A Cell Culture Model for T Lymphocyte Clonal Anergy

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T lymphocytes respond to foreign antigens both by producing protein effector molecules known as lymphokines and by multiplying. Complete activation requires two signaling events, one through the antigen-specific receptor and one through the receptor for a costimulatory molecule. In the absence of the latter signal, the T cell makes only a partial response and, more importantly, enters an unresponsive state known as clonal anergy in which the T cell is incapable of producing its own growth hormone, interleukin-2, on restimulation. Our current understanding at the molecular level of this modulatory process and its relevance to T cell tolerance are reviewed.

MATURE T LYMPHOCYTES THAT EMERGE FROM THE ADULT mammalian thymus migrate to peripheral lymphoid organs such as the spleen and lymph node. There, these naïve T cells encounter breakdown products of foreign proteins (antigens) on the surface of specialized antigen-presenting cells (dendritic cells and macrophages), usually in the form of peptides bound to self molecules encoded by genes of the major histocompatibility complex (MHC) (Fig. 1). MHC class II molecules display peptides derived from proteins internalized through the endocytic pathway and are recognized predominantly by inducer T lymphocytes expressing the CD4 surface molecule. MHC class I molecules display peptides derived from proteins synthesized inside the antigen-presenting cell (for example, viral proteins) and are largely recognized by cytotoxic T lymphocytes expressing the CD8 surface molecule (*1*).

The frequency of T cells specific for any given foreign antigen is initially small. If these cells are to play a central role in host defense, they must selectively increase in number. Thus, activation of the T lymphocyte upon recognition of foreign antigen leads to autocrine growth in which the stimulated naïve cells proliferate in response to their own production of the polypeptide growth hormone interleukin-2 (IL-2) and the receptor for IL-2 (Fig. 2) (*2*). In addition, the cells differentiate, acquiring the ability to produce other lymphokines, such as interleukin-4 (IL-4) and gamma interferon (IFN-γ) for $CD4^+$ cells (*3*). These proteins serve as effector molecules for activating other cells in the immune system. IL-2 also plays a critical role in this recruitment function, as it can act in a paracrine fashion to help activated B lymphocytes and $CD8^+$ cytotoxic T lymphocytes expand in number. Finally, in some cases, repeated stimulation with foreign antigen (and possibly other signals) causes the T cell to specialize in its function (Fig. 2). For example, $CD4^+$ T cells lose their ability to produce certain lymphokines, thus becoming specialized in either delayed-type hypersensitivity for T helper cells type 1 (T_H1 cells) or B cell help for T_H2 cells (*4*). One consequence of this specialization is that certain subsets of T cells (T_H2) no longer show IL-2–dependent autocrine growth. Nonetheless, they can still be influenced by other T cells that produce IL-2, since they retain the ability to express IL-2 receptors on activation. Until this late stage, however, the primary growth hormone for all T lymphocytes is IL-2, and the ability of a $CD4^+$ T cell to produce IL-2 is a key factor in determining its full participation in an immune response.

In this review, I will summarize the known biochemical and molecular events that lead to IL-2 production. I will also discuss the biological state known as T cell clonal anergy, in which the capacity to produce IL-2 is repressed, thus preventing the cell from proliferating in response to an antigenic challenge and from helping other immune cells to proliferate and differentiate. The change of state that follows the induction of clonal anergy is not strictly a differentiation event in the classical sense. The cell maintains its morphology and does not gain the ability to produce new products or perform new functions. The change is also not an activation process, as the cell does not return to the same state from which it started. The process is more akin to long-term potentiation in the nervous system (*5*) and represents a modulation in the reaction of the cell to external stimuli, which has important consequences for its function. Thus, an understanding of the molecular events taking place in the model system that has been developed for clonal anergy could give insights for other biological systems.

The T Cell Antigen Receptor

The minimal requirement for an antigen-specific immune response is effective binding of the peptide and MHC molecule on the antigen-presenting cell by a clonally distributed T lymphocyte surface receptor. The T cell protein complex that is responsible for this recognition event and subsequent signal transduction is known as the T cell antigen receptor (Fig. 1) (*6*). For most T cells, this is composed of two disulfide-linked proteins (α and β) that make physical contact with the peptide and MHC molecule. Each of these proteins is divided into a variable (V) and constant (C) portion, the former differing in structure between any two individual T cell clones and thus giving the cell its unique recognition specificity. This receptor diversity arises during T cell development in the thymus from DNA rearrangements in the gene segments encoding the α and β chains (*7*). These chains are noncovalently associated

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with three integral membrane proteins (γ, δ, and ε), referred to as the CD3 complex and thought to be involved in signal transduction (*6*). In addition, the receptor complex contains one of two forms of a disulfide-linked molecule, either a homodimer composed of two ζ chains, or a heterodimer composed of one ζ chain and one η chain (*8*). The ζ chain contains a consensus sequence for a nucleotide-binding site (*9*), which might be involved in signal transduction. The two forms of the receptor are present in different amounts on the cell surface. For mature T cells, the ζζ form constitutes 90 to 95% of the molecules, the ζη form only 5 to 10% (*10*). Each form of the receptor is believed to couple to different signal transduction pathways (discussed below).

Finally, on engagement of the α and β chains of the T cell antigen receptor, another protein is brought into the receptor complex: the CD4 or CD8 molecule (Fig. 1) (*11*). CD4 binds MHC class II molecules and, therefore, participates in the response to antigen by inducer T cells, whereas CD8 binds MHC class I molecules, and, therefore, participates in the response to antigen by cytotoxic T cells (*12*). These "accessory" molecules are not always essential for antigen responsiveness (based on blocking experiments with antibodies against CD4 or CD8) (*13*). Instead, they are thought to facilitate the response of T cells bearing receptors with low affinity for the peptide-MHC complex by increasing the avidity of the T cell for the antigen-presenting cell, by altering the signaling portion of the antigen receptor to decrease the occupancy required for signal transduction, or by transmitting a separate synergizing signal (*14*).

