Commentary

Models of T Cell Anergy: Is There A Common Molecular Mechanism?

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Anergy is a cellular state in which a lymphocyte is alive but fails to display certain functional responses when optimally stimulated through both its antigen-specific receptor and any other receptors that are normally required for full activation. The term was initially used by Nossal and Pike (1) to describe an unresponsive state induced by the injection of soluble protein antigens in vivo, in which the antigen-specific B cells were still found to be present in the animal, but these cells could not be reactivated by antigen or mitogen to make Ig. The first observation of proliferative unresponsiveness induced in purified T cells using peptide antigens was made on human $CD4^+$ clones (2). The results were initially interpreted as a direct inactivation of the T cells through recognition of free antigen; however, subsequent blocking studies with anti-Ia antibodies revealed the involvement of MHC class II molecules (expressed on the T cells; 3). Downregulation of T cell antigen receptor expression was noted after the stimulation and postulated to be the molecular mechanism for the blocking of reactivation (4). Studies with mouse $CD4^+$ T cell clones uncovered other ways of inducing an unresponsive state, which at first appeared to be similar to the nonproliferating state seen with human T cell clones (5, 6). Presentation of peptide antigens either on chemically fixed APCs (5) or in planar lipid membranes containing only MHC class II molecules (6) was successful, as was stimulation of highly purified T cells with either concanavalin A (7) or anti-CD3 antibodies coated on a plastic surface (8). These results suggested that occupancy of the T cell antigen receptor alone, in the absence of other signals, was responsible for inducing the unresponsive state. Proof for this came from so called "allogeneic add-back" experiments in which live APCs bearing allogeneic MHC class II molecules were used to reconstitute the ability to stimulate a proliferative response and prevent the induction of unresponsiveness, even though the allogeneic cells themselves could not present the antigen to the T cell clone (9). The allogeneic APCs were postulated to be delivering a costimulatory signal(s) needed for both effects.

When similar allogeneic add-back experiments were carried out with purified human T cell clones inactivated by exposure to high concentrations of soluble peptides, addition of either allogeneic or syngeneic APC failed to prevent the induction of the unresponsiveness (10). This puzzling observation was further compounded when other laboratories were able to set up mixed leukocyte responses (MLRs) using transfected human cell lines as APCs (11) and essentially reproduce as well as extend the earlier findings made in the murine systems (12). Finally, the recent discovery of partial peptide agonist ligands has led to a new mouse model for anergy in which these peptides were used to induce an unresponsive state, even in the presence of costimulation (13). The goal of this commentary is to sort through these various models of anergy to try and find some common underlying molecular mechanism(s) and then to apply these thoughts to the new work of Groux et al. (in this issue). This study adds yet another twist to the induction process, the cytokine IL-10, which with TCR occupancy produces an anergic state in freshly isolated human $CD4^+$ T cells that appears to be more profound than any of the previous in vitro models.

Murine and Human T Cell Anergy Induced by TCR Occupancy in the Absence of Costimulation

Molecular Characterization. Anergized cells generated by TCR occupancy in the absence of costimulation fail to proliferate when restimulated with normal APC and antigen (4–8, 11, 12). In $CD4^+$ Th1 clones and $CD8^+$ CTL clones, this is caused by a block in IL-2 production (14, 15; see Fig. 1). Transcription of the IL-2 gene is decreased about 8-fold, and IL-2 secretion is decreased ~20-fold (14). Recent work suggests that the transcriptional block results from a failure to activate $p21^{ras}$ after TCR occupancy (16). This leads to a decrease in the activities of two of the MAP (mitogen-activated protein) kinase pathways, ERK (extracellular signal-regulated protein kinases) and JNK (c-Jun NH_2-terminal kinases; 17), as well as a failure to activate a critical transcription factor of the IL-2 gene, AP-1 (14). In addition, increased amounts of a negative regulatory factor, Nil-2a, have been found in anergic human T cells (18). This factor has been shown to block AP-1–induced transactivation of reporter constructs (19).

