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CLONAL EXPANSION VERSUS FUNCTIONAL CLONAL INACTIVATION: A Costimulatory Signalling Pathway Determines the Outcome of T Cell Antigen Receptor Occupancy[1]

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INTRODUCTION

The rapid development of a T-cell proliferative response to foreign antigens is one essential determinant of the capacity of the immune system to eliminate a pathogen that has invaded an organism. The expansion of antigen-specific T cells of the $CD4^+$ inducer phenotype is critical in the generation of delayed-type hypersensitivity responses (DTH) as a result of the ability of these cells to secrete lymphokines (including interferon-γ) that attract and activate macrophages at the site of antigen deposition. $CD4^+$ cells are also critical for the development of cytotoxic responses to virus-infected cells, malignant cells, and allogeneic tissue, either directly through lymphotoxin release (TNF-β), or by the lymphokine-mediated expansion of cytotoxic T lymphocytes. Finally, these T cells also provide help for antibody responses through the secretion of interleukins during interactions with antigen-specific B cells.

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Because of the pivotal role of the T cell in these situations, T lymphocyte proliferation must be tightly regulated to protect against the development of inappropriate immune responses to self-antigens, or exaggerated, pathologic responses to foreign antigens. Deletion of autoreactive T-cell clones during maturation in the thymus, and induction of antigen-specific regulatory or suppressive T-cell populations both appear to represent control mechanisms capable of modifying the potential for T-cell responsiveness to antigen. In addition, the antigen responsiveness of any particular mature T cell may be dependent upon its previous experience with antigen and accessory cells, such that subsequent responses could vary in intensity and nature.

In this review we examine the control of T-cell proliferation at the level of the antigen-presenting cell (APC). In particular, we focus on the nature of the critical biological and biochemical signals required to activate the T cell, as well as the consequences of incomplete signalling. Finally, we propose a three-signal model to explain our observations, and we suggest how this model could be applicable to clonal deletion and clonal anergy mechanisms of tolerance induction in vivo.

A BIOLOGICAL TWO-SIGNAL MODEL OF T-CELL ACTIVATION

A solution for the problem of self-nonself discrimination was one of the driving forces behind the development of clonal selection theories in immunology (1–3). The existence of preformed cells—each with a unique receptor—allowed the system to purge itself of autoreactive clones by eliminating cells bearing anti–self receptors. The earliest explicit model to speculate on how this deletional process might occur was put forth by Lederberg (4). He postulated a temporal model of lymphocyte signalling in which antigen receptor occupancy was identical in both immature and mature cells, but coupling of receptor engagement to more distal biochemical pathways varied with the state of maturity of the cell. Receptor engagement by self-antigens during early development gave a negative signal to the cell, causing it to die. Subsequent maturation of surviving lymphocytes changed the biochemical coupling mechanism inside the cells in such a way that later receptor engagement by foreign antigen was perceived as a positive signal for the cell to respond (i.e. with secretion of antibody or lymphokine).

Bretscher & Cohn (5) subsequently recognized that this model was not sufficient to account for B-cell self-tolerance, given the propensity of the immunoglobulin genes in activated B lymphocytes to undergo somatic

hypermutation. Through this process, receptors on mature B cells could acquire the capacity for recognition of self-antigens, which upon engagement would lead to autoreactivity in the Lederberg model. There could be no escape by reversion of the cells to an immature state, because in the adult this would allow foreign antigen the opportunity to induce tolerance. As a solution to this problem, Bretscher & Cohn proposed the first two-signal model of lymphocyte activation. Signal one was postulated to be occupancy of the antigen-specific receptor (membrane Ig on B cells). Alone, this signal led to inactivation of the lymphocyte. If, however, the cell received a second signal, simultaneous with antigen-receptor occupancy, then the combination of signals would activate the cell and induce a positive response to the antigen. The second signal in their model was formulated in terms of the emerging concept of T-cell help (hapten-carrier effects) (6); today it might take the form of T cell–derived lymphokines (7). This model was capable of dealing with the problem of continually arising autoreactive clones in the mature B-cell population, as such cells would be turned off when they encountered self-antigens alone, in the absence of autoantigen-stimulated T-cell help. Foreign antigens, however, would remain immunogenic, as simultaneous T-cell stimulation by carrier determinants would provide the responding B-cell population with a second signal. Thus, the model predicted a form of competition at the level of antigenic stimulation between activation and inactivation of the mature, responding lymphocyte. Despite the fact that tolerance at the B-cell level no longer appears absolutely necessary—i.e. T-cell receptor genes do not undergo somatic hypermutation (8), and, therefore, T-cell tolerance should be sufficient to control B-cell responses, the general outlines of the model have been substantiated for multivalent antigens (reviewed by Nossal in *Annu. Rev. Immunol.*; 9).

The extension of the concept of a two-signal model to T-cell activation was primarily the work of Lafferty and coworkers (10, 11). They postulated from their studies on the generation of cytotoxic T lymphocyte (CTL) responses that the alloantigens found on most cells in a transplanted tissue were incapable of eliciting an immune response; only hematopoietic stimulator cells carried within the transplanted tissue provided both allogeneic major histocompatibility complex (MHC) antigens and what they termed *an inductive stimulus* (second signal) required for the initiation of a T-cell response. Consistent with this model was the work of Bach et al (12), which demonstrated that UV-irradiation of allogeneic stimulator cells resulted in a failure to elicit a CTL response to class-I determinants in a primary mixed lymphocyte culture (MLC). Although Bach suggested that the UV-sensitivity of the stimulator cell was at the level of presentation of class-II determinants (as the addition of normal class-II disparate stimu-

lators could restore the response), Lafferty & Woolnough interpreted the result as evidence that UV-irradiation reduced the metabolic activity of the stimulator cell population and prevented the delivery of a costimulatory signal (signal two). They went on to suggest a soluble nature for the second signal, given the ability of concanavalin A (Con A)-stimulated spleen cell supernatants to restore the response to UV-treated stimulator cells. Our current understanding of CTL generation would suggest that $CD4^+$ T cell–derived lymphokines such as interleukin 2 (IL-2) make up the soluble factors within a Con-A supernatant capable of supporting CTL growth (13, 14). Nonetheless, we might predict that the failure to produce IL-2 by $CD4^+$ helper T cells in a mixed lymphocyte culture directed against UV-irradiated stimulator cells stems from an inability of these treated stimulatory cells to deliver a second signal.

The role of the accessory cell in T-cell activation has also been defined through the use of mitogens such as Con A and phytohemagglutinin (PHA). These molecules are capable of stimulating T-cell proliferation in the absence of MHC molecule recognition by the antigen-specific receptor, presumably by interacting directly with the CD3 complex (15). When accessory cells are carefully depleted from T-cell populations, a significant reduction in mitogen responsiveness is observed, consistent with the need for accessory cell–derived second signals to stimulate a proliferative response (16). The ability of IL-1 in many cases to replace the requirement for accessory cells in both the generation of CTL in mixed lymphocyte cultures and in mitogen-induced proliferation has led to the hypothesis that this monokine may represent the soluble second signal (17, 18). As information accumulates, however, to suggest (*a*) that IL-1 receptors are not present on all murine $CD4^+$ T-cell populations (19), (*b*) that the effects of IL-1 on the responsive population of murine T cells may be limited to enhancement of lymphokine response rather than lymphokine production (20), and (*c*) that the effects of IL-1 may in some cases be limited to actions on the accessory cells in the population (21), it seems unlikely that IL-1 alone plays a direct role in alloreactive or mitogen-induced responses as the actual second signal for many T cells. Nevertheless, IL-1 may augment the ability of accessory cells within the population (or contaminants within an accessory cell–depleted population) to deliver the second signal to T cells.

All of the studies described above support the notion of a general biological two-signal model for T-cell activation. The model of Bretscher & Cohn, however, makes a unique prediction regarding the potential effects of isolated T-cell antigen receptor (TCR) occupancy in the absence of an adequate accessory cell–dependent signal two, i.e. signal one alone should inactivate the cell. Experimental evidence consistent with this was

first reported by Claman and coworkers (22, 23). In their studies, intravenous injection of haptenated spleen cells induced a state of tolerance, as measured by the subsequent failure of the animal to develop contact sensitivity upon reexposure to the hapten. The ability of simultaneous Con-A administration to prevent this induction of antigen-specific unresponsiveness and, in fact, to lead to the sensitization of the animal to the hapten suggested that two signals were operative in the control of contact sensitivity. In their model, the haptenated spleen cells provided one signal (antigen/MHC) to the responding T cells, which alone was tolerogenic, and the Con A provided a second signal that converted the first signal to a positive stimulus.