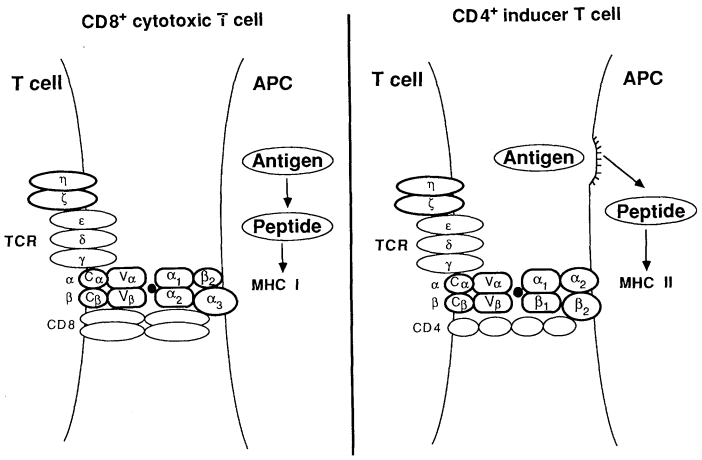

Fig. 1. Antigen recognition by the two major subpopulations of T lymphocytes. $CD8^+$ cytotoxic T lymphocytes (left side) recognize peptides (●) derived mainly from antigens made and degraded in the cytoplasm. These peptides bind to MHC class I molecules in the endoplasmic reticulum and are presented to T cells on the surface of the antigen-presenting cell (APC). The MHC class I molecule is composed of two proteins: a large transmembrane chain consisting of three external domains (α_1, α_2, and α_3) and a smaller chain known as β_2-microglobulin. The peptide binds in a groove formed by the external α_1 and α_2 domains. $CD4^+$ inducer T lymphocytes (right side), mediating help and delayed-type hypersensitivity, recognize peptides derived mainly from antigens internalized by endocytosis and bound to MHC class II molecules. The MHC class II molecule is composed of two transmembrane proteins (α and β) whose outer NH_2-terminal domains (α_1 and β_1) form the peptide binding groove. The T cell antigen receptor (TCR) is the same seven-chain structure (α, β, γ, δ, ε, ζ, η) on both subpopulations of lymphocytes, differing only in the variable (V) regions of the α and β chains that contact the peptide-MHC molecule complex. C_α and C_β are the constant portions of these chains. Note that the actual stoichiometry of the seven proteins in the receptor has not been definitively established. Concomitant with antigen recognition, the "accessory" molecules also become involved in the binding. The two-chain CD8 molecule interacts with the MHC class I molecule, and the one-chain CD4 molecule interacts with the MHC class II molecule. Recent evidence suggests that at least one chain of CD8 interacts with the α_3 domain of MHC class I (*90*); however, the exact three-dimensional structure of this interaction, as well as whether a similar interaction occurs for CD4, is unknown. Therefore, the two-dimensional geometry shown in this figure should not be taken too literally.

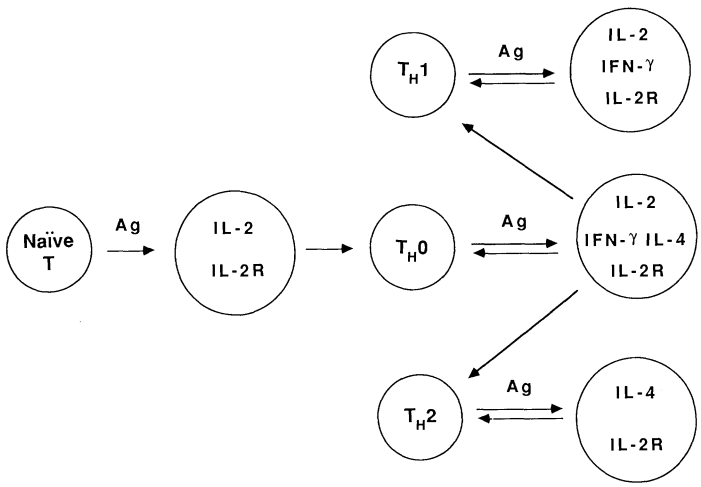

Fig. 2. Antigen-driven changes in lymphokine production by $CD4^+$ T lymphocytes. Recent evidence (*3*) suggests that naïve T cells are capable of making IL-2 and expressing a high-affinity receptor for IL-2 (IL-2R) during their first encounter with antigen (Ag). This allows the cells to proliferate and differentiate into T_HO cells (*91*), which are now competent to make other lymphokines, such as IFN-γ and IL-4, when reactivated with antigen. In the mouse, further stimulation with antigen can drive the cells to turn off selective lymphokine genes, producing phenotypes such as the T_H1 cell, which can make IFN-γ and IL-2 on antigen activation but not IL-4, and the T_H2 cell, which can make IL-4 but not IFN-γ and IL-2 (*4*). The former appears specialized for mediating delayed-type hypersensitivity, whereas the latter appears specialized for helping B cells make antibody. The conversion of T_HO to T_H1 or T_H2 cells has only been inferred from temporal relationships after antigen priming and has not been directly demonstrated. Therefore, other schema for the lineage relationships among these three cell types are possible. $CD8^+$ T cells also exist in forms that do and do not produce IL-2 (IL-2–dependent) (*92*). Again, indirect experiments suggest that the former may be a precursor of the latter (*93*).