IL-2 is not the only cytokine whose production is diminished in anergic T cells (20). For example, IL-3/GM-CSF production is decreased 10-fold. The production of other cytokines, such as IFN-γ, however, is hardly affected at all. One of the most interesting cytokines is IL-4. In murine Th0 cells, IL-4 production is unaffected by anergy, yet the cells are prevented from proliferating (21). This is because — in addition to blocking IL-2 production — anergy blocks the ability of the cells to become competent to proliferate to an IL-4 signal (22). A similar phenomenon oc-

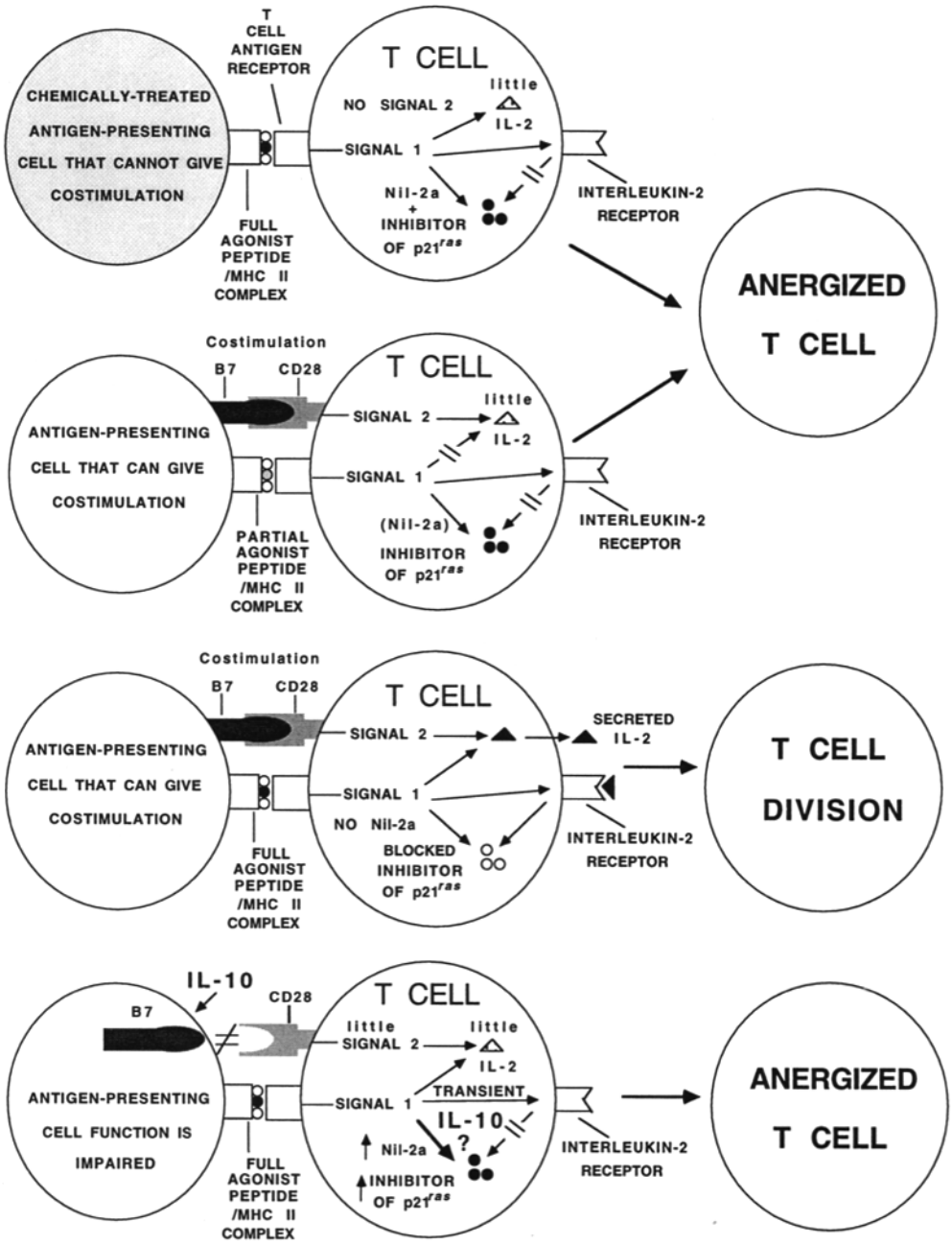

Figure 1. A way to unify anergy induced by TCR occupancy in the absence of costimulation with anergy induced by altered peptide ligands through a common underlying molecular mechanism. A speculative model for how IL-10 may augment anergy induction is also presented. See the text for a detailed description.

curs in the subset of Th2 cells that can be anergized (23). The ability of IL-12 to augment the proliferative response of Th1 cells is also blocked by anergy induction (24). Furthermore, the ability of anergic T cells to induce B cells to proliferate is impaired because the $CD4^+$ T cells are blocked in their expression of the CD40 ligand (25). Finally, anergic $CD8^+$ T cells are blocked for IL-2 production, but not for TCR-dependent cytotoxicity (15).

The common theme in all these observations is that anergy blocks the ability of the cell to produce and/or respond to proliferative signals. Even the block in IL-3 production fits into this pattern as it was recently shown that T cells that acquire the ability to express high affinity IL-3 receptors can proliferate when stimulated with a combination of IL-3 and IL-4 (26). In contrast, the production of the effector cytokines IL-4 and gamma-IFN—critical for the differentiation process of Th1 and Th2 cells—is unaffected. Thus, anergy appears to be a negative feedback process akin to a growth arrest state.

