Down Regulation of Human T-Lymphocyte Activation by Antibody to the 50 Kilodalton Surface Protein Associated with the Sheep Erythrocyte Receptor

by

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ABBREVIATIONS USED:

CHX	Cycloheximide
Con A	Concanavalin A
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
DRB	5,6-dichloro-1-β-Diribofuranosyl benzimidazole
E-Receptor	Sheep erythrocyte receptor
HBSS	Hank's balanced salt solution
IL1	Interleukin 1
IL2	Interleukin 2
J32	Human leukemic T cell line Jurkat subclone 32
kd	Kilodalton
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
NK	Natural killer cells
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
SRBC	Sheep erythrocytes
SSC	Standard saline citrate
Tac	IL2 receptor determinant on activated T lymphocytes
TPA	12-O-tetradecanoyl-phorbol-13-acetate
U/ml	Unit per milliliter
Δ FL	Relative mean fluorescence intensity

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STATEMENT OF THE PROBLEM

1. Introduction

Recent developments in our understanding of T lymphocyte proliferation have shown that T-cell response to antigen or mitogen involves a series of cellular and lymphokine-mediated events which culminate in T cell proliferation. Antigen or mitogen binding to its specific T cell receptors, in the presence of an accessory cell, triggers the production of interleukin 2 (IL2) and the generation of cell surface IL2 receptors (1-6). Subsequent T cell proliferation is dependent on the interaction of IL2 with IL2 receptors (7-8).

Mature human T cells form spontaneous rosettes with sheep erythrocytes at 4°C. The receptor responsible for this phenomenon (E-Receptor) has been thought to be involved in T cell activation (9-12). This association was based on early observations that stimulated T cells, unlike resting T cells, form stable E-rosettes at 37°C (9-13). Recently a 50 kd surface protein associated with the sheep erythrocyte receptor has been defined by monoclonal antibodies. Using these antibodies, it was shown that PHA- stimulated lymphocytes express 3-6 times more 50 kd protein per cell than resting T cells (14-15). More recently, it was shown that this protein, also known as T11, can serve as an integral component of the T cell activation pathway resulting in Interleukin 2 (IL2)-dependent proliferation (15). Several monoclonal antibodies to certain epitopes on the 50 kd determinant have been shown to suppress the T-cell proliferative response to antigen and lectin mitogen PHA (16-19). The mechanism(s) of this antibody-mediated suppression is poorly understood. Initial investigation showed that monoclonal antibodies OKT11A inhibited the proliferation of T lymphocytes induced by calcium

ionophore A23187, but not by Phorbol ester TPA (17), and that antibody OKT11A reduced levels of IL2 in the supernatant of lymphocyte cultures stimulated with mitogen or antigen (17). However, the mechanism(s) by which anti-50 kd antibodies suppress T lymphocyte proliferation vis-a-vis its effect on IL2 production or secretion, the mechanism by which it regulates IL2 production, IL2 receptor expression, and the nature of signal(s) transduction being modulated by these antibodies have not been well determined. This may be attributed to unavailability of direct methods, and reagents to address these questions. With the availability of monoclonal antibody to the IL2 receptor anti-Tac (20-21) and cDNA probes for IL2 gene (22-23), I have examined the role of the 50kd molecule in T cell proliferation. My main goal was to determine the role of this molecule in IL2 production, IL2 receptor acquisition induced by mitogens, and to define at what level of IL2 gene expression it exerts its function.

2. Specific Objectives:

A. To determine the regulatory role of the 50 kd molecule in T cell proliferation, I examined:

1. the effect of monoclonal antibody 9.6 to the 50 kd molecule on:
   a) lymphocyte proliferation stimulated with antigen, lectin mitogens and the phorbol ester TPA.
   b) IL2 elaboration in cultures of lymphocytes stimulated with mitogen or antigen.
   c) IL2 receptor acquisition on lymphocytes stimulated with mitogen and/or phorbol ester TPA.
2. the ability of IL2 preparations to overcome the effect of anti-50 kd antibodies on lymphocyte proliferation and IL2 receptor acquisition.