Can a biological two-signal model explain both the requirement for accessory cells in the induction of T-cell responses and the control of T-cell responsiveness to subsequent stimulation? Work in our laboratory over the past three years has directly demonstrated that some T cells can enter a state of reduced responsiveness upon encountering processed antigen presented by Ia molecules in the absence of a functional accessory cell (24). Incubation of murine Type I $CD4^+$ T-cell clones with peptide antigens presented in association with purified Ia molecules in a planar membrane (25) or presented by chemically fixed APC (26) failed to stimulate proliferation and instead induced a state of proliferative nonresponsiveness. The effect was both antigen and MHC specific, suggesting that TCR occupancy was required. The state of clonal anergy induced by the incubation was long-lived, lasting greater than seven days, and was not associated with accelerated mortality, as the cells remained capable of proliferating if stimulated with exogenous IL-2. Flow cytometric analysis of the cells stained with anti-TCR monoclonal antibody demonstrated comparable levels of receptor expression on normal and nonresponsive T cells (25). Thus, reduced expression of the TCR at the cell surface was not the reason for the nonresponsiveness. Analysis of T-cell lymphokine production revealed that the inability of these lymphocytes to proliferate in response to further antigen stimulation appeared to stem from a lymphokine production defect—in particular, the failure to secrete sufficient IL-2 to drive proliferation (25, 27). Production of IFN-γ and IL-3 were also reduced by the incubation; however, the defect was not as profound as that observed for IL-2 synthesis (M. K. Jenkins, unpublished observations). Thus, occupancy of the TCR with antigen in the presence of fixed APC resulted in a functional inactivation of the clone at the level of IL-2 production.

Lamb & Feldmann and their colleagues (28, 29) have observed what appears to be a similar state of antigen unresponsiveness in normal human T-cell clones incubated with high doses of free peptide antigen in the

absence of accessory cells. In contrast to murine T cells, human T-cell clones express class-II MHC molecules capable of presenting peptide antigens. Such antigen/Ia complexes could occupy the TCR on these cloned cells (signal one) and, in the absence of an exogenous accessory cell population capable of providing a second signal, induce an unresponsive state. Experiments by their group, however, have suggested that the unresponsive state results from down regulation of antigen receptor expression (30, 31). This is clearly not the case for the murine clones. In addition, others have found some Ia-bearing human T-cell populations competent to present antigen and induce the proliferation of T cells specific for the antigen (32). Possibly this inconsistency is related to the state of activation of the T cell presenting the antigen. An activated T cell may possess costimulatory activity, whereas the rested, cloned T cell may not (see below). Further studies will be required to determine how applicable this human in-vitro model of T-cell tolerance is.

Many details regarding the nature of the unresponsive state in the murine system have yet to be explored. Nonetheless, its induction appears to depend on the presence or absence of an accessory cell–derived costimulatory activity. Several laboratories have found that during stimulation of T cells with peptide antigen and chemically fixed syngeneic APC, the addition of a normal allogeneic accessory cell population (itself incapable of presenting the antigen because of the lack of an appropriate Ia molecule) allows a proliferative response to the antigen (33–35) and antagonizes the induction of unresponsiveness (36). Figure 1 demonstrates this ability of normal accessory cells to induce a proliferative response to peptide antigen presented on paraformaldehyde-fixed APC. Neither fixed APC alone nor fixed APC in the presence of lymphokine (in this case rIL-6) are sufficient to induce proliferation to the antigen in the absence of a normal accessory cell population. In our hands, low-density splenic accessory cells have been the most potent source of this costimulatory activity. The high-density fraction of T-depleted spleen (resting B cells) works to a lesser extent, and its activity is radiosensitive (37). Resting splenic T cells have no costimulatory activity, although activated T cells may acquire it (see below). Unlike the soluble activity suggested by Lafferty, culture supernatants (and recombinant IL-1 and IL-6) do not replace the costimulatory activity of intact accessory cells, and separation of the viable accessory cell population from the responding T cells by a permeable millipore membrane prevents the addback effect (36). IL-2 is capable of driving the T-cell clone to proliferate in the presence of peptide antigen plus fixed APC or planar membranes (as the clone constitutively expresses high affinity receptors for IL-2); however, the presence of exogenous IL-2 can not prevent the induction of the antigen-unresponsive state (25). Thus, these

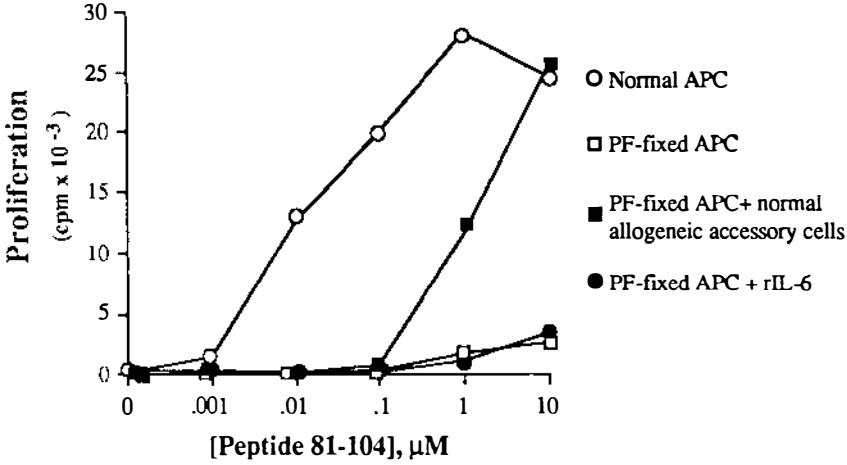

Figure 1 Normal allogeneic accessory cells reconstitute the ability of fixed APC and antigen to stimulate a proliferative response. The murine Type I CD4$^+$ T cell clone A.E7 (2×10^4 per well) was incubated with the pigeon cytochrome *c* peptide 81–104 in the presence of either normal syngeneic B10.A spleen cells (5×10^5) (open circles), or in the presence of 0.5% paraformaldehyde-fixed (PF) syngeneic spleen cells alone (5×10^5) (open squares) or in combination with normal allogeneic B10 T-depleted low-density spleen cells (2×10^5) (closed squares) or recombinant IL-6 (100 u/ml) (closed circles). Thymidine incorporation was measured at 60 hr following a 16 hr pulse and is expressed as the mean cpm of duplicate samples.

experiments appear to define a cell-bound or short-range acting costimulatory activity required for the delivery of signal two.

What is the nature of this costimulatory activity? At the present time we only know what it isn't: IL-1, IL-2, IL-3, IL-4, IL-6, IFN-γ, GM-CSF, TGF-β, and TNF-α all fail to reconstitute a proliferative response, and none is capable of blocking the induction of unresponsiveness. Kurt-Jones et al (38) have described a membrane-bound form of IL-1 capable of providing a costimulatory signal in the proliferative response to antigen of Type II CD4$^+$ T cells. However, we have found that receptor occupancy of Type II CD4$^+$ T cells in the absence of IL-1 or costimulatory signals does *not* induce a state of unresponsiveness (M. K. Jenkins, unpublished observations). Only Type I CD4$^+$ T cells show this effect. Furthermore, membrane IL-1 is incapable of substituting for intact accessory cells in our system. Recently, Weaver et al (39) have described experiments demonstrating that although antigen and fixed resting B cells fail to stimulate proliferation in a Type I CD4$^+$ T-cell population (identical to our system), proliferation is induced with antigen presented by chemically fixed B cells pre-activated with IFN-γ and anti-IgM antibody. Presumably the

pretreatment of the APC population resulted in the elaboration of a surface molecule with costimulatory activity, resistant to fixation, that was capable of providing the second signal to T cells responding to the peptide antigen/MHC complexes. Thus, the molecule(s) is not always constitutively expressed on the APC surface, and probably normally it requires a T-cell interaction to induce it.

From all these experiments, it seems reasonable to suggest that the Bretscher & Cohn model of B-cell activation and inactivation is applicable to Type I CD4$^+$ T cell clones (Figure 2). Signal one is defined by occupancy of the T-cell antigen receptor by a complex formed between antigenic peptide and an Ia molecule on the APC surface, and this signal alone is capable of inducing a nonresponsive state. Signal two is a costimulatory factor or cell interaction molecule, rapidly induced on the APC surface, which then binds to a distinct receptor on the T-cell surface and acts in synergy with signal one to stimulate the T cell to make IL-2 and to proliferate. How then are these two signals transduced into the cell, and what are the biochemical consequences?

A BIOCHEMICAL TWO-SIGNAL MODEL OF T-CELL IL-2 GENE ACTIVATION

Fisher & Mueller (40) showed in 1968 that phyto hemagglutinin stimulation of normal human peripheral blood lymphocytes induced the rapid incorporation of radioactive orthophosphate into phosphatidyl inositol (PI). Based on substantive work at that time in other hormone/receptor systems (41; also reviewed by Hokin in 42), the authors suggested that PHA stimulates the interconversion of phosphatidic acid and PI in lymphocyte membranes. Later work in other systems emphasized the importance of

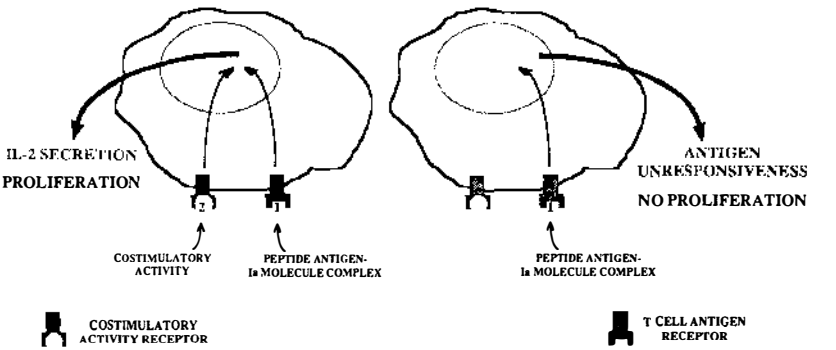

Figure 2

PI breakdown as the initial event in cell activation, rather than the observed reaccumulation of PI in the membrane (43).