Costimulation. At the time of receptor occupancy, costimulation blocks the induction of anergy (9, 27; see Fig. 1). This second signal can be given up to 2 h after TCR occupancy and still have a full effect. Two hypotheses have been put forth to explain how this works. One is that signal transduction through receptors for costimulation blocks the production of the molecular inhibitors that cause anergy.

For example, in human clones, B7 engagement of CD28 blocked the increased production of Nil-2a stimulated by TCR engagement (18). The second hypothesis is that the large amount of IL-2 produced in the presence of costimulation (30–100-fold more than without it) either prevents the inhibitor from being made by signal transduction through the IL-2 receptor, blocks it once it has been made, or dilutes it out by stimulating multiple rounds of division after IL-2–induced proliferation. Evidence for these latter ideas includes prevention of anergy in human clones by signaling through antibody cross-linking of the common γ chain of the IL-2 receptor (12) and induction of anergy in mouse clones after normal stimulation, if antibodies to IL-2 and IL-2 receptor are added (28) or if the IL-2 is washed out 12 h after induction (20). These different mechanisms are not mutually exclusive.

The molecules involved in delivering costimulatory signals are thought to be predominantly the CD28 receptor on the T cell and the B7 molecules (CD80 and CD86) on the APC (29, 30). B7 engagement of CD28 leads to tyrosine phosphorylation of the receptor's cytoplasmic tail and binding of phosphatidylinositol 3-kinase (31), although this event has recently been shown to be nonessential for costimulation (32, 33). This is followed eventually by activation of JNK, which in concert with MAP kinase activation via the TCR, augments IL-2 gene transcription via phosphorylation of the Jun protein of the AP-1 transcription factor and possibly also CD28RC (34, 35). CD28 signaling also stabilizes IL-2 mRNA posttranscriptionally by an unknown mechanism (36), and this is the major mechanism of costimulation in the mouse (37).