B. To define the level of IL2 gene expression at which the 50 kd molecule regulates IL2 production in stimulated lymphocytes:

a) The effect of antibody 9.6 on IL2 mRNA accumulation in lymphocytes stimulated with PHA and TPA was examined.

b) The effect of antibody 9.6 on IL2 mRNA accumulation was compared to the effect of immunosuppressant cyclosporin A and a transcription inhibitor.

c) The mechanism(s) regulating the transient expression of IL2 was investigated

d) The effect of antibody 9.6 on IL2 mRNA superinduction induced by CHX was examined

INTRODUCTION AND LITERATURE REVIEW

A. The Sheep Erythrocyte Receptor on Human T Cells (E-receptor)

1) Discovery of Rosette Formation

In 1970 several investigators observed that human peripheral blood lymphocytes when incubated with sheep erythrocytes (SRBC) formed spontaneous rosettes (E-rosettes) (24-26). The reaction is specific to sheep erythrocytes. Erythrocytes from horse, ox, rabbit, guinea pig, and mouse do not form rosettes with human lymphocytes (27). Further examination of the rosetting phenomenon demonstrated that it involved only a fraction of human peripheral lymphocytes (28). Wybran et al (29) were the first to suggest that these cells were chiefly thymic derived. Further support to this conclusion was given by the findings that E-rosettes are formed by 90-100% of human thymocytes and by 51-81% of peripheral blood lymphocytes but not by immunoglobulin carrying lymphocytes, i.e., B cells (27). Further studies indicated that the overwhelming majority of lymphocytes which form E-rosettes are T lymphocytes (30). The actual percentage of E-rosettes observed in different studies varied, depending on the details of the technique employed (24,30). Furthermore, a small fraction of E-rosette positive lymphocytes appear to have functional and cell surface characteristics of natural killer cells (31).

2) Biophysical and Biological Parameters that Affect E-Rosette Formation

Despite the apparent simplicity, the reaction between sheep erythrocytes and human lymphocytes is a complex one. E-rosette formation requires a viable and metabolically active lymphocyte; it is dependent on

the presence of divalent cations (27). The treatment of lymphocytes or SRBC with neuraminidase increases the number of SRBC bound per cell as well as the total number of cells participating in the reaction, suggesting that neuraminidase reduces the electric surface charge of the cells, thereby enabling a more effective interaction between receptors exposed on the membrane of PBL and sites on SRBC (32). The reaction is optimal at pH 7 to 8 and decreases at acid pH. Temperature has a very characteristic effect on the stability of the rosettes. It was found that maximal adherence between SRBC and human peripheral blood lymphocytes (PBL) occurred when first contact between cells was established at 37°C followed by incubation at low temperature (28,30). With further incubation at 37°C the rosettes disintegrated (28,30). However, thymocytes and activated lymphocytes form stable E-rosettes at 37°C (9-13). Rosette formation was inhibited in the presence of sodium azide (30). This observation suggested that metabolic processes involving the plasma membrane are essential for E-rosette formation, and if the process is analogous to the capping phenomenon, movement of certain membrane components may be required for the reaction. The two-temperature incubation required is thought to be consistent with this interpretation. Cytoskeletal microfilaments play a critical role in the E-rosette formation; thus prior incubation of the lymphocytes in Cytochalasin B inhibits rosette formation (33). This inhibition has been attributed to the binding of Cytochalasin B to actin-containing microfilaments (34). Pretreatment of the lymphocytes with trypsin (30,35) or phospholipase A removed their E-binding capacity (35). In contrast, treatment of lymphocytes with papain enhances E-rosette-formation (35).

