2), conventional anergy (signal 1 alone), and rapamycin-induced anergy. Interestingly, this same pattern is observed when examining the effect of costimulation on cytokine production (Ref. 34 and our unpublished observations). That is, the production of IL-2 by A.E7 is costimulation dependent, while IL-3 and, to a greater degree, IFN-γ are produced in response to signal 1 alone (34). This hierarchy may ultimately provide insight into the precise molecular mechanisms of cytokine induction and inhibition in Th1 cells.

Fig. 9 depicts a general model for anergy induction. We have adapted concepts from the kinetic proofreading model of TCR signal transduction to incorporate a number of observations concerning anergy induction process (35, 36). TCR engagement leads to a series of reactions (A → B → C) that ultimately lead to full signal 1-induced changes. At reaction B, the negative regulatory pathway leading to anergy is initiated. It has been shown that certain variant peptides with lower affinities for a particular TCR can induce anergy even in the presence of costimulation, a so-called partial agonist anergy (2, 37, 38). A partial agonist may have an affinity for the TCR that only permits signaling up to B, never leading to full signal 1. Thus, in this case, the negative regulatory factors are produced but there is insufficient signaling to produce IL-2 and the subsequent G1 to S phase transition. This is consistent with the data of Madrenas et al., who showed that anergy induction for partial agonists could be overcome by the addition of exogenous IL-2 (39). In the case of conventional anergy, TCR engagement results in both the initiation of the negative regulatory pathway as well as full signal 1. However, in the absence of costimulation, little or no IL-2 is produced, and, as a result, there is no entry into S phase. In rapamycin-induced anergy, there is production of the negative regulatory factors as well as full signal 1 and signal 2. This leads to IL-2 production, release, and IL-2R engagement. However, the cell is blocked from proceeding through the cell cycle by rapamycin. As a result, there remains a buildup of negative regulatory factors, and the cell is hyporesponsive upon rechallenge. In this model, hydroxyurea blocks proliferation distal to the events responsible for the abrogation of anergy, and, as a result, even though cells incubated in the presence of signal 1 plus 2 and hydroxyurea do not proliferate, signaling through the IL-2R leads to the prevention of anergy. Finally, signal 1 plus 2 followed by the removal of IL-2 results in a small anergic effect. We postulate that this is due to the fact that the cells are continuously being stimulated through the TCR and thus the IL-2 produced during the 16-h culture period is unable to accomplish the complete dissipation of the negative regulatory factors.

Several groups have shown that anergic T cells display a block in the MAP kinase pathway upon rechallenge (27, 28, 40). Our current studies have demonstrated a similar block in cells that have been anergized in the presence of costimulation and rapamycin. However, in previous studies using IL-2 promoter-driven reporter constructs, our laboratory has shown that the block in the Ras pathway alone cannot fully account for all of the inhibition of IL-2 production seen in anergy (23). Rather, this inhibition appears to be due in part to cis-dominant repression mediated at the level of the IL-2 promoter. The target of this repression appears to be centered around the −180 and −150 regions of the enhancer/promoter (23) and may be mediated by cAMP-response-element-binding protein (CREB) family member proteins (41) and our unpublished observations. We believe that persistent TCR engagement leads to the induction/activation of such negative transcriptional regulators. Upon rechallenge of anergic cells (in the setting of decreased induction of positive transcription factors such as Jun and Fos), these unopposed negative factors would serve to shut down IL-2 transcription. Recently, Bodor and Habener have suggested that the decrease in IFN-γ and IL-2 production secondary to increases in cAMP is the result of the up-regulation of the negative transcription factor inducible cAMP early repressor (ICER) (42). ICER is an isoform of cAMP-response-element modulator (CREM) that has the ability to bind to DNA, but lacks a transactivating domain (43). It remains to be seen if this protein contributes to the decrease in IL-2 and IFN-γ production seen in anergy.

Because rapamycin does not appear to inhibit IL-2-induced p21^waf1 activation or the induction of c-myc mRNA, presumably these biochemical consequences of IL-2R engagement do not play

FIGURE 9. Model of anergy induction. This model adapts the kinetic proofreading model of T cell activation (35, 36) to incorporate a number of observations concerning anergy induction in CD4^+ T cell clones. See Discussion for details.
a role in inhibiting anergy (44). A role for signaling via the common γ-chain of the IL-2R is supported by findings in human T lymphocytes that activating Abs against this chain can antagonize anergy induction (12). The prevention of anergy induction by IL-2 must involve mTOR, the serine/threonine kinase blocked by rapamycin (17, 44). At this time, it is unclear if mTOR is directly or indirectly involved in the degradation or inactivation of negative factors up-regulated by TCR engagement. In this regard, mTOR is an obligate participant in the activation of p70s6k, which is proposed to be essential for G1 cyclin-cdk activation and progression of the cell from G1 into S phase (44). Although the precise mechanism whereby p70s6k promotes this transition is not known, it is believed that progression through the cell cycle is mediated in part by the kinase’s ability to enhance translational initiation (44). This is mediated in part by its ability to phosphorylate and inactivate translational repressors. Likewise, p70s6k might serve the same function in terms of inactivating the negative regulatory factors that maintain anergy. In addition, p70s6k has been shown to phospho-rylate and increase the transcriptional activity of CREM+ (45). It remains to be seen if this kinase has additional effects on other CREB family members, for example those that might be involved in the cix-dominant repression of IL-2 transcription.

TCR engagement results in the transition of the cells into the late G1 phase of the cell cycle (17). Further progression is blocked by the accumulation of the inhibitor Kip-1 (44). Signaling through the IL-2R results in the degradation of Kip-1 through the ubiquitin-proteasome pathway and the subsequent assembly of G1 cyclin-cdk complexes (46). This in turn leads to progression into S phase and cellular proliferation. The presence of rapamycin inhibits IL-2-mediated degradation of Kip-1. Similarly, we propose that rapa- mycin promotes anergy by blocking cell cycle progression and promoting the build up of negative regulatory factors. As is the case for the inhibitor Kip-1, it might be that progression through the cyclin-dependent kinase pathway leads to the degradation/in-activation of the negative regulatory factors that mediate anergy. In this regard, it has been shown that the transcriptional repressor ICER is degraded by the ubiquitin-proteasome pathway (47). If such is the case, then Kip-1 itself may be central to the mainte-nance of the anergic state by its ability to inhibit the cyclin-de-pendent kinase cascade. Thus, anergy might be viewed as part of the mechanism of check point cell cycle growth arrest adopted by the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose. Current studies are focused on determining the level at which anergy is prevented in the lymphocyte for an immunological purpose.