Soon thereafter, lymphocyte proliferative responses to PHA were found to be inhibited by nontoxic concentrations of citrate, EDTA or EGTA, consistent with a dependency of the response on calcium ions (44, 45). PHA-induced uptake of radioactive calcium into lymphocytes was demonstrated about the same time (46, 47), and in recent years numerous studies have demonstrated the correlation between increases in the intracellular calcium free ion concentration ($[Ca^{+2}]_i$) and the induction of IL-2 secretion and/or T-cell proliferation by various stimuli including antigen and antigen-presenting cells (27, 35, 48–50). The development of the calcium ionophore, A23187, subsequently allowed one to directly test the mitogenic properties of increasing $[Ca^{+2}]_i$. It was initially reported that the ionophore alone was capable of inducing some degree of proliferation by human lymphocytes (51); however, the general consensus was that increased $[Ca^{+2}]_i$ alone was insufficient for a full proliferative response. More recent studies have demonstrated that calcium ionophores in combination with protein kinase C (PKC)-activating phorbol esters have potent mitogenic activity (52). More importantly, phorbol esters have appeared to substitute for accessory cells in the response of purified T cells to mitogens or treatment with anti-CD3 antibody (49, 53–55). The requirement for PMA in the response to mitogen was found to be at the level of IL-2 gene transcription and protein synthesis, as well as at the level of IL-2 receptor expression (56).

Recently, Berridge & Irvine (57) and Nishizuka (58, 59) have proposed a hypothesis that explains the associations between PI metabolism, increased $[Ca^{+2}]_i$, and PKC activation. Agonist-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) results in the production of the two second-messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-sn-diacylglycerol (DAG), both of which would play a critical role in the induction of cell proliferation. Utilizing permeabilized cells or microinjection techniques, IP_3 and its metabolic products were demonstrated to be capable of increasing the $[Ca^{+2}]_i$ by initiating an influx of Ca^{+2} into the cytoplasm, both from intracellular stores within the endoplasmic reticulum and from the extracellular milieu. DAG is thought to be active in the stimulation of the Ca^{+2}-dependent protein kinase C (PKC), based on in vitro studies with DAG analogs. The importance of PIP_2 hydrolysis and the initiation of this bifurcating pathway of signal transduction in the induction of cellular processes is underscored by multiple observations of synergy between calcium ionophores and PKC-activating phorbol esters in different biological systems (recently reviewed by Berridge in 60).

Experiments performed in T-cell systems have in general supported such

a hypothesis. Imboden & Stobo (50) stimulated the human T-cell tumor line Jurkat with anti-CD3 antibody and demonstrated the production of IP_3 as well as increases in $[Ca^{+2}]_i$, consistent with the model that T cell receptor occupancy results in the hydrolysis of PIP_2. Similar increases in inositol phosphates have been demonstrated in normal T cells in response to antigen plus syngeneic APC (27, 61). Kuno, Gardner, and their colleagues (62, 63), following their finding of a PHA-activated, non-voltage gated, calcium channel in normal T-cell plasma membrane, used a patch-clamp technique to show that IP_3 (or a metabolite) could open these channels. Finally, several laboratories have demonstrated PKC translocation to the plasma membrane (a marker of PKC activation) (64, 65) or PKC-dependent phosphorylation of the CD3 gamma chain following stimulation of T cells with antigen and APC, mitogen, or anti-CD3 antibody (66–68), suggesting the endogenous production of DAG.

The concordance of the T-cell findings with other biological systems led Weiss et al (69) to propose a biochemical two-signal model for activation of the IL-2 gene in T lymphocytes. They suggested that accessory cells present antigen and occupy the T cell antigen receptor (TCR) in a manner that can be mimicked by mitogen or anti-CD3 antibody plus a phorbol ester. The result of the perturbation of the receptor is the hydrolysis of PIP_2 and generation of IP_3 and DAG. The accumulation of these second messengers then leads to a rise in $[Ca^{+2}]_i$ and activation of PKC, respectively. Taken in light of the observation that the combination of calcium ionophore plus phorbol ester is sufficient to induce T-cell proliferation, this model defined increases in $[Ca^{+2}]_i$ and PKC activation as the requisite second messengers for IL-2 gene transcription. For purposes of general discussion, this biochemical two-signal model will be considered the current dogma (Figure 3).

Does this biochemical model suffice to explain signal transduction within the framework of the biological two-signal model of T-cell activation described earlier? We have demonstrated that TCR occupancy in the absence of the accessory cell-derived costimulatory signal is not sufficient for the induction of IL-2 synthesis and that in fact it renders the cell unresponsive to further stimulation. Others have demonstrated a requirement for a phorbol ester in T cell proliferative responses to anti-CD3 antibody or mitogens in the absence of accessory cells. The biochemical two-signal model, on the other hand, suggests that TCR occupancy is competent to induce the hydrolysis of PIP_2 and the production of both IP_3 and DAG. This would predict that TCR occupancy by antigen/Ia complexes alone (in the absence of the biological second signal) should be competent to induce T-cell IL-2 secretion. Weiss and his colleagues are aware of this paradox and have offered two possible explanations: (*a*)

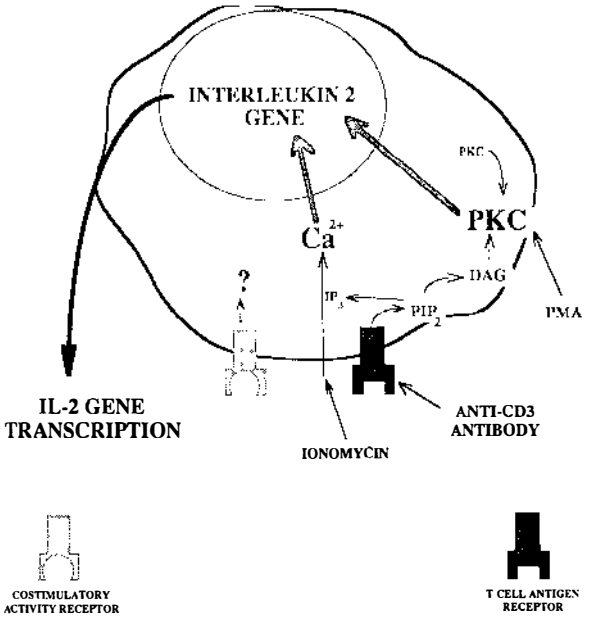

Figure 3

TCR occupancy alone may never generate the degree of PKC activation induced by phorbol ester; if so, then the accessory cell costimulatory signal would be required to obtain the high level of PKC activity needed prior to the initiation of IL-2 gene transcription. In support of this, recent studies have suggested that IL-1 can act by increasing the level of DAG in T lymphocytes through the induction of phosphatidylcholine hydrolysis (70). (*b*) PMA stimulation of T cells may result in effects independent of PKC activation; it is one of these other effects that mimicks the second messenger cascade initiated by the accessory cell costimulatory activity. In the remainder of this review we address this question and propose that increases in $[Ca^{+2}]_i$ and PKC activation are in fact insufficient second messengers for the induction of IL-2 secretion and proliferation by Type I $CD4^+$ T cell clones. A third biochemical signal appears to be required; its identity is at present unknown.

A BIOCHEMICAL THREE-SIGNAL MODEL OF T-CELL IL-2 GENE ACTIVATION

Increases in $[Ca^{+2}]_i$ Can Occur Independently of the Costimulatory Signal

As discussed earlier, stimulation of murine Type I $CD4^+$ T cells with antigen and chemically modified syngeneic APC fails to elicit a normal

proliferative response and instead induces the T-cell population into a state of antigen unresponsiveness. This effect is both antigen and MHC specific, implying that physiologic occupancy of the TCR and some form of receptor signalling is required. The ability of the same receptor to signal the development of a vigorous proliferative response or to induce a state of unresponsiveness in the absence of proliferation, suggests either that the quality of the signalling can vary, and in turn determine the cell's response, or that some other intracellular signal is involved. In either case, the accessory cell–derived costimulatory activity would appear to be responsible for determining the outcome.