Reversal of Anergy and In Vivo Models. One of the intriguing and most puzzling aspects of anergy is its ability to be reversed by stimulation of the T cell clones with IL-2 (20). This is possible because the clones abnormally express low levels of the high affinity IL-2 receptor on their surface. The reversal is not an outgrowth of small numbers of cells that failed to be anergized with the initial stimulus, since limiting dilution cloning showed that all of the cells reverted with the same plating efficiency as normal cells after stimulation and growth in IL-2. The reversal was demonstrated at both the level of cytokine production (20) and transcriptional activation of the IL-2 gene (14). In the human model for anergy involving TCR occupancy in the absence of costimulation, the anergic state could not be totally reversed by the addition of IL-2 alone. In this system, the cells had to be stimulated also with anti-CD2 antibody (38).

The ability to reverse anergy raises the question of the in vivo relevance of this state. If anergy is a form of self tolerance, why keep these cells around, where they might be reactivated through IL-2 produced by T cells specific for foreign antigens, leading to autoimmunity? This line of reasoning has led some investigators to dismiss anergy as an in vitro artifact of T cell clones. However, in vivo studies with superantigens (39–41) and adoptive transfer of T cells from TCR-transgenic mice (42, 43) have demonstrated that anergy can be induced in vivo. After the initial expansion and deletion phases induced in Vβ expressing T cell subsets by superantigens, there remains a cohort of Vβ^+ cells that appears to be refractory to restimulation in a proliferation assay. This population does not merely represent cells expressing particular Vα chains that prevent superantigen activation in the first place, since on restimulation of the population, one can observe early tyrosine phosphorylation events that are indicative of an anergic state (41, 44). Furthermore, restimulation with an anti-CD3 antibody also revealed a significant decrease in IL-2 production and a block in proliferation (41). Interestingly, addition of IL-2 to the restimulation cultures only partially reversed this proliferative block. This observation suggests that the anergic state induced in vivo also includes inhibitors of IL-2 receptor signaling, something that has never been seen in in vitro models with mouse clones.

Given this strong evidence for the existence of anergy in vivo, one now needs to consider the involvement of this state in the process of self tolerance. Naive T cells of the standard lineages (i.e., excluding NK1.1$^+$ T cells) make predominantly IL-2 in large amounts when first activated. If this first encounter with antigen is a TCR occupancy event in the absence of costimulation, the amount of IL-2 produced is very low and it is conceivable that an anergic state ensues. If such cells are specific for self antigens in the peripheral tissues, could they serve a useful role? Recent models of immunity promulgated by Janeway (45) and Matzinger (46) have emphasized the critical importance of costimulation in the decision of the immune system to make a response. Particularly in the "danger" model, autoreactive cells to peripheral self-antigens are viewed as a possible constituent of the normal immune response to harmful antigens. After serving this function and eliminating the danger signals, they are postulated to rest down and eventually be deleted. In such a model, anergic cells could represent a quiescent form of these autoreactive cells, waiting for IL-2 produced by the antigen-specific response of other cells to be called into play.

T Cell Anergy Induced by TCR Occupancy and IL-10 in Human CD4$^+$ PBLs

The induction of unresponsiveness in freshly isolated PBMCs when stimulated in an MLR with allogeneic cells in the presence of inhibitors of costimulation, such as CTLA4-Ig, has been reported (47). The failure to observe a secondary proliferative response when these cells were stimulated with the same allogeneic cells (and to give a proliferative response to third party allogeneic stimulators) has been referred to by others as anergy. As pointed out by the authors, however, because the frequency of responding cells in these cultures is low (1–5% of T cells), it is really impossible to be certain that the cells are actually alive and nonfunctional, as opposed to having been deleted. The same is true for the Groux et al. report in this issue, in which they use IL-10 as an inhibitor to help induce unresponsiveness in an MLR involving purified human CD4$^+$ T cell responders. These authors, however, also studied stimulation