Signal Transduction

The generation of second messengers inside the T cell after antigen receptor occupancy or cross-linking involves at least two main pathways: (i) activation of a phospholipase C (PLC) that cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,2-*sn*-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) and (ii) activation of one or more tyrosine kinases (Fig. 3) (*15*). The former pathway leads to activation of the serine/threonine kinase, protein kinase C (PKC), by means of DAG, and an increase in intracellular free calcium ion concentration by means of IP_3 (*16*). Most of the calcium comes from outside the cell through ion channels activated by IP_3 or the inositol 1,3,4,5-tetrakisphosphate derived from it (*17*). The tyrosine kinase pathway, in contrast, is poorly understood. One postulated mechanism involves the CD4 and CD8 accessory molecules. The cytoplasmic domains of these molecules bind the tyrosine kinase $p56^{lck}$ (*18*). Movement of this kinase into close proximity with the receptor when CD4 or CD8 enters the complex might account for the tyrosine phosphorylation of the ζ chain that has been detected during activation (*15*). This phosphorylation, however, is first seen only several minutes after receptor engagement, a rather late event. Other cell proteins have been detected that are phosphorylated on tyrosine residues at much earlier times (5 s) (*19*). Thus, it is presumed that other tyrosine kinases are involved in initiating the signaling cascade. One candidate is $p59^{fyn}$ (*20*).

Which second messenger pathway is critical for T cell activation?

Initially, it was thought that the PLC pathway was the major limb responsible for IL-2 production, because pharmacological stimulation of this pathway with a calcium ionophore (to raise intracellular calcium) and a phorbol ester (to activate PKC) is sufficient to elicit transcription of the IL-2 gene in T cell tumor lines (*21*). More recently, however, tumor variants have been selected in which PLC can no longer be activated (*22*). These cells cannot increase intracellular calcium or generate water-soluble inositol phosphates when stimulated through the T cell antigen receptor. Nonetheless, they produce IL-2 on stimulation and phosphorylate tyrosine residues on their ζ chains. Thus, PLC activation is not necessary for IL-2 production. Interestingly, these variants are defective in η chain expression, and there appears to be a direct correlation between the level of ζη receptor expressed on the T cell and the amount of PIP_2 hydrolysis (*23*). These results suggest that it is the ζη form of the receptor that couples to the PLC (Fig. 3). The biochemical basis of this coupling is not clear. In other systems, a guanine nucleotide–binding protein (G protein) serves as the coupling agent (*24*). To date, however, no good evidence for G protein involvement in T cell activation has been found. An alternative mechanism involving tyrosine kinases has recently been described for activating PLC-γ in fibroblasts and epidermal cells (*25*). This mechanism has yet to be demonstrated in T cells. Furthermore, results with the η-negative variants suggest that the ζζ form of the receptor is adequate for activation of at least one tyrosine kinase, and yet its stimulation is not adequate for PLC activation (*23*). On the other hand, it is possible that the tumor variants may contain more than one mutation.

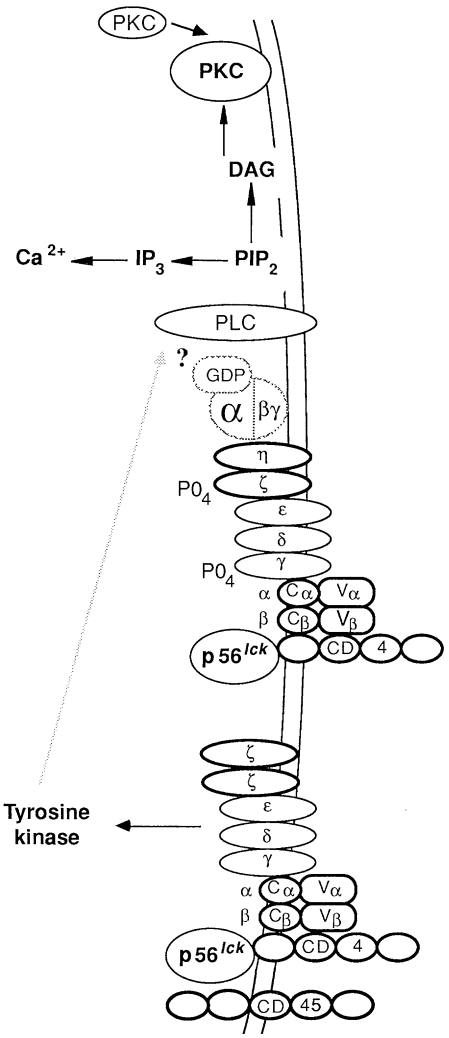

Fig. 3. Signal transduction through the T cell antigen receptor. The T cell antigen receptor exists in two forms (*6*). Each has an α and β chain responsible for antigen recognition and a CD3 complex composed of γ, δ, and ε chains. The difference lies in the complexing to either a ζζ homodimer or a ζη heterodimer. On antigen recognition, the latter form of the receptor is thought to signal by coupling to a phospholipase C (PLC) in the membrane. The mechanism of the coupling is unknown, but may involve a guanine nucleotide–binding protein (GDP, shown in dotted lines) or a tyrosine phosphorylation event (stippled arrow). Activation of PLC leads to the hydrolysis of PIP_2 into 1,2-*sn*-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). In turn, DAG activates the serine/threonine kinase, protein kinase C (PKC), mobilizing it to the plasma membrane, whereas IP_3 raises intracellular calcium ion concentrations (Ca^{2+}) by releasing calcium from intracellular stores and opening calcium channels in the plasma membrane. PKC activation results in phosphorylation (PO_4) of the γ chain of the T cell antigen receptor. The ζζ form of the receptor is capable of activating one or more tyrosine kinases on antigen recognition. The molecular events leading to tyrosine phosphorylations are unknown. One proposed model leading to phosphorylation of the ζ chain of the T cell antigen receptor involves $p56^{lck}$, a cytoplasmic tyrosine kinase that is known to bind to the cytoplasmic portion of the CD4 molecule. This mechanism could be used by either form of the receptor (ζζ or ζη) and the same may be true for other tyrosine phosphorylation events. CD45 is a membrane protein whose cytoplasmic domains have tyrosine phosphatase activity. Its role in signal transduction is currently not fully understood, but it might act on $p56^{lck}$ (*94*).