3) E-receptor on Activated T Lymphocytes

Although mature resting T lymphocytes form E-rosettes at 4°C, they do not rosette with sheep erythrocytes at 37°C. However, when lymphocytes are stimulated by PHA, calcium ionophore A23187 (12) or alloantigen (9,13), they form E-rosettes which are stable at 37°C. Therefore, formation of stable E-rosettes at 37°C had been implicated as an early indicator of lymphocyte activation (9-13). This stability has been attributed to an increase in the expression of E-receptor and/or the "unmasking" of a new receptor on activated lymphocytes. Lymphocytes stimulated by PHA express a higher number of E-receptors as suggested by an increased number of SRBC on each cell and an increase in the number of lymphocytes forming E-rosettes (36). Presumably an increased number of binding sites permits more cross-linking of sheep erythrocytes, thereby enhancing the stability of E-rosettes at 37°C. This interpretation is supported by electron microscopic studies showing an increase in the number of attachment points between cell surface on activated or neuraminidase-treated lymphocytes and SRBC (37). However, changes in avidity of the E-receptor upon activation could not be ruled out. An increase in membrane fluidity of PHA-activated T lymphocytes due to higher thermodynamic energy has been reported (38). Such changes could lead to conformational alteration and enhancement in E-receptor avidity to SRBC.

Stable E-rosette formation at 37°C has also been associated with human disease. Patients with acute lymphocytic leukemia (39), chronic hepatitis (40) or sarcoidosis (41) are reported to have higher than normal levels of spontaneous stable rosettes at 37°C. In contrast, some patients with malignancies, autoimmune disease, viral infection (42), as well as aging, are reported to have diminished levels of stable E-rosetting T

lymphocytes in the peripheral blood. These studies suggest that the E-receptor molecule is important in the function of T cells in a wide range of activities of the immune response.

4) Factors Associated with the E-receptor Function

Defective T lymphocyte E-rosette function has been observed in association with several diseases, including cancer and viral diseases (42), autoimmune diseases (43). However, the specific mechanisms responsible for the observed defect of E-rosette function in these diseases has not been established. In one study, defective E-rosette formation in viral hepatitis B was attributed to the presence of a serum factor(s) designated Rosette Inhibitory Factor (RIF). It was identified as lipoprotein of the low-density lipoprotein (LDL) class which binds lymphocytes and reduces E-rosette formation and the capacity of T lymphocytes to respond in mixed lymphocyte reaction (44). This inhibition is not due to steric hindrance at the lymphocyte surface. The requirement of 13-24 hrs after binding of RIF to lymphocyte to achieve maximal inhibition of rosette formation is consistent with a metabolically induced event. This RIF inhibited mixed lymphocyte reaction; however, equivalent lipoprotein fractions from normal serum are equally inhibitory, suggesting that this effect is not directly attributable to RIF activity.

Furthermore, malignant ascites fluid from ovarian and colonic cancer contain an immunosuppressive moiety (50 kd) that appears to be related to the E-receptor (45). It inhibits E-rosette formation, PHA proliferation response, mixed lymphocyte reaction and natural killer cell activities.. More recently this factor has been shown to inhibit cell free DNA polymerase α activity of human PBL (46).

B. The 50 kd Surface Protein Associated with the E-Receptor

1) Identification and the Structural-Functional Complexity

With the advent of somatic cell fusion techniques, Kamoun et al developed a murine monoclonal antibody designated 9.6 that reacts with a 50 kd polypeptide on the surface of human T cells (47). This antigen is identical to or closely associated with the human T cell receptor for sheep erythrocytes. Evidence in support of this conclusion was derived from three observations. First, there was a close correlation between expression of 50 kd and the ability of cells to form E-rosettes. Second, cells preincubated with antibody 9.6 did not form E-rosettes. Third, cells lysostripped of 50 kd with antibody 9.6 did not form E-rosettes. Several other investigators have reported monoclonal antibodies capable of blocking rosette formation, including ATM 1.1 (leu 5), 1.2, 3.1 and 3.2 (48), OKT11, OKT11A (49), D66 (50), LFA2 (18), and 35.1 (19). Each of these antibodies has been reported to react with a 50 kd cell surface membrane polypeptide.