of the freshly isolated $CD4^+$ PBLs with cross-linked anti-CD3 mAbs on a plate in the presence of IL-10. The unresponsiveness induced in this case can clearly be called anergy, since all the T cells are affected. The features of the state described bear a strong resemblance to the anergy induced in mouse and human clones with TCR ligation in the absence of costimulation, although there are some important differences. The cells appear to be blocked in their IL-2 and GM-CSF production, as expected from other models, but they are also blocked in their IFN-γ production. Signal transduction on restimulation revealed an intact calcium mobilization pathway and an ability to bypass the anergic block with phorbol esters and ionomycin. This is consistent with the recent localization of the biochemical block in murine anergy to a failure to activate $p21^{ras}$ (16, 17). In contrast to the murine model, however, the cells failed to reexpress high affinity IL-2 receptors after an appropriate rest period of 10 d followed by restimulation. As a consequence, they could not be stimulated with exogenous IL-2, and thus the anergic state could not be reversed. This is a striking difference with the clonal models, although as mentioned earlier, there is some evidence from mouse in vivo TCR-transgenic models that IL-2 receptor expression or signaling is also impaired (41). These results suggest the possibility that there are different degrees or levels of anergic unresponsiveness, possibly relating to the degree of block of ras activation. A partial block may prevent transactivation of AP-1 bound to phorbol ester response elements, whereas a complete block may prevent AP-1 from participating with NF-AT at other sites in the enhancer. Support for this idea can be found in the recent studies of Sundstedt et al. (44), in which repeated injections of superantigens in vivo created an anergic population, which upon restimulation revealed impaired NF-AT and NF-κB p65/p50 binding, as well as impaired AP-1 binding to IL-2 enhancer response elements. Another possibility is an earlier block at the level of tyrosine phosphorylation of the TCR ζ chain, which has recently been proposed (48).

The concept that tolerance might exist at different levels in vivo was first proposed by Arnold et al. (49). In this scheme, anergy was considered as a single stage from which the cells could be rescued. Other stages included receptor downmodulation and deletion. The new data using IL-10 suggest that anergy itself may have different levels. This in turn raises questions about the biological function and fates of these intermediates. It has been argued that anergy is a slightly slower form of T cell death by apoptosis. This is clearly not the case at a biochemical level. Peripheral deletion of T cells involves engagement of Fas by Fas ligand or the TNF receptor by TNF (50). The subsequent FADD-mediated signal transduction events activate ICE-like proteases that carry out the programmed cell death (51). This pathway can be blocked by protease inhibitors (52). Such blockers have no effect on the induction of anergy in mouse T cell clones (Chiodetti, L., and R.H. Schwartz, unpublished observations). Nonetheless, the half-lives of anergic T cells have not yet been measured. Because the homeostatic half-life of naive T cells appears to be controlled independently from that of memory T cells (53), it would be interesting to know whether conventionally anergized cells had a longer half-life than those anergized either in the presence of IL-10 or by repeated injection of superantigens. It is conceivable that deeper states of anergy, representing repeated antigen stimulation, might lead to a more rapid elimination of the cells by homeostatic mechanisms other than FAS- or TNF-mediated killing.

What is IL-10 doing to facilitate anergy induction? The simple model is that it is blocking the delivery of costimulation (see Fig. 1). IL-10 has been reported to have a major effect on macrophages (54). It can convert activated cells to a resting monocyte-like state in which the expression of proinflammatory cytokines (such as IL-1, IL-12, and TNF-α) and costimulatory molecules is downregulated (55). Other APC, such as dendritic cells, can also be affected (56). In addition, one experiment has suggested that exposing freshly isolated Langerhans cells (the APCs of the skin) to IL-10 overnight can convert them from stimulators of proliferation to inducers of anergy for murine T cell clones (57). Thus, even in the cross-linked anti-CD3 experiments of Groux et al., where contaminating APC in the purified $CD4^+$ T cell population may play a key role in providing costimulation, the IL-10 could be blocking their function. On the other hand, even in the IL-10–treated groups in this experiment, a small amount of proliferation was noted, suggesting that some costimulation for IL-2 production may have been getting through in these cultures. Thus, the profound anergy brought about by IL-10 could result from other sites of action, particularly in the T cell (58, 59). IL-10 could augment the production of Nil-2a or the inhibitor of $p21^{ras}$ activation. Alternatively, it could block the downregulation of these components by signal transduction through the IL-2 receptor. In fact, a combination of all these effects is possible. Whatever the molecular mechanism(s), however, it is clear that the observations in this paper suggest that IL-10 may be useful in clinical situations in which it is desirable to induce profound T cell unresponsiveness.