At this point, it appears that either second messenger pathway is adequate for signaling to the IL-2 gene. Whether they are united at some final common biochemical pathway or act independently through effects on different gene response elements is not clear. Certain inhibitors, such as cyclosporin A, appear to block IL-2 production stimulated by either pathway (*26*). The mechanism of action of this drug, however, is not understood; it could either block a final common pathway or have effects on two different pathways. Where these pathways lead in T cells also remains unknown. Increases in intracellular calcium presumably activate calmodulin, and the major calmodulin-binding protein in T lymphocytes has been identified as the phosphatase calcineurin (*27*); however, whether this protein participates in signal transduction to the nucleus is unclear. Mutants lacking the CD45 glycoprotein fail to divide properly (*28*). The cytoplasmic domain of this protein has tyrosine phosphatase activity (*29*), but how or if the tyrosine kinase and PLC pathways interface with CD45 is not known. Thus, the biochemical connections between second messengers and gene activation remain to be elucidated.

Gene Activation

Resting T lymphocytes are transcriptionally silent for all of the lymphokine genes discussed above as well as for most of the cellular oncogenes required for cell division. Signaling through the T cell antigen receptor leads to activation of many of them (*30*). Some oncogenes (for example, c-*fos*) are transcribed very early, within the first 15 min, suggesting a direct activation of preexisting transcription factors by the biochemical signals coming into the nucleus (*31*). Others [for example, tumor necrosis factor–α (TNF-α)] are transcribed slightly later, within the first hour, and also appear to be directly activated, because inhibitors of protein synthesis do not block the induction of transcription (*32*). Finally, the activation of certain genes (for example, IFN-γ and IL-2) requires new protein synthesis, suggesting that new regulatory proteins (for example, a nuclear factor that binds to the octamer ATTTGCAT, NF-AT) or new modifiers of existing regulatory proteins need to be made in order to activate transcription (*32, 33*).

Activation to cell division requires a second receptor-ligand interaction similar to the progression phase in the stimulation of other cell types (*34*). For the T lymphocyte, this is provided by the interaction between IL-2 and the IL-2 receptor (*2*). Since both of these molecules are synthesized by the same cell, the stimulation is autocrine. The second messenger system for the signal transduction event between IL-2 and the IL-2 receptor is not known, but a tyrosine kinase pathway has been suggested (*35*). The consequence of the signaling is the activation of transcription of genes such as c-*myb* and of the transferrin receptor required by the cell to enter S phase (*31, 36*).

Currently, we know of over 75 molecules that change with T cell activation (*30*). Most are involved in cell division and lymphokine

production and appear to be reversibly activated. A few molecules, however, undergo prolonged changes that are more characteristic of differentiation or modulation events. For example, the induction of cell interaction molecules (integrins) known as very late antigens (VLA) takes place 1 week after stimulation, and these molecules remain expressed on the cell surface (*37*). Such molecules increase the avidity of interactions between the T cell and the antigen-presenting cell and possibly serve a role in memory, a functional state of the immune system in which the cells respond more quickly to a repeated challenge with the same antigen. One of the VLA molecules (VLA-5) is a receptor for the extracellular matrix protein, fibronectin (*37*). A recent study (*38*) suggests that fibronectin may act as a costimulatory molecule for T lymphocytes, signaling through VLA-5 and synergizing with antigen-receptor occupancy to fully activate the T cell (discussed below).

Competence for lymphokine gene activation can also be induced on antigen stimulation. The conversion of naïve T cells to T_H0 cells requires antigen and results in the appearance of IL-4 and IFN-γ after activation (Fig. 2) (*3*). Once turned on, the genes for these proteins remain transcriptionally active for many rounds of cell division. Under certain conditions, however, they become inactive when the cells differentiate and become more specialized in their lymphokine production. The regulatory mechanisms for this are unknown, but a recent study has suggested that the reduction of IL-2 production in T_H2 cells may be through a labile regulatory protein that represses transcription of the IL-2 gene (*39*).

Costimulatory Signals

Occupancy or cross-linking of the antigen-specific receptor is sufficient for the full activation of certain lymphokine genes (for example, IL-4) (*39*). In contrast, many of the other lymphokine genes require additional signaling events through other receptors in order to manifest full activity (*40*). These other signals are referred to collectively as costimulatory activities. There is some confusion in the literature as to the nature of the molecule or molecules involved. In some cases, costimulation can be provided by soluble ligands such as IL-1 (*41*). In other cases, cell-cell interactions are required (*42*). These two situations are difficult to distinguish, as close proximity is sometimes required to deliver high concentrations of labile soluble ligands, whereas soluble ligands can act indirectly to increase the activity of interacting cells (*43*). The best way to identify a soluble costimulatory molecule is to show that it acts on a single T cell. This has never been done successfully.