A number of experiments were performed to examine signalling events in T cells following stimulation with antigen in the presence of either normal or chemically modified APC. Measurements of intracellular calcium ion concentration following stimulation with antigen and fixed APC demonstrated increased $[Ca^{+2}]_i$, consistent with intact T cell receptor signalling under these conditions (27). However, the antigen dose response curve demonstrated a requirement for 100-fold more antigen to achieve the same average $[Ca^{+2}]_i$ that one sees upon stimulation with normal APC. In addition, the kinetics of T cell receptor signalling (as measured by calcium influx) were delayed relative to stimulation with normal APC. These findings were compatible with the idea that fixed APC are defective in their ability to present antigen and occupy the TCR; however, the defect does not prevent the induction of an initial increase in $[Ca^{+2}]_i$. It should be noted that recent experiments suggest that a persistence of the increase in $[Ca^{+2}]_i$ is a critical factor in the induction of IL-2 secretion (71); we have not yet fully explored the possibility that fixed APC are defective in maintaining the increased $[Ca^{+2}]_i$ beyond the 20 min period examined in our experiments.

Experiments were also performed to determine the effect of antigen and fixed APC on T-cell PI metabolism (27). In this case, IP generation was barely detected, in contrast to the large accumulations of total IP seen in T-cell populations stimulated with normal APC plus antigen. Similarly, IP generation could not be detected in T cells responding to antigen in the presence of planar lipid membranes containing Ia-molecule (M. K. Jenkins, unpublished observations).

These calcium and IP results initially suggested the interpretation that (*a*) the development of an increase in $[Ca^{+2}]_i$ is dependent on the generation of only small amounts of IP_3; (*b*) chemically modified APC and isolated Ia molecules in the presence of antigen are incapable of occupying the TCR in a manner that results in a rapid hydrolysis of PIP_2; and (*c*) a failure to activate PKC (secondary to poor DAG production) in the face of a significant rise in $[Ca^{+2}]_i$ results in a pattern of T-cell activation that

is insufficient to induce IL-2 production and proliferation but is sufficient to induce the unresponsive state. This led to the hypothesis that the balance achieved between PKC activation and $[Ca^{+2}]_i$ increase would determine the response of the T cell to the stimulus.

A number of features made this hypothesis attractive. First, incubation of the T cells with antigen and fixed APC in the presence of EGTA failed to result in the induction of the unresponsive state, consistent with a requirement for increases in $[Ca^{+2}]_i$ in the response (27). Second, the addition of cyclosporin A during stimulation with antigen and fixed APC prevented the induction of unresponsiveness (36). This result also suggested a role for calcium ions in the response, as cyclosporin A is a potent inhibitor of multiple calcium-dependent early activation events, possibly by binding and inactivating calmodulin (72–74). Third, the calcium ionophore, ionomycin, was found to be a potent inducer of the unresponsive state, suggesting that a nonmitogenic intracellular calcium rise without PKC activation was sufficient to induce the state (27). Finally, the hypothesis was entirely consistent with the biochemical two-signal model described above. Such a model would predict that accessory cell–derived costimulatory activity is necessary for maximum TCR-induced PIP_2 hydrolysis, DAG production, and PKC activation. In other words, PKC could never be adequately activated in the absence of normal accessory cells.

Further experiments, however, turned out not to support this model.[2] Careful examination of the kinetics of IP generation suggested that the formation of conjugates between T cells and APC played a key role in the observed rate of accumulation of IP. In addition, the delay in calcium rise noted in T-cell populations stimulated with antigen and fixed APC was also the result of a reduced ability of fixed APC to form conjugates with T cells. Therefore, in subsequent experiments the generation of IP was measured following pelleting of the cells by centrifugation to assure rapid conjugate formation. These experiments then demonstrated a significant rate of IP generation following stimulation with fixed APC and antigen, although there did remain a clear shift in the antigen dose response curve to higher antigen concentrations when compared to antigen responses in the presence of normal APC. Finally, the addition of a normal allogeneic accessory cell population (incapable of occupying the T cell receptor in the presence of antigen because of the lack of a relevant Ia molecule), during the incubation with antigen and syngeneic fixed APC, resulted in

[2] Mueller, D. L., Jenkins, M. K., Schwartz, R. H. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T-cell proliferation and to prevent the induction of unresponsiveness. Submitted.

no enhancement of IP generation, despite significant augmentation of the proliferative response to the antigen and prevention of the development of the unresponsive state. Attempts to correct the proliferative defect in the response to antigen and fixed APC with a phorbol ester, on the other hand, met with only partial success (38).

A similar need for accessory cells prior to the induction of proliferation in our T-cell clones occurs during activation in response to Con A[2] (75). The rate of PIP_2 hydrolysis following stimulation with this mitogen in the absence of accessory cells, however, was quite high. The addition of accessory cells to these cultures again had no effect on this rate. Recent experiments by Nisbet-Brown et al (35) also demonstrated an inability of accessory cells to influence the effect of anti-CD3 monoclonal antibody or antigen and fixed APC on the $[Ca^{+2}]_i$ in human T-cell clones. Thus, occupancy of the TCR alone is sufficient for PIP_2 hydrolysis, and the costimulatory signal induced by accessory cells appears to be independent of TCR signalling.

Several important concepts regarding the costimulatory effect of accessory cells have come out of these recent experiments.[2] It appears that a relatively low rate of PIP_2 hydrolysis is sufficient to induce a vigorous proliferative response, if an excess of costimulatory activity is present. Experimentally, this is demonstrated by a relative shift of the proliferative dose response curve to lower antigen concentrations, compared to the antigen-induced generation of IP. Under conditions of limited costimulatory activity (either by lowering the density of accessory cells in the culture, or by reducing the costimulatory activity within the accessory cell population with gamma or UV irradiation), proliferation appears more heavily dependent on very high rates of PIP_2 hydrolysis. Finally, chemically modified APC appear to possess two functional defects at the level of antigen-presentation. The first is a defect in TCR occupancy by antigen/Ia molecule complexes. Perhaps fixation-induced damage to the Ia molecule itself or to accessory molecules, responsible for the formation of T-cell/APC conjugates and proper cross-linking of the occupied TCR, results in the observed reduction in antigen-induced signalling. The second defect is the loss of the costimulatory activity normally provided by the APC and required by the T cell for proliferation. Only the second defect can be corrected by allogeneic accessory cells.

PKC Activation Occurs Independently of the Costimulatory Signal

Our observation that the rate of PIP_2 hydrolysis is not predictive of the ability of a T cell to proliferate in response to any given stimulus is difficult

to reconcile in terms of the biochemical two-signal model of activation of the IL-2 gene we have described. One would expect this rate to be directly related to the level of DAG and the degree of PKC activation, as well as levels of IP_3 and $[Ca^{+2}]_i$ The activities of these second-messengers should then have determined the proliferative response. To address this question directly, we examined the level of DAG in a T-cell clone treated with Con A in the absence of accessory cells.[2] Stimulation of these cells resulted in significant increases in DAG by 1 hr of incubation. As predicted by stoichiometry, the Con A–stimulated levels of DAG correlated well with the observed increased rates of IP production. We concluded from these data that if a costimulatory signal were to play an important role in the proliferative response, and yet act within this two-signal model, its effects would have to be beyond PIP_2 hydrolysis, perhaps at the level of direct PKC activation.

To test this, we used the system of endogenous T-cell receptor phosphorylation to assess the activity of PKC.[2] It has been shown that the CD3-γ chain is phosphorylated, in response to T-cell stimulation with antigen, at a serine residue indistinguishable from that phosphorylated in response to treatment with a DAG analog such as phorbol dibutyrate or PMA (76–78). In addition, long-term incubations with phorbol ester under conditions that tend to deplete the cell of PKC activity result in a failure to phosphorylate the CD3-γ chain upon stimulation with antigen (77). By examining the level of CD3-γ polypeptide phosphorylation following stimulation of the T cell, we have been able to determine the relative activity of PKC under conditions that either favor the induction of proliferation or the loss of antigen responsiveness. The T-cell clone A.E7 responded to stimulation with antigen in the presence of normal syngeneic APC with the phosphorylation of CD3-γ, demonstrating a rise in PKC activity during early activation (Figure 4). Fixation of the APC population with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) prior to stimulation significantly reduced the antigen-induced phosphorylation; this was consistent with the reduction seen at the level of PIP_2 hydrolysis. This reduced level of CD3-γ phosphorylation was not affected by the addition of normal allogeneic accessory cells. This independence of PKC activity from the costimulatory signal was confirmed by the observation that Con A stimulation of T cells in the absence of accessory cells resulted in high levels of CD3-γ phosphorylation, without much proliferation. Thus the low level of PKC activity induced following stimulation with antigen and fixed APC was sufficient to allow the induction of proliferation, if a source of costimulatory activity was present. These results demonstrated that the costimulatory signal is not being delivered through any mechanism that increases TCR occupancy or its observed consequences, and thus it must

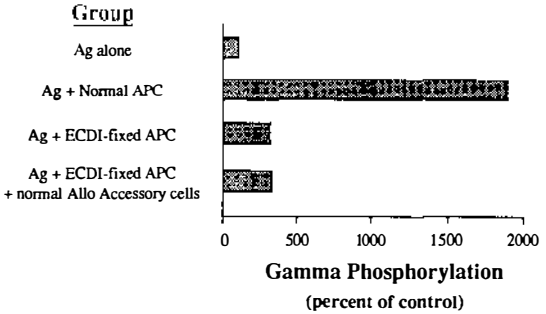

Figure 4 PKC-dependent CD3-γ-chain phosphorylation is independent of the costimulatory signalling pathway. [^{32}P]orthophosphate-labeled A.E7 T cells (2.5×10^7 per group) were incubated 45 min with the pigeon cytochrome *c* synthetic analog DASP (100 μM) either alone or in the presence of 5×10^7 normal syngeneic B10.A APC, 5×10^7 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI)-fixed B10.A APC, or 5×10^7 ECDI-fixed B10.A APC plus 2.5×10^7 normal allogeneic B10 low density T-depleted spleen cells. Cells were lysed in detergent and the CD3-complex was immunoprecipitated with 145-2C11 monoclonal antibody (127) and protein A-sepharose. Two-dimensional SDS-polyacrylamide gel electrophoresis analysis of the CD3-γ chain was performed and phosphorylations were detected by autoradiograpy of the dried gels. Densitometry of autoradiograms was performed, and results are expressed as the percent increase in gamma chain phosphorylation over that seen in the absence of APC (antigen alone).

be acting via an independent intracellular second-messenger pathway in the cell.