Anergy Induced with Altered Peptide Ligands

The discovery that single amino acid substitutions in T cell peptide determinants could create antagonist and partial agonist ligands led to the discovery of some analogues that could induce a proliferative unresponsive state in murine T cell clones (13). Stimulation with these molecules blocked IL-2 production by the clones after restimulation, but other responses remained intact, such as upregulation of the IL-2 receptor α chain (see Fig. 1). The limited pattern of responsiveness observed resembled that of other murine anergy models, but the surprising observation was that the induction of this state could be achieved in the presence of functional APCs. Even the addition of anti-CD28– or B7-expressing transfectants failed to prevent the induction of unresponsiveness. Thus, costimulation, at least as delivered to the outside of the cell, was not missing. Inside the cell, however, some interesting biochemical events were found to be going on (60, 61). Examination of phosphotyrosine

blots revealed that stimulation with partial agonists led to three differences in the pattern of phosphorylation compared to stimulation with full agonists: (*a*) Zap-70 was not detectably phosphorylated; (*b*) CD3 ϵ chain phosphorylation was significantly reduced; and (*c*) the 23-kD phosphorylated form of the ζ chain was greatly reduced. This diminished signaling pattern is compatible with the idea that the TCR has low avidity for the altered peptide ligand–MHC complex, although other models are possible (61). Sloan-Lancaster and Allen interpreted these results to mean that the partial signaling pattern was responsible for the induction of anergy (60).

Further studies, however, have suggested that this is probably not the case (60a). The unusual tyrosine phosphorylation pattern was not found when chemically fixed APC and full agonist peptides were used to induce anergy. Thus, the partial signaling is either not necessary for anergy induction or the two unresponsive states are not equivalent. The former conclusion is suggested by the finding that unresponsiveness induced by altered peptide ligands could be prevented by the addition of IL-2 during the first 24 h of culture, similar to what has been observed by Boussiotis et al. (12) for anergy induced by TCR occupancy in the absence of costimulation. The IL-2 addition did not alter the unusual tyrosine phosphorylation pattern. Thus, this pattern per se does not determine anergy induction; rather, downstream events such as the production of IL-2 are critical. Nonetheless, if the anergic state is comparable to that in other models, events such as the induction of an inhibitor of ras activation must be induced under these signaling conditions (see Fig. 1). Whether Nil-2a would also be induced is unclear. The defect in the partial agonist stimulation would then be the inability to provide enough signaling through the calcium/calcineurin and/or MAP kinase pathways to synergize with an intact CD28 signaling pathway to produce sufficient IL-2 to block the induction or function of the inhibitor of $p21^{ras}$ activation. Thus, as shown in Fig. 1, the anergic state achieved with partial agonists in the presence of costimulation could be similar to the anergic state induced by TCR occupancy in the absence of costimulation, but different from normal activation, where all the inhibitors are blocked.

Human T Cell Clones Stimulated in the Presence of High Concentrations of Soluble Peptides

This model does not exist in the mouse because activated mouse T cells do not express MHC class II molecules, which are required for the induction process (3). Like the partial agonist model, the presence of professional APCs does not inhibit the induction of unresponsiveness (10, 62). In fact, the presence of APCs leads to a large proliferative response. Thus, IL-2 can be produced by the cells, and still the unresponsive state is induced. Single cell studies in agarose matrices suggest that antigen presentation is required by one T cell to another, but antigen-pulsing experiments do not work, indicating that some interaction of the soluble antigen with the responding T cell is required for the effect (62). The state of unresponsiveness is deep, i.e., the cells make few detectable lymphokines on restimulation, similar to the IL-10 model (10). Induction of this state is accompanied by a down modulation of both CD28 and the TCR complex (4, 63). Molecular studies have suggested an impaired intracellular calcium response (62) and a diminished binding of the NF-AT transcription factor to the distal response element in the IL-2 enhancer, with no significant effect on AP-1 (64). The block in IL-2 production can be overcome by a calcium ionophore.