A second problem arises in defining the mechanism by which a costimulatory activity operates during a cell-cell interaction. There appear to be three ways in which the interaction of a "costimulatory" receptor with a ligand on the surface of the presenting cell can augment a particular activation event. One is through functional or physical interaction (or both) of this receptor with the antigen-specific T cell receptor and enhancement of signal transduction by way of the second messenger pathways described earlier. The lymphocyte function–associated antigen-3 (LFA-3)–CD2 interaction appears to operate through this mechanism (*44*). Second, cell interaction molecules that increase the avidity of the interactions between T cells and antigen-presenting cells will increase antigen receptor occupancy by prolonging the time the two cells remain together. The LFA-1–intercellular adhesion molecule–1 (ICAM-1) interaction is an example of this type of mechanism (*45*). In the final form of costimulation, the ligand-receptor interaction initiates its own second messenger cascade, which synergizes with the antigen receptor signals at some distal biochemical step or at the level of gene activation (*46*). This last mechanism of costimulation can be distinguished from the other two by experiments in which the two signals, antigen receptor occupancy and costimulation, are provided by ligands on physically separate particles, cells, or surfaces. Any synergy requiring the interaction of the two receptors would be prevented by such a maneuver, whereas intracellular pathway interactions would not.

In this review, detailed studies on the costimulatory activity required to activate murine T_H1 clones will be described (*42*). In this system, no soluble molecules have yet been found that will provide costimulation [IL-1 through IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor–β (TGF-β) have been tested]. The costimulation, however, can be provided by a separate cell incapable of interacting with the T cell antigen receptor, incapable of acting across a 0.45-μm membrane, and incapable of affecting levels of second messengers associated with T cell antigen receptor signaling. Thus, this costimulation appears to represent a cell-cell interaction operating through an independent receptor.

In the murine T_H1 clones, lymphokines such as IFN-γ and IL-3 are partially induced by antigen-receptor occupancy alone (*40*). Addition of costimulatory activity from the antigen-presenting cell results in full activation. In contrast, IL-2 production appears to be completely dependent on costimulatory activity. Without some form of this signal, no detectable IL-2 mRNA is observed. This concept is still somewhat controversial, since antibodies to CD3 (anti-CD3) on a solid surface, or the combination of phorbol ester and calcium ionophore, are known to readily stimulate T cells to make IL-2 and divide (*21, 47*). Recent evidence, however, suggests that such responses are only observed at high cell density (*48*). Single murine T_H1 cells cultured in microwells do not proliferate to such stimuli. Furthermore, the proliferative response of T cells at high density falls off with decreasing cell number in a nonlinear manner. The slope of a plot of the logarithm of response versus the logarithm of T cell number is approximately 2, suggesting that a cell-cell interaction is required for the T cells to produce the IL-2 they need to divide. This cell interaction requirement can be eliminated by adding IL-2 or satisfied by adding a sufficient fixed number of antigen-presenting cells to provide a constant source of costimulatory activity needed for IL-2 production.

The molecular nature of the costimulatory activity for murine T_H1 cells is unknown, as are the receptor and the signal transduction pathway. Costimulation can be studied, however, by means of presenting cells that are incapable of displaying antigen to a particular T cell clone because they express the wrong allelic form of the MHC molecule (allogeneic cells) (*42*). These cells are added as a source of costimulatory activity to any system in which a pure antigen receptor stimulation is given, for example, monoclonal antibody to CD3 (*49*) or chemically fixed antigen-presenting cells plus peptide (*42*). In such systems, addition of the allogeneic cells is necessary for the production of IL-2 by the T cell clone. This is achieved, however, without any augmentation of PIP_2 hydrolysis, any increase in protein kinase C activity, or any increase in phosphorylation of tyrosine residues in the ζ chain of the T cell antigen receptor (*46*). Thus, no effect is observed on antigen-receptor–induced early signaling events, suggesting that the costimulatory signal is delivered through a different pathway.

Is the costimulatory signal synergizing at the level of the IL-2 gene? The IL-2 enhancer-promoter region has been mapped 5′ of the transcription start site (*50*). Most of the regulatory activity [as defined in transfection experiments with assays measuring chloramphenicol acetyltransferase (CAT) activity] is contained in the 300-bp region immediately upstream. At least four different response elements have been identified in this segment by deletion mapping or sequence analysis (*51*): the phorbol ester response element (*52*),

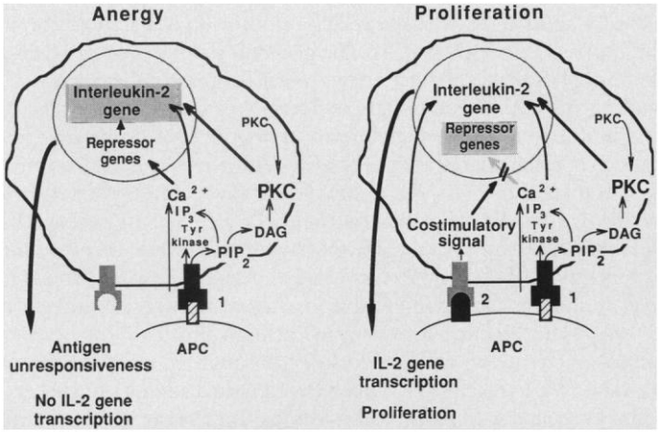

Fig. 4. The costimulatory signal determines the outcome of T cell receptor occupancy. Occupancy of the antigen-specific T cell receptor by a peptide–MHC molecule complex (1) leads to activation of tyrosine kinases and phospholipase C as described in Fig. 3. These signal transduction events alone are not sufficient for activation of the IL-2 gene (left drawing). Instead, the calcium signal appears to activate one or more repressor genes that cause the cell to enter an anergic state. In contrast, if a simultaneous costimulatory signal is initiated (2) by a ligand on the antigen-presenting cell (APC), then the induction of the repressor genes is inhibited and the IL-2 gene is activated, leading to a proliferative response by the T cell (right drawing).