Increases in $[Ca^{+2}]_i$ and PKC Activation are Insufficient Second Messengers in the Induction of T-Cell Proliferation

Our biochemical data clearly support a role for a costimulatory signalling pathway in the T cell that is independent of increases in $[Ca^{+2}]_i$ and the activity of PKC. This pathway must be active during antigen stimulation for IL-2 secretion to occur and antigen responsiveness to be maintained. In other words, TCR-mediated increases in $[Ca^{+2}]_i$ and PKC activation alone are insufficient to induce T-cell proliferation. In an additional series of experiments, we wished to test this theory, as well as try to reconcile it with the observation that purified T cells will proliferate in response to treatment with the combination of ionomycin and PMA. In particular, we attempted to establish whether there was a true independence of proliferation under these conditions from accessory cell–derived costimulatory signals.

Our results argue against such an independence. Incubation of T-cell clones with the combination of ionomycin and PMA at saturating doses consistently resulted in only suboptimal proliferative responses. In

contrast, incubation with these agents in the presence of an accessory cell population caused maximal proliferation of the T cells (comparable to that achieved with antigen and APC), suggesting a synergistic relationship between costimulatory signals and both increasing $[Ca^{+2}]_i$ and PKC activation[3]. We have also observed relatively low yet significant levels of proliferation in response to stimulation with *either* ionomycin or PMA in the presence of syngeneic accessory cells. This finding suggested the possibility that intense stimulation of two of the three biochemical pathways is sufficient to induce a limited degree of IL-2 gene transcription; however, we could not assess the importance of basal PKC activity or $[Ca^{+2}]_i$, respectively, in these responses. It is important to note, though, that the proliferative response to accessory cells and phorbol ester has been found to be completely resistant to inhibition by cyclosporin A. This is consistent with the hypothesis that the costimulatory signalling pathway operates independently of the effects of $[Ca^{+2}]_i$. More importantly, this effect suggests an identifying characteristic for use in experiments designed to delineate the costimulatory signalling pathway. Work in the human system with the anti-CD28 monoclonal antibody 9.3 has suggested that the CD28 molecule is coupled to an alternate activation pathway that, in concert with PKC activation, is capable of inducing IL-2 synthesis in the absence of a detectable calcium rise and in a cyclosporin A-resistant fashion (79). Thus it is intriguing to suggest that a molecule such as CD28 could be the receptor for the costimulatory activity.

Having demonstrated a synergistic relationship between the costimulatory signalling pathway, increased $[Ca^{+2}]_i$ and PKC activation, it was necessary to isolate the T cells completely from any potential costimulatory activity to determine if costimulatory signals were absolutely required for proliferation. Single T cells were found to be incapable of dividing in response to stimulation with only the combination of ionomycin and PMA. This failure to proliferate apparently resulted from a failure to produce IL-2, because cellular division could be induced with the addition of an exogenous source of IL-2. To reconcile this difference with ionomycin plus PMA-induced proliferation at high T-cell density, T-cell titrations were performed in the presence of ionomycin plus PMA (Figure 5). Log-log plots of thymidine incorporation versus T-cell number established that proliferative responses to ionomycin plus PMA are dependent on cell interactions in the culture. The slope of the plot was approximately two, suggesting that at least two cells must interact to generate the proliferative

[3] Mueller, D. L., Jenkins, M. K., Chiodetti, L, Schwartz, R. H. 1989. Increased $[Ca^{+2}]_i$ and PKC activation are insufficient second messengers for T-cell proliferation to ionomycin and PMA. In preparation.

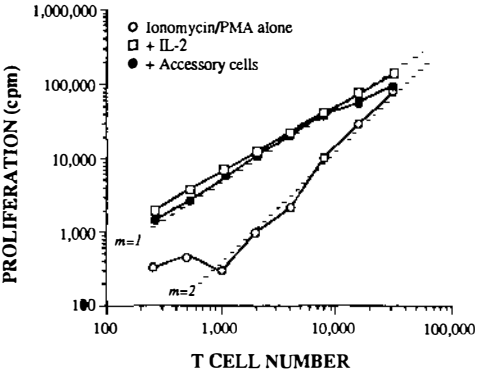

Figure 5 Proliferation of T cells in response to ionomycin and PMA is dependent on cell-cell interactions. A.E7 T cells were titrated into a microtitre plate in 2-fold dilutions from 2^{15} cells/well. Ionomycin 0.5 μM and PMA 10 ng/ml were added to all cultures. Incubations were carried out either without addition (open circles), in the presence of exogenous IL-2 (2% MLA supernatant) (open squares), or together with 5×10^2 cells of the B cell hybridoma LS (following 10,000R gamma irradiation and mitomycin C treatment) (closed circles). Cultures were pulsed at 48 hr and thymidine incorporation was measured at 60 hr. Results are expressed as the mean cpm of six replicates and plotted in log-log fashion. Theoretical curves with slope of one or two are indicated by the dashed lines.

response. This requirement for cell interactions could be satisfied by the addition of an accessory cell population (at constant density) that contained a good source of costimulatory activity. It also could be overcome by the addition of exogeneous IL-2. Finally, in a separate series of experiments, we found we could inhibit ionomycin plus PMA-induced proliferation with the addition of a fixed splenic B-cell population. These cells appeared to be capable of competing with the delivery of costimulatory signals by interacting with T cells in a nonstimulatory fashion—in essence behaving like cold-target inhibitors in a CTL assay. These results suggest that the cell interactions required for proliferation to ionomycin plus PMA involve the delivery of the costimulatory signal to the T-cell population. Similar results have been obtained with T cells stimulated with an anti-CD3 monoclonal antibody coated on a plate.

The identity of the costimulatory activity donor in this response appears to be the activated T cell itself, although we can not rule out the possibility of an extremely potent contaminating accessory cell. Resting splenic T cells are not a source of costimulatory activity; however, we have found that the Type II $CD4^+$ T-cell clone D10.G4 will allow our Type I $CD4^+$ T cell clone A.E7 to proliferate in response to concanavalin A or anti-CD3 antibody, in the absence of any other accessory cell population. Therefore, there is reason to think that an intensely activated T cell might

be capable of providing a nearby cell with the costimulatory signal it requires to proliferate. This interpretation could also reconcile the observation that some Ia^+ human T cells induce T-cell unresponsiveness in the presence of antigen, while others induce proliferation.

Given our observations that T-cell proliferative responses to both anti-CD3 antibody alone and to the combination of ionomycin and PMA require the generation of a costimulatory signal, it becomes necessary to reassess the biochemical two-signal model for T-cell activation of the IL-2 gene. In particular, one must determine if these data support either of the two hypotheses proposed by Weiss and his colleagues to account for the role of the accessory cell (mimicked by phorbol ester) in the induction of IL-2 gene transcription. The first hypothesis stated that the ability of phorbol ester to substitute for an accessory cell population in the response to mitogen or anti-CD3 antibody results from the fact that the accessory cells are required for sufficient PKC activation. Our data demonstrate that this is not the case. PKC activation is detectable upon stimulation with fixed APC and antigen or Con A, in the absence of normal accessory cells, and the addition of accessory cell–derived costimulatory activity has no effect on the degree of PKC activation. There is no evidence that IL-1 acts in our system to increase the level of DAG and to enhance the activity of PKC, as we should have noted such effects at the level of CD3-γ chain phosphorylation. The second hypothesis suggested that the effects of phorbol esters were not limited to PKC activation, and that one of these other activities mimics the costimulatory signal. We have found, however, that under conditions of limited costimulatory activity (purified T cells at low density), PMA cannot mimic the costimulatory signal. Thus, it appears that PMA acts indirectly (presumably through intense PKC activation in the presence of $[Ca^{+2}]_i$) to induce the costimulatory activity within the purified T cell population and allow the generation of the costimulatory signal as a consequence of T-T interactions.