Although not all the desired biochemical studies have been done in this system (e.g., measuring the activation ability of $p21^{ras}$), I do not perceive a clear way to unify this model with all the others I have discussed. It appears to represent a block in the calcium/calcineurin pathway rather than the ras/MAP kinase pathway. It is a reproducible effect having been demonstrated in several laboratories. It can be induced in activated as well as resting T cells and, therefore, could be of great importance in manipulating clinical states during acute onset of diseases (63). The closest mouse model that I have seen to this state is the unresponsiveness of T cell clones studied by Otten and Fitch (65). This also appears to be a block in the calcium-signaling pathway; however, it is induced by signaling through the IL-2 receptor and appears to be a mechanism to block signaling through the TCR when the cell is in cycle. This mechanism possibly exists as a buffer on the propriocidal effect (66). Unlike the human clonal anergy, however, it can be overcome by stimulating with high concentrations of antigen, and the state spontaneously decays when the T cells are rested in culture for 5–7 d. Another possible unresponsive state that could be a model for this human clonal anergy is the veto effect (67). I have discussed this idea before (68), and recent studies with mouse $CD8^+$ T cell clones provide support for a model in which reciprocal recognition of one T cell by the antigen-specific receptor of the other inactivates both cells (69). Nothing is known, however, about the biochemistry of the unresponsive state that is induced, so it is difficult to rigorously compare the two effects. Further studies are required to clarify this situation. At the present time, I would suggest that this form of anergy be kept distinct from other models in the literature, perhaps by referring to it as calcium-blocked anergy in contrast to ras-blocked anergy.

Conclusions

Since the initial discovery of an anergic state in T cell clones, a variety of models of unresponsiveness have been developed that have been given the same descriptor. At one point in time, almost every tolerance model published was attributed to anergy, without any clear attempt to ascertain whether the minimal requirements had been met of a live cell that was functionally unresponsive in at least some assay. In more recent times, with the shift in fashion to apoptosis as the universal mechanism for tolerance, the number of models of unresponsiveness attributed to anergy has returned to realistic proportions. Although some peripheral

deletion addicts would like to herd anergy models into their corral as a slow form of cell death, the recent characterizations of the underlying biochemical mechanisms of the two processes have clearly delineated the pathways. In this overview of the various currently accepted models for anergy, I have attempted to find a unifying molecular mechanism to comment on the new paper by Groux et al. in this issue, which introduces IL-10 as a tool for facilitating the induction of the state. I think that all but one of the models can be understood in the same way. The state is induced by a TCR occupancy event that stimulates the production of several inhibitors, one that blocks $p21^{ras}$ activation and another (Nil-2a) that blocks cytokine transcription. These inhibitors prevent transcription of IL-2 and other cytokines, and they block proliferative pathways when the cell is reactivated. The induction of these inhibitors is normally antagonized by costimulation involving signaling through receptors such as CD28, and proliferation induced by signaling through the IL-2 receptor. The anergic state is stable and seems to exist at different levels depending possibly on the concentrations of the molecular inhibitors that are induced. Cells in this state have been found in vivo after injecting mice with superantigens. In contrast to this "ras-blocked" anergy, high concentration of peptides administered to human T cell clones produces inhibition of the calcium/calcineurin signaling pathway ("calcium-blocked" anergy) accompanied by a downmodulation of the TCR and CD28. The critical biological question remaining to be answered is, what role, if any, do these anergic cells play in an immune response and/or in tolerance induction?

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