the nuclear factor κB (NF-κB) response element (*53*), the octamer response element (*54*), and the antigen receptor response element 2 (which binds NF-AT) (*33*). Some of the nuclear proteins that bind to these sites have been well characterized and are known to be affected by receptor-transmitted signals. For example, NF-κB is normally found in the cytoplasm in an inactive form complexed to an inhibitor (*53*). Activation of PKC with a phorbol ester modifies the inhibitory protein, allowing it to disengage and the NF-κB protein to move to the nucleus. There, NF-κB binds to its response element and contributes to gene activation. Phorbol esters also activate the AP-1 complex by increasing the synthesis of c-*fos* messenger RNA (mRNA) (*54*). T cell antigen-receptor occupancy has a similar effect (*55*). A report on the consequences of costimulation with IL-1 provides a good model for how one type of costimulation may synergize with antigen-receptor occupancy at the level of transcriptional control (*56*). IL-1 signaling was shown to increase message levels for c-*jun*, which encodes the second protein component of the AP-1 complex. The combined increase in the c-*jun* and c-*fos*–encoded proteins is proposed to lead to a synergistic interaction, since the latter combines with the former to enhance the c-Jun protein's binding affinity for the phorbol ester response element (*52*).

T Cell Clonal Anergy

One of the most important biological discoveries in the field of T cell activation during the past few years was the finding that occupancy of the antigen-specific receptor in the absence of a costimulatory signal is not a neutral event for the T cell (*57*) (Fig. 4). Instead, this form of signaling induces in the T cell a state of unresponsiveness characterized by an inability to produce IL-2 when the cells are subsequently exposed to both antigen-receptor occupancy and a costimulatory signal. This state was first discovered by investigators using murine T_H1 (*58*) and human (*59*) T cell clones, but since then has been demonstrated with freshly isolated T cell populations (*60*). These systems provide a tissue culture model for the state of in vivo tolerance known as T cell clonal anergy, in which self-reactive lymphocytes are not deleted during development, but somehow cannot be activated to divide upon stimulation with antigen and presenting cells (*61*). The lack of T cell division can have many causes. For example, occupancy of the antigen-specific receptor at the time that IL-2 interacts with the IL-2 receptor blocks the cell from entering S phase (*49, 62*). In the converse situation, stimulation of T_H1 murine clones with high doses of IL-2 induces a refractory period during which the cell cannot be stimulated by antigen and presenting cells (*63*). Both of these nonresponsive states, however, are transient. Removal of the stimulus leads to a full restoration of proliferative competence within 1 week. In contrast, the nonresponsive state to be detailed in this review persists indefinitely after removal of the induction signal. The critical change in this modulated state is a dramatic reduction in the ability of the T cell to produce IL-2. It is the lack of IL-2 production that prevents the cell from dividing. Several of the in vivo models for T cell clonal anergy (*61, 64, 65*) also suggest that impairment of IL-2 production is a critical element in their mechanism of nonresponsiveness. Thus, I will refer to the murine T_H1 tissue culture model to be discussed as a type of T cell clonal anergy.

The induction phase for anergy in the cell culture model is an active process, requiring new protein synthesis, as it can be blocked by cycloheximide (*66*). The critical second messenger event for induction appears to be a rise in intracellular calcium, because exposure of the cells to the calcium ionophore, ionomycin, is sufficient to induce the state, and because chelation of calcium ions with EGTA blocks the induction (*40*). The dose of ionophore required for a maximum effect must be maintained for 6 to 8 hours, and the mean level of intracellular free calcium ions achieved is equivalent to that sustained in normal activation. Addition of a source of costimulatory activity within the first 2 hours of antigen receptor occupancy also blocks the induction of anergy. Between 2 and 8 hours, there is only a partial rescuing effect, and after 8 hours there is none (*46*).

Induction of the anergic state in cell culture has been achieved by a variety of different stimuli. Initially, it was found that chemical pretreatment of the antigen-presenting cell with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) or paraformaldehyde prevented antigen-induced proliferation of normal T cell clones and led to clonal anergy (*42, 58*). This technique was complicated by the concomitant chemical damage of the MHC class II molecules, various adhesion molecules, or both, on the cell surface, and therefore required increased antigen concentrations (10- to 100-fold) to form sufficient antigen–MHC molecule complexes for adequate T cell antigen-receptor occupancy. Under these conditions, increases in intracellular calcium could be achieved in the absence of costimulation, because the costimulatory activity is usually not constitutively expressed on the surface of antigen-presenting cells, and thus chemical fixation prevents its induction. Recent studies suggest that if one activates the presenting cell to express the costimulatory activity before fixation, then the cells are capable of stimulating a T cell proliferative response (*67*).

The cleanest system for inducing clonal anergy, from a molecular point of view, is the planar membrane system (*68*). Purified MHC class II molecules are inserted into liposomes, and these are spread out on a glass or plastic surface. Antigen is then added in the form of a chemically processed peptide so that antigen–MHC molecule complexes form, and the T cells are added for 48 hours (*66*). During this time, the T cells enlarge but do not divide. Subsequent attempts at stimulation with normal antigen-presenting cells and antigen reveal that the T cells have become anergic. This result suggests that a pure antigen-receptor occupancy event is sufficient to induce anergy.