Activation of the IL-2 Gene Requires Three Biochemical Signals

We think that only a three-signal biochemical model is compatible with all of the biological observations (Figure 6). Occupancy of the TCR with an antigen/Ia complex results in the hydrolysis of PIP_2 leading to a subsequent rise in $[Ca^{+2}]_i$ and activation of PKC. In the absence of the biological second signal (costimulatory activity), these intracellular second messengers are capable of inducing a state of antigen unresponsiveness and are insufficient to induce significant IL-2 gene transcription or proliferation. Our data would suggest that increased $[Ca^{+2}]_i$ is both necessary and sufficient for the induction of antigen nonresponsiveness. Relatively

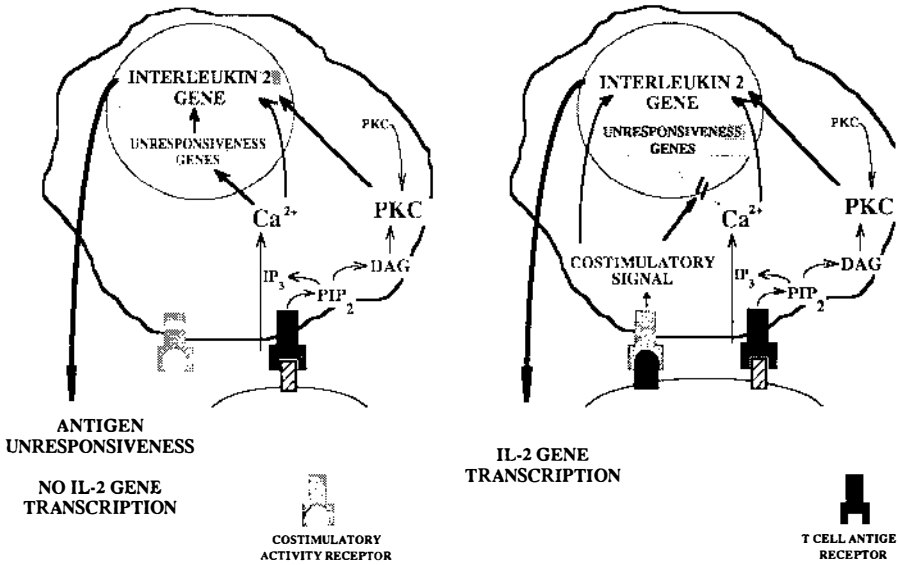

Figure 6 The CD4+ T cell on the left is stimulated with antigen/Ia molecule complexes in the absence of the costimulatory activity. The T cell on the right is stimulated in the presence of the costimulatory activity.

low PKC activity may be permissive in the development of this state, whereas intense PKC activity acts (with increased $[Ca^{+2}]_i$) to increase the costimulatory activity within the T-cell population and antagonize the development of unresponsiveness.

In the presence of the biologic second signal, a separate costimulatory pathway is activated in the T cell which acts to induce the transcription of the IL-2 gene in concert with increases in $[Ca^{+2}]_i$ and PKC activation. All three signalling pathways must be active for maximum lymphokine secretion and proliferation. In addition, the costimulatory signal is capable of abrogating the induction of unresponsiveness.

The biochemical events that occur in the induction of the nonresponsive state have not yet been elucidated. Cycloheximide addition blocks the induction of unresponsiveness, suggesting that new protein synthesis may be required (25). The simplest model would be to postulate that the calmodulin-dependent pathway activated by increases in $[Ca^{+2}]_i$ induces the transcription and synthesis of a series of proteins capable of either directly or indirectly inhibiting the transcription of the IL-2 gene (although the cycloheximide experiment does not differentiate this from the possible effects on mRNA stability or the translation of DNA regulatory proteins). The costimulatory signalling pathway would then block the

synthesis or activities of these inhibitory proteins as well as facilitate transcription of the IL-2 gene in concert with the effects of PKC activation and increased $[Ca^{+2}]_i$.

The synergistic interactions observed in the stimulation of Type I $CD4^+$ T-cell clones by PMA, ionomycin, and accessory cells support the concept of a three signal model. The fact that these three stimuli can mimic full T-cell activation, however, does not prove that the second messenger cascades they induce must occur following antigen-induced activation via receptor occupancy. More complex models, involving other receptor-mediated signals, can be envisioned. For example, recent studies of T-cell hybridomas expressing receptor mutations have demonstrated that anti-Thy 1 antibody-mediated induction of IL-2 gene transcription can occur in the absence of PIP_2 hydrolysis (80, 81). In this case, the natural biochemical signals for inducing IL-2 gene transcription may not be PKC activation and/or increases in $[Ca^{+2}]_i$. Our incomplete understanding of second messenger generation following TCR occupancy, however, does not reduce the importance of the costimulatory signalling pathway in the regulation of the IL-2 gene. TCR occupancy may normally lead to both the rise in $[Ca^{+2}]_i$ necessary for the induction of the unresponsive state, and the stimulation of another unidentified signalling pathway capable of acting on the IL-2 gene. The costimulatory signal would still be required to block the effects of calcium ions, in order to prevent the induction of unresponsiveness, as well as to synergize in the induction of IL-2 gene transcription.

Most of the data supporting our three signal model come from experiments involving Type I $CD4^+$ T cell clones. These cells do not represent small resting T lymphocytes. They constitutively express low levels of IL-2 receptors and often contain chromosomal rearrangements. How applicable, then, is the model to small resting T lymphocytes? Preliminary studies on freshly isolated $CD4^+$ T cells suggest that some of the major features of the model hold true (M. K. Jenkins, D. L. Mueller, unpublished observations). For example, the proliferative response of antigen-primed draining lymph node T cells can be completely eliminated by an 18 hr exposure to ionomycin. Furthermore, ionomycin, PMA, and accessory cells synergize in the stimulation of a proliferative response from mesenteric lymph node T cells. In particular, the expression of the IL-2 receptor also appears to be optimal only in the presence of all three signals. Whether this is a direct effect on the IL-2 receptor gene or an indirect effect via activation of the IL-2 gene, to give IL-2–induced up-regulation of its receptor, remains to be determined. These experiments suggest that the three signal model may be applicable to the activation of IL-2-producing, small resting T cells.

ROLE OF THE COSTIMULATORY SIGNAL IN THE REGULATION OF T-CELL RESPONSES IN VIVO

Experimentally Induced T-Cell Tolerance to Foreign Antigens

A number of experimental observations in vivo support the idea that responsiveness to a foreign antigen by mature T cells can be blocked. As early as 1946, Chase (82) demonstrated that contact sensitivity to dinitrochlorobenzene in guinea pigs could be inhibited by the prior oral administration of the chemical. Battisto & Bloom (83) later showed that the development of a DTH response to bovine gamma globulin (BGG) in guinea pigs was significantly reduced by pretreatment of the animals with an intravenous infusion of BGG-coupled spleen cells. This experimentally induced tolerance was both antigen-specific and long-lasting. Although it now appears certain that this type of unresponsiveness to foreign antigens develops at the level of the $CD4^+$ T-cell population, the actual mechanism of tolerance can not be ascertained from these data (23). Some have suggested a role for antigen-specific suppressor cells in the maintenance of this form of experimentally induced T-cell tolerance. A discussion of suppression is beyond the scope of this review. It should be noted, however, that Miller et al (84) were able to demonstrate that suppression could not completely account for the tolerance induced with hapten-modified spleen cells. They showed that elicitation of 2,4-dinitro-1-fluorobenzene contact sensitivity was blocked by prior intravenous infusion of dinitrophenylated spleen cells. The induction of tolerance was rapid, antigen-specific, and MHC-restricted, and initially not associated with transferable suppression, suggesting a state of clonal anergy. Furthermore, treatment of the animals with cyclophosphamide prior to the infusion of hapten-modified spleen cells completely abrogated the induction of suppressor cells; yet, tolerance to the hapten was still induced.

Based on these observations, and as an extension of previous studies examining the specificity of T-cell proliferative responses, our laboratory examined the influence of intravenous administration of pigeon cytochrome *c*, covalently cross-linked with ECDI to the surface of syngeneic antigen-presenting cells (APC), on the priming of a T-cell proliferative response to the antigen (26). Four days following intravenous challenge with the cross-linked antigen/APC, subcutaneous immunization with antigen in complete Freund's adjuvant was found to be ineffective at priming the T-cell population, i.e. the in vitro secondary T-cell proliferative response to peptide fragments of the antigen was greatly reduced. Immunity to another antigen (purified protein derivative of tuberculin) was not affected. A detailed analysis of the requirements for inducing the non-

responsive state revealed that the same antigen and Ia molecule specificities were needed as those required for T-cell priming.

These results suggest that TCR signalling occurs during both T-cell priming and the induction of specific T-cell unresponsiveness to antigen in vivo. In the case of antigen covalently cross-linked to chemically fixed APC, we assume that the intravenous infusion of antigen/Ia complexes (at the surface of the APC) results in the occupancy of the TCR on antigen-specific lymphocytes in the absence of a source of costimulatory activity. Consistent with the biological two-signal model, antigen responsive clones would not be expanded by this type of stimulation; rather, these T cells would be functionally inactivated, resulting in a failure to detect them upon priming in the standard fashion.