Immunoprecipitation and pre-clearing experiments with OKT11, LFA-2, leu 5, and 9.6 monoclonal antibodies demonstrated that these antibodies recognize the same molecule. Moreover, binding of these antibodies has similar cell distribution (18,48). They all bind to T cells and thymocytes but not to B cells. The 50 kd determinant is expressed in greater amounts on thymocytes than on peripheral T cells. Within the T cell population the level of 50 kd molecule partially resolves peripheral T lymphocytes into two subpopulations. One expresses more 50 kd determinant (9.6^+, leu 5^+) than the other; T cells with higher density correlate with the cytotoxic/suppressor cells, whereas the lower density 50 kd cells correlate with helper/inducer cells (48, and unpublished

observation). It is interesting to note that peripheral T cells show low and high E-rosette stabilities and thymocytes also form high stability rosettes (51). Thus a strong correlation exists between the quantitative expression of the 50 kd determinant and the stability of E-rosette formation, further supporting that the 50 kd determinant is either identical to the E-receptor or very closely associated with it. However, the identify of the E-receptor would be established only by purification of the appropriate molecule(s) and demonstration of specific binding to SBRC and/or direct inhibition of E-rosette formation.

Functional studies revealed that antibody 9.6 blocks cytotoxic T lymphocyte (CTL) and natural killing (NK) function without affecting cells that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (52). With the further development of more monoclonal antibodies to the E-receptor and functional studies utilizing these antibodies as analytic probes, the structural and functional complexity of the 50 kd surface protein became evident. Although a systematic study of all antibodies to the 50 kd has not been reported, several distinct epitopes of 50 kd determinant have been recognized. Antibody 35.1 recognized an epitope in close spacial proximity to, but distinct from, the epitope recognized by antibody 9.6. Both antibodies 9.6 and 35.1 were shown to bind to 50 kd protein, inhibit proliferative response to mitogen and antigen, and cytotoxic T lymphocyte (CTL) activity. However, only antibody 9.6 was found to inhibit natural killer (NK) activity and E-rosette formation (19). These results were not attributable to differences in antibody class or binding characteristics because Scatchard analysis demonstrated that these two IgG2a antibodies have comparable avidity and that T cells bind each antibody in equivalent amounts (19). This pattern of

specificity of inhibition by antibody 9.6 and antibody 35.1 appears to provide a basis for linking E-rosette formation and NK lysis; however, antibodies LFA2 (18), OKT11A (49), Leu 5 (48), D66 (50) like antibody 9.6, block E-rosette formation but do not block NK activity. Moreover, antibody D66 which blocks E-rosette formation does not block 9.6 binding (50). Thus these findings support the notion that various functional properties of the 50 kd molecule could be attributed to distinct epitopes defined by various monoclonal antibodies. This conclusion is corroborated by the phylogenetic studies discussed below. The overall picture of functional distribution among epitopes on the 50 kd structure suggests that some of the functions studied, such as cytotoxic T lymphocyte effector function, T cell proliferation and E-rosette function, are not strictly epitope dependent, whereas the function of NK is restricted to the 9.6 epitope.

More recently, Meuer et al (15) developed yet another group of monoclonal antibodies to the 50 kd E-receptor-associated protein. On the basis of qualitative cellular distribution and competitive blocking experiments, three distinct epitopes termed $T11_1$, $T11_2$, and $T11_3$ were defined on the 50 kd structure by means of monoclonal antibodies. $T11_1$ is associated with the sheep erythrocyte binding site since antibodies to this anti-$T11_1$ epitope block rosette formation with SBRC. In contrast, antibodies to $T11_2$ and $T11_3$ epitopes did not block SBRC rosetting. Moreover, the $T11_3$ epitope was expressed on activated but not on resting T lymphocyte or thymocytes. Analysis of $T11_3$ epitope expression indicates that it appears within 30 min of binding by antibody to $T11_2$ even at 4°C; or following T cell activation by PHA. Incubation of T cells or T cell clones with monoclonal antibodies to both the $T11_2$ and $T11_3$ epitopes