This concept was confirmed by inducing anergy with an anti-CD3

monoclonal antibody specific for the ε chain of the T cell antigen receptor (*49*). Coating the plastic surface of a tissue culture well with the antibody, incubating the T cells for 18 hours in these wells, and removing them from the stimulus induced the anergic state. Interestingly, these cells made a small proliferative response during this form of stimulation, suggesting that some IL-2 was being produced. As mentioned earlier, the magnitude of this response is density dependent, and the proliferation is thought to result from the induction of costimulatory capacity in the T cells (*48*). The importance of this observation is that it suggests a dissociation of anergy from IL-2 production and demonstrates that the absence of IL-2 is not the reason for the induction of the nonresponsive state. A similar conclusion was reached in the chemically fixed and planar membrane systems by showing that the addition of exogenous IL-2 did not prevent the induction of anergy (*58, 66*). Whether the difference in IL-2 induction versus the prevention of anergy represents a quantitative difference in the amount of costimulatory signal required for each event, or is simply the successful activation of a small subpopulation of the T cells, is not clear at the present time.

The final method that has been used to induce T cell clonal anergy is stimulation with the lectin concanavalin A (Con A), added under conditions in which the cloned murine T_H1 population is rigorously depleted of antigen-presenting cells (*46*). This molecule binds to specific sugars on a number of different cell surface proteins and is thought to have its stimulatory effect by interacting with the T cell antigen receptor. Its importance in the study of T cell anergy stems from the insights gained in the area of early biochemical activation events. In particular, all of the other systems showed impairment in the generation of inositol phosphates during anergy induction, compared to normal activation. No inositol phosphates were detected after stimulation with ionomycin, while suboptimal amounts were detected after stimulation with chemically fixed cells or anti-CD3. With Con A, however, the same amount of inositol phosphates was generated without antigen-presenting cells (conditions that lead to anergy) as with antigen-presenting cells (conditions that lead to a proliferative response and no anergy). These results were critical to our conclusion that the difference in the fate of the cell (IL-2 production and division versus anergy) depends on the presence or absence of a costimulatory activity acting independently of PIP_2 hydrolysis.

T cells in the anergic state are best characterized by their lack of IL-2 production (*40, 66*). No biologic activity is found in the culture supernatants of cells stimulated with normal antigen-presenting cells and antigen. Weak proliferative responses are often observed, however, at antigen concentrations that are 30- to 100-fold higher than required for activation of the same T cell in its normal resting state. The maximum response achieved is also suboptimal, usually 5 to 30% of normal. These results suggest that a small amount of IL-2 is being produced, but that it is not being detected in the culture supernatants because the cells are utilizing it. Examination of the anergic T cell population for IL-2 message by Northern (RNA) blot analysis, to avoid the problem of IL-2 consumption, showed a 93 to 95% reduction in net levels of mRNA 4 hours after stimulation, compared to activated normal cells (*69*). In situ hybridization revealed that all the cells were deficient (*70*). Thus, the normal increase in IL-2 mRNA after T cell activation is greatly blunted in anergized cells, and only those cells that at high antigen concentrations make enough IL-2 for autocrine growth are able to proliferate. Whether the block prevents IL-2 gene transcription or affects posttranscriptional stability of the mRNA is not yet known.

Most of the signaling events at the surface of anergized T cells are normal. These cells have equivalent numbers of T cell antigen receptors to those found on normal cells (*49, 66*). Signal transduction through the antigen receptor stimulated by Con A or anti-CD3 appears normal in anergized cells as measured by increases in intracellular calcium ions (*71*). The generation of total water-soluble inositol phosphates in the presence of LiCl is comparable for normal and anergized cells, although differences in loading of [^{3}H]*myo*-inositol, as well as potential differences in pool sizes, make interpretations of small quantitative differences difficult. The overall impression is that no differences are consistently observed, suggesting that anergy is a block in signaling more proximal to nuclear events. The one discrepant observation for this hypothesis is the ability of the combination of the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate and the calcium ionophore ionomycin to stimulate anergized T cells to proliferate when cultured at high density. Whether this represents a bypass of the block by reversing a kinase defect, a reversal of a transcriptional block by a pharmacologic phosphorylation of a nuclear transcription factor, or some other event requires further elucidation.

Interleukin-2 is not the only lymphokine whose activity is modulated in anergic T cells. The biologic activity of IL-3 recovered in the supernatant after stimulation with antigen-presenting cells and antigen is decreased about 87% (*71*); that for IFN-γ is down about 33%. In contrast to IL-2 production, however, the antigen dose-response curves for IL-3 and IFN-γ production are not shifted to higher concentrations. Thus, the anergic T cell, when stimulated with low concentrations of antigen, is mainly deficient in its ability to proliferate. This creates an interesting state in which the cell can produce small amounts of some lymphokines, but cannot divide.

The anergic state, once induced in vitro, routinely lasts for several weeks (24 days is the longest time interval examined) (*58, 66*). For human T cell clones, this state has been reversed by stimulating the cells with high concentrations of IL-2 (*72*). Preliminary observations with mouse T cell clones suggest a similar outcome (*69*). Cell recoveries suggest that this is not the result of outgrowth of a subpopulation of cells that were not anergized. This reversibility raises the possibility that the anergic state is maintained by a stable negative regulatory factor or factors that are diluted out with multiple rounds of division. Thus it is conceivable that anergic cells could be rescued in vivo by IL-2 from neighboring cells that are responding to other antigens, provided occupancy of the antigen receptor on the anergic T cell simultaneously led to the expression of its IL-2 receptor.