This simple application of our in vitro model of T-cell inactivation to the in vivo findings, however, is not sufficient to explain a number of puzzling observations. First, fixed APC require preprocessed antigen in vitro in order to form antigen/Ia molecule complexes, yet coupling the whole molecule to spleen cells was adequate for the in vivo effects. How does the antigen get processed? Are some T-cell determinants generated by denaturation during coupling or by the action of serum proteases at the surface of the fixed cell? Another paradox is that in vitro, fixed APC can be prevented from inducing T-cell nonresponsiveness by the addition of normal allogeneic accessory cells. Why don't the normal accessory cells in vivo do the same thing? Is this a quantitative effect and does it depend heavily on where the fixed cells home? These and other questions are currently being investigated.

The experimental tolerance studies described above do appear to suggest a model in which the route of entry of the antigen and the physical state of the APC influencc the outcome of T-cell activation in vivo (clonal expansion versus functional clonal inactivation). Possibly the two factors—location of the antigen and attributes of the available APC—are associated, i.e. the site at which antigen is encountered under ordinary conditions could determine the type and number of accessory cells available for antigen presentation. The subcutaneous appearance of antigen, with subsequent transport via veiled cells through tissue lymphatics to the lymph node, might assure a rich supply of potent bone marrow–derived accessory cells (e.g. dendritic cells, macrophages) capable of providing the costimulatory activity required for the induction of a proliferative response within the antigen-specific T-cell population (85, 86). In contrast, the oral or intravenous introduction of antigen might result in the presentation of antigenic peptides by APC that possess little costimulatory activity (87).

The examination of Ia molecule expression in various tissues has deter-

mined that many nonhematopoietic cells can express Ia molecules; furthermore, in vivo treatment with IFN-γ induces the expression of Ia molecules on such cells (88). Ia^+ fibroblasts, keratinocytes, and thyroid follicular epithelial cell lines all fail to induce normal T-cell proliferative responses to peptide antigens in the absence of a costimulant such as PMA, normal bone marrow–derived accessory cells, or the anti-CD28 antibody 9.3 in vitro (34, 89–91). In the case of Ia^+ keratinocytes, Gaspari et al (92) find that incubation with Type I $CD4^+$ T-cell clones in the presence of peptide antigen results in the induction of proliferative unresponsiveness in the T cell. Recently, Markmann et al (93) have shown that pancreatic islets taken from transgenic mice that express the I-E MHC molecule transgene exclusively on their beta cells are also capable of inducing proliferative unresponsiveness in T-cell lines in the presence of antigen. These results are consistent with the idea that some MHC-bearing cells may occupy the TCR in the absence of a costimulatory signal under physiologic conditions and so may induce clonal anergy.

In vivo experimental evidence in support of the critical role for accessory cells in the regulation of T-cell responsiveness can be found in studies of tolerance induction in adults by the parenteral administration of monomeric preparations of mammalian serum proteins. Dresser (94) showed that immune paralysis to BGG could occur after intraperitoneal injection of a deaggregated preparation of the antigen. Thorbecke and her colleagues (95) later confirmed the result by demonstrating that bovine serum albumin (BSA) that had been "filtered" by passage through a rabbit (and subsequently recovered in the serum) would induce tolerance to BSA in a naive animal following intravenous administration. They suggested that "filtered" BSA is depleted of the "phagocytizable" components necessary for the recruitment of macrophages into the response, an event required for sensitization to the antigen. Chiller et al (96, 97) demonstrated that a similar state of tolerance to human gamma globulin (HGG), following the intravenous infusion of the deaggregated-form of the antigen, resulted from the development of unresponsiveness in both the B-cell and T-cell compartments. Weigle et al (98) have since found that the induction of T-cell unresponsiveness by treatment with deaggregated HGG can be prevented by the simultaneous administration of IL-1. These results are consistent with the notion that the parenteral introduction of antigen in a form that neither induces its uptake and presentation by potent accessory cells of the reticuloendothelial system, nor induces the costimulatory activity within the APC population it encounters, results in presentation of antigen to T cells in the absence of a costimulatory signal. The ability of IL-1 to modulate the T-cell response to antigen in this form appears to confirm the relative lack of costimulatory activity available to the T cell.

IL-1 treatment in vivo could potentially augment the delivery of the costimulatory signal by increasing the expression of surface adherence molecules and upregulating T-cell/accessory cell interactions (99) and/or enhancing accessory cell potency (21). Taken together, these data suggest that the APC population encountered at the site of antigen introduction may play an important role in determining the response of the antigen-reactive T cell by up- or down-regulating antigen responsiveness.

T-Cell Unresponsiveness in the Maintenance of Self-Tolerance

The models of experimentally induced tolerance to specific antigens in adult animals discussed above suggest that functional T-cell inactivation might play an important role in the generation of this in vivo state. For tolerance to self-antigens, however, recent experiments using the Vβ17a-specific anti-TCR antibody KJ23 have established that deletion of autoreactive clones (anti-I-E^k, in this particular case) occurs during maturation of T cells in the thymus (100). Similar evidence for clonal deletion has been found in the maintenance of self-tolerance to Mls determinants (101, 102) and the male (H-Y) antigen (103). Thus, the costimulatory signal would be important in natural tolerance only if it played a role in clonal deletion or in the functional inactivation of autoreactive clones that escaped deletion.

Within the framework of a biological two-signal model of T-cell activation, one could propose that clonal deletion is the direct result of thymocyte antigen receptor signalling in the absence of activation of the costimulatory signalling pathway. This situation could result either from an inability of the immature T cells to respond to a costimulatory signal at an early stage in their development, or because of an inability of the thymic APC to deliver a costimulatory signal. Recently, Matzinger & Guerder (personal communication) have examined the induction of T-cell tolerance in explanted thymic lobes and found that the addition of normal allogeneic splenic dendritic cells, a potent APC in the generation of in vitro T-cell proliferative responses, resulted in tolerization of the allo-specific CTL precursors. T cells that matured in organ cultures responded to third-party allogeneic stimulator cells but failed to develop a CTL response to stimulator cells syngeneic with the donor dendritic cell population. If the biological two-signal model is applicable, this result suggests that immature thymocytes are incapable of responding to the costimulatory activity, or that the thymic microenvironment prevents the delivery of such a signal.

Since $CD4^+CD8^+$ (double positive) thymocytes (presumably the $CD3^{lo}$ subset) are capable of responding to antibody-induced TCR cross-linking with a small rise in $[Ca^{+2}]_i$ (105, 105a), antigen receptor occupancy in the absence of a costimulatory signal might be sufficient to kill the cell, con-

sistent with the role of calcium ions in the functional inactivation seen with our mature T cclls, in vitro. Recent work in our laboratory has demonstrated that clonal deletion in the thymus is sensitive to cyclosporin A treatment in vivo (106). Strains of mice that bear a *Vβ17a* T cell receptor gene and express an *I-E* encoded Ia molecule normally fail to generate significant numbers of mature Vβ17a$^+$ T cells because these T cells are often specific for determinants associated with I-E molecules and are deleted. Treatment with cyclosporin A during syngeneic bone marrow reconstitution of lethally irradiated C57Br (*Vβ17a*$^+$,E_α^+) mice resulted in a failure to delete entirely the newly arising, Vβ17a-expressing mature thymocyte population. Similar observations have been made by Gao et al (107) for Vβ11-expressing cells. Since many of these thymocytes should bear autoreactive T-cell receptors, these results may explain the previous observation that cessation of cyclosporin A therapy following irradiation and bone marrow reconstitution of rats (108, 109), mice (110), and humans (111) results in the development of graft-v-host disease. Autoreactive thymocytes developing in the presence of cyclosporin A would fail to be deleted and would then move to the periphery and subsequently mediate the disease, once they were released from the cyclosporin A signalling block. In terms of our discussion, such data suggest that signalling via increases in $[Ca^{+2}]_i$ within the thymocyte population may be necessary for deletion of autoreactive T-cell clones. In support of this notion, Wyllie et al (111a) have shown that the calcium ionophore, A23187, can induce DNA degradation and cell death in rat thymocytes, in a manner that is blocked by cycloheximide. Given the concordance of these data with the in vitro model of T-cell unresponsiveness, it is possible that this mechanism of thymic tolerance induction may rely on T-cell receptor occupancy in the absence of a second signal.

Experiments performed with $F_1 \rightarrow$ parent bone marrow chimeras have demonstrated the elimination of thymocytes reactive with any of the MHC antigens expressed on the surface of donor-derived cells, suggesting an important role for the bone marrow–derived thymic dendritic APC in the normal induction of self-tolerance within the T-cell population (112–114). In contrast, auto-MHC antigens expressed only on non-bone marrow–derived tissues appear to elicit a different and, at times, incomplete form of tolerance. For example, adult-thymectomized SJL mice (*Vβ17a*$^+$,E_α^-) that have been lethally irradiated and reconstituted with syngeneic bone marrow and then engrafted with a deoxyguanosine-treated (BALB/c × SLJ)F_1 thymus, fail to delete the Vβ17a$^+$ population of newly developed T cells (114). In this case, expression of an I-E^d molecule exclusively on the thymic stromal elements was not sufficient to delete physically the potentially autoreactive Vβ17a$^+$ T cell population; nevertheless, these

animals did tolerate their thymic graft. Similar evidence for peripheral tolerance to tissues that bear MHC molecules not expressed on bone marrow-derived cells has been observed in other systems: in (*a*) chicken chimeras resulting from the transplantation of embryonic quail thymic stromal elements into a chick embryo (115), in (*b*) *Xenopus* chimeras created by joining the anterior one-third of a 24 hr old embryo to the posterior two-thirds of an allogeneic embryo (116), in (*c*) parent → F_1 bone marrow cimeras (117) and (*d*) in transgenic mice that express a particular Ia molecule exclusively on thymic epithelium (114, 118) or peripheral tissues (pancreatic beta cells and kidney tubular epithelium) (119).

The basis for this peripheral tolerance in the absence of clonal deletion could be clonal inactivation. It is possible that the T cells, specific for these MHC molecules expressed only on non-bone marrow–derived elements, have had their TCR occupied by the autoantigen, and a failure of these MHC-bearing cells to provide the costimulatory activity resulted in the functional inactivation of the clones, instead of activation and expansion. This model would predict a reduced capacity of T cells from these animals to display autoreactivity to the MHC antigens in mixed lymphocyte reaction (MLR) or cell-mediated lympholysis (CML) assays.

In the case of the transgenic mice described above, an MLR failed to demonstrate any significant reactivity to the Ia molecule encoded by the transgene, consistent with a complete functional inactivation of autoreactive clones (in the demonstrated absence of clonal deletion of at least the $V\beta17a^+$ population) (114, 118, 119; personal communication, L. Burkly). The parent → F_1 bone marrow chimeras demonstrated significantly reduced MLR reactivity relative to third party stimulators and an absent CML response (117). In several of the other models, however, a full response in MLR or CML was reported (116, 120–122). Thus, although functional inactivation may be playing some role in the peripheral tolerance, it is clear that other regulatory mechanisms are also likely to be involved.

Finally, the "veto cell" phenomenon (124, 125), observed in vitro and in vivo, may also be mediated by a functional inactivation mechanism. In experiments performed by Rammensee & Bevan (126), infusion of class I–incompatible spleen cells into mice resulted in the specific loss by the recipient of class I–alloreactivity to the donor strain. The mice were found to be chimeric, as donor-derived CTL could be demonstrated in an MLC response to third-party allostimulators (but not to stimulators syngeneic with the host). These results suggest that T cells from each strain, specific for the other haplotype, were functionally inactivated. It is possible that this occurred as a result of allospecific T cells recognizing the class-I MHC antigen on the T cell of the other strain, as originally proposed by Miller

(124). Since we have found T cells to have no detectable costimulatory activity in the resting state, recognition of antigen on the surface of such a cell would result in TCR occupancy of the responding T cell in the absence of a second signal, the prerequisite for functional inactivation.

Thus, the experiments described above suggest that in situations where the physical deletion of T cells specific for self-antigens has not occurred, tolerance to these autoantigens can still be maintained. While it is likely that clonal deletion of autoreactive T cells during development represents the most important means for achieving self-tolerance, functional inactivation could provide one type of 'second level' protection against the development of a pathological autoimmune response by clones that escape deletion.

Potential Clinical Relevance

T-cell unresponsiveness may also be important in the maintenance of peripheral tolerance in clinical situations. Experiments performed with the in vivo administration of anti-CD3 monoclonal antibody by Bluestone and his coworkers demonstrated that mice given intravenous anti-CD3 antibody develop rapid suppression of allogeneic skin graft rejection (127, 128). The long-lasting immunosuppression observed in this model was not simply due to a loss of alloreactive T cells or CD3 modulation, but instead appeared to reflect some functional defect in the T-cell population. It is possible that this element of immunosuppression following anti-CD3 therapy represents the development of an antigen-unresponsive state in the T-cell population, following TCR signalling by the monoclonal antibody in the absence of a costimulatory signal.

Support for this notion comes from findings in vitro on the induction of antigen-nonresponsiveness in T-cell clones treated with anti-TCR monoclonal antibody in the absence of accessory cells (129). In recent experiments performed in our laboratory[4], Type I $CD4^+$ T-cell clones stimulated with anti-CD3 monoclonal antibody (in solid-phase, coated to a plate) proliferated suboptimally and entered a state of reduced antigen-responsiveness following the stimulation. The series of inductive events at both the biochemical and lymphokine production levels appears similar to what has been observed with antigen and ECDI-treated APC or Ia molecules in planar membranes. Thus, coating of T cells in vivo with anti-CD3 could lead to the cells being sequestered at sites where Fc receptor-mediated cross-linking would trigger their functional inactivation.

[4]Jenkins, M. K., Chen, C., Jung, G., Mueller, D. L., Schwartz, R. H. 1989. Inhibition of antigen-specific proliferation of Type I murine T cell clones following stimulation with immobilized anti-CD3 monoclonal antibody. In preparation.

The effects of donor blood transfusions on allograft survival might also work through a functional inactivation mechanism (130, 131). Infusions of hematopoietic cells, depleted of costimulatory activity either by treatment of the population with an inhibitor of metabolic activity (e.g. UV light, chemical fixatives), or by removal of potent accessory cells from the population, have been shown to suppress MLR responses and to prolong allogeneic graft survival (132–135). Donor resting T cells and red blood cells may inactivate class I–specific alloreactive T cells in a "veto"-like manner; resting B cells, recently shown to be poor stimulators in a primary response in vivo (136), might inactivate class II–specific alloreactive cells.

Finally, studies by Lafferty and others (137–140) have demonstrated that depletion of donor APC from allogeneic tissue prolongs the survival of the graft. This can be interpreted in terms of the biological two-signal model as a failure of this transplanted tissue to activate an immune response. It is conceivable, however, that the success of this manipulation also depends on the process of inactivation. CTL precursors entering the nonspecific inflammatory sites (induced by the surgical procedure) may encounter allogeneic class-I molecules on parenchymal cells incapable of delivering costimulatory signals, and may thus become functionally inactivated. Similarly, if IFN-γ is released at these sites from sources such as natural killer cells, it is possible that endothelial and epithelial cells would begin to express class-II molecules and thus provide a means of inactivating $CD4^+$ T cells. If these mechanisms do play a role, then elimination of "passenger leukocytes" with anti-class II antibodies (139) might prove to be a less effective means of reducing graft rejection than administering antibodies directed against molecules specifically expressed on the surface of potent APC, or depleting these APC in some other specific fashion (138).

CONCLUSION

Proliferation of T lymphocytes relies on the simultaneous occupancy of the T-cell antigen receptor and delivery of an accessory cell–derived costimulatory signal. T-cell receptor occupancy alone is read as a signal to down-regulate further antigen responsiveness and possibly to maintain self-tolerance. The presence of bone marrow–derived accessory cells capable of delivering a costimulatory activity during occupancy of the antigen receptor results in the proliferation of the T cell and development of effector cell function.

At the biochemical level, T-cell receptor occupancy by peptide antigen/Ia complexes is sufficient to induce the hydrolysis of PIP_2. Increases in $[Ca^{+2}]_i$, as well as activation of PKC (as measured by phosphorylation

of the CD3-γ polypeptide) are directly correlated with the induction of PIP_2 hydrolysis and occur independently of the presence of accessory cell-induced costimulatory signals. Thus, there is no evidence for a direct involvement of the costimulatory signalling pathway in the generation of increased $[Ca^{+2}]_i$ and PKC activation.

Increased $[Ca^{+2}]_i$ and PKC activation are *not* sufficient biochemical signals to induce T-cell proliferation. Proliferation requires the generation of a costimulatory signal acting independently on the IL-2 gene. Proliferation of T cells in the absence of accessory cells in response to stimulation with anti-CD3 monoclonal antibody or the combination of ionomycin and PMA results from the induction of costimulatory activity within the T-cell population and delivery of the activity during T-T interactions. Thus, T-cell IL-2 gene activation and subsequent proliferation are dependent on at least three biochemical signals.

Increased $[Ca^{+2}]_i$ (following T cell receptor occupancy) is sufficient to functionally inactivate Type I (but not Type II) $CD4^+$ T cell clones. These clones develop a state of proliferative unresponsiveness that is long-lived and stems from an inability to produce IL-2 upon antigen restimulation. The cells maintain their viability in vitro and remain capable of proliferating in response to exogenous IL-2. In contrast, the presence of an accessory cell-induced costimulatory signal in parallel with antigen receptor occupancy prevents the calcium-dependent induction of unresponsiveness and synergizes with the rise in $[Ca^{+2}]_i$ and increase in PKC activity to induce the transcription of the IL-2 gene.

These observations suggest that the original biological two-signal model proposed by Bretscher & Cohn for B-cell activation is applicable to T-cell activation. Signal one is occupancy of the antigen-specific receptor and alone is capable of inducing the functional inactivation of mature T cells and possibly clonal deletion of immature thymocytes. Signal two is the costimulatory signal; it functions as a gate on the first signal to determine its outcome. The presence of signal two leads to a full proliferative response by the T-cell following occupancy of its antigen receptor; the absence of signal two leads to a state of proliferative nonresponsiveness.

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