In Vivo Relevance of the Model

From the previous discussion, it is clear that many mature T lymphocytes are programmed in such a way that the costimulatory signal controls the outcome of T cell antigen-receptor occupancy (Fig. 4). In the presence of costimulation, recognition of antigen and the MHC molecule induces expression of both IL-2 and the IL-2 receptor, leading to cell division. In the absence of costimulation, antigen-MHC complex recognition elicits partial IL-2 receptor and lymphokine (for example, IFN-γ) production, but little or no IL-2. Instead of dividing, the cell is induced into a state of anergy. This physiology of T cell activation is similar to the two-signal model for B cell activation proposed in 1970 by Bretscher and Cohn (*73*). In their scheme, signal one (immunoglobulin receptor occupancy) alone led to tolerance, while signal one plus signal two (a costimulatory signal delivered from another antigen-specific cell) caused the B cell to make antibody.

The reason for two signals in the Bretscher and Cohn model was to have a mechanism for tolerizing mature B cells whose immunoglobulin receptors had somatically hypermutated to anti-self specificity after encountering foreign antigen. The reason for having two signals to activate mature T cells is not obvious. Such a mechanism

might provide mature T cells a means to be tolerized to peripheral antigens (that is, those found exclusively in other tissues of the body) (*57, 74*). This may be the case for naïve $CD8^+$ T cells immediately after they emerge from the thymus, as most peripheral tissues express MHC class I molecules but probably lack the ability to deliver a costimulatory signal. The circumstances under which $CD4^+$ T cells would normally encounter such antigens in the absence of costimulation is less clear. Certain cells, such as keratinocytes in the skin, express MHC class II molecules when activated by IFN-γ (*75*). This usually occurs during an inflammatory response. Although activated keratinocytes have been shown to induce anergy in T cell clones in vitro (*76*), presumably because they lack the ability to deliver a costimulatory signal, one might expect that during an inflammatory response the invading monocytes would be able to provide this signal to a T cell being stimulated by a keratinocyte.

One cell type that does appear to be capable of inducing anergy in the peripheral tissues is the resting B lymphocyte. This circulating cell is a poor stimulator in the primary mixed-lymphocyte response in vitro (*77*) and cannot initiate an antibody response in vivo (*78*), although it does participate in the priming of proliferative T cells in vivo (*79*). More importantly, B cells have been shown to induce clonal anergy in $V_\beta6^+$ T cells in vivo when minor lymphocyte-stimulating antigen type 1^a (Mls-1^a) is the antigen (*64, 80*). B cells may also be involved in the partial tolerance to allogeneic MHC molecules induced by injecting adherent cell-depleted spleen cells intravenously (*81*). On the other hand, if resting B cells can induce anergy, it would seem to present problems for the host in responding to foreign antigens. B cells, with their high-affinity immunoglobulin receptors, should pick up low concentrations of circulating foreign antigens and, after processing and presentation, induce tolerance instead of immunity. A possible solution to this paradox, as discussed by Janeway (*82*), is that most relevant foreign antigens enter the body as parts of infectious organisms. These organisms might bring with them their own costimulatory activity. Such molecules are most likely different from the endogenous costimulatory activity, for example, molecules such as lipopolysaccharide. Presumably, the immune system has evolved to recognize such molecules as a signature of foreign invaders. Janeway has even argued that receptors for these foreign costimulatory molecules represent the primitive immune system, which only subsequently became linked to an antigen-specific receptor recognition system in a two-signal model. Alternatively, the bacterial products could simply serve to induce endogenous costimulatory signals in resting B cells by activating them. For example, lipopolysaccharide-stimulated B cells become more potent antigen-presenting cells (*80, 83*). In this context, then, peripheral self antigens would induce tolerance by virtue of their inability to activate a costimulatory pathway, while foreign antigens would induce an immune response.

Another mechanism that the immune system might use to avoid the problem of tolerance to foreign antigens is the spatial distribution of its responding cells. The outcome of a challenge to the system (tolerance versus response) is strongly influenced by the route through which the foreign antigen enters the body (*84*). Intravenous administration favors tolerance induction (*85*), whereas subcutaneous injection favors an immune response (*86*). The type of response is thought to relate to the cell types that first encounter the antigen. In the skin, Langerhan's cells appear to be ideally designed to pick up the antigen, to carry it to the draining lymph nodes, and to present processed peptides to the T lymphocytes there (*87*). What happens on intravenous injection is not understood, but may involve the preferential encounter of antigen with B cells and subsequent induction of anergy. Thus, "professional" antigen-presenting cells, capable of delivering costimulatory signals, are strategically located so as to encounter foreign antigens when they first enter the body and to initiate an immune response to bring about the elimination of the antigen.

A role for clonal anergy in thymocyte development has also been described. These results are reviewed extensively in another article in this issue (*88*). Most of the models for which this has been demonstrated utilize radiation-induced bone marrow chimeras (*65*) or nude mice (*89*). It could be argued that these experimental systems represent artificial situations in which the emergence of clonal anergy is a consequence of the manipulation or the mutation. Nonetheless, I think these models give some insight into natural mechanisms. For example, clonal anergy might exist as a fail-safe mechanism that will neutralize T cells with receptors of too low an affinity for self antigens to be eliminated by clonal deletion in the thymus.

Whether or not clonal anergy proves to be a natural mechanism for producing peripheral or thymic tolerance, knowledge of its existence and of ways to induce it should prove useful in clinical situations. The selective neutralization of antigen-specific immune responses has been the ultimate goal of immunologic intervention for years. Interfering with the delivery of costimulatory signals, for example, with monoclonal antibodies against the ligand or the receptor, could at last provide a means for selectively treating autoimmune diseases and facilitating organ transplantation.

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