induced IL2 production, IL2 receptor acquisition and lymphocyte proliferation (15). In contrast, incubation of human thymocytes with the combination of anti-$T11_2$ plus anti-$T11_3$ antibodies induced IL2 receptor expression on all thymocytes but failed to induce IL2 production and proliferation. Consequently, thymocyte proliferation in response to anti-$T11_2$ plus anti-$T11_3$ triggering required exogenous IL2 (53). These findings suggest that IL2 receptor expression does not require IL2 elaboration. The ability of anti-$T11_2$ plus anti-$T11_3$ to induce IL2 receptor expression on thymocytes which do not express the antigen receptor complex ($T3/Ti^-$) suggest that activation of human thymocytes via the 50 kd pathway is independent and not necessarily regulated by the antigen receptor-mediated activation pathway. If the T11 activation pathway is the major means by which thymocytes are induced to proliferate, and this in turn requires exogenous IL2, then additional factors would be necessary to initiate thymocyte growth before the esablishment of a mature T cell IL2 source. In this regard, various lines of thymic epithelium have been reported to secrete factors that intiate IL2 production (54).

2) Phylogenetic Distribution of the 50 kd Protein Associated with the E-Receptor

Phylogenetic studies in primates of the reactivity of SRBC with primate T cells and with monoclonal antibody 9.6 demonstrated a remarkable structural conservation of E-receptor on T cells during primate evolution. Other T-cell determinants are less well conserved. SRBC and antibody 9.6 reacted with T cells of all primates studied, including gorillas, chimpanzees, gibbon, old world monkeys, and new world monkeys (55). A more recent study used OKT11A monoclonal antibody confirmed the remarkable structural conservation of the 50 kd molecule in species as

distant phylogenetically as lemur and man (56). Whereas one monoclonal antibody did not bind to T cells from two distinct subspecies of owl monkeys, other anti-50 kd monoclonal antibodies which recognize different epitopes on the 50 kd determinant were found to be reactive with these subspecies of owl monkey PBL (56). Similarly it was found that antibody 35.1 does not react with some baboon T cells, even though these cells were isolated by E-rosetting and reacted with antibody 9.6. Cells from other baboons or from two other nonhuman primate species were also found to react with antibody 9.6 but not with antibody 35.1. These findings clearly indicated that 35.1 and 9.6 antibodies bind to distinct epitopes of the 50kd (19). The difference in binding of T cells from various primate species to a panel of monoclonal antibodies which define different epitopes of the same surface structure indicates that the expression of various epitopes of a single surface antigen defining T cell populations are separable. The conservation of structure suggests that this antigen, a molecule which fortuitously binds to sheep erythrocytes must play a crucial, though still undefined, role in the function of T cells.

3) Ontogeny of the 50 kd Surface Protein Associated with the E-Receptor

The ability of lymphocytes to form E-rosettes appears in the human thymus between 11 and 14 wks and it increases gradually between 14 and 20 wks of gestation (57). It is interesting that the onset of thymocyte response to PHA appears at about 10 wks and to Con A at 13-14 wks of gestation. Recently discrete stages of intrathymic ontogeny have been defined on the basis of monoclonal antibody probes directed at unique T lineage specific surface proteins. Thus the earliest identifiable T-lineage cell expresses the sheep erythrocyte binding protein 50 kd, T11

and no other marker (Stage I). With further maturation thymocytes acquire T4, and T6, T8 determinants (Stage II), and in Stage III thymocytes lose T6 and acquire the T3-Ti antigen receptor molecule (58,59). The level of 50 kd determinant decreases in density as thymocytes mature to peripheral T lymphocytes (48). In the peripheral lymphocytes, functionally distinct subpopulations of T cells express different levels of 50 kd determinant. The cytotoxic/suppressor subset expresses more of the determinant than does the helper/inducer subset. Thus the differential expression during T cell development suggests a role for the 50 kd protein in maturation and/or function.

C. Human T-Cell Activation

1) Current Growth Model

T cell proliferation occurs as a series of complex and precisely orchestrated events. Resting T cells express few or no interleukin 2 receptors. However, stimulation of T lymphocytes by antigen or mitogen leads to rapid induction of surface IL2 receptor and to endogenous IL2 production, secretion and subsequent binding to the IL2 receptor. Once a critical number of IL2 receptors have bound IL2, DNA synthesis and cell mitosis occurs. Finally in the absence of continuous antigen stimulation the IL2 receptor levels are reduced to the resting stage levels (60,61).

Recent advances in the dissection of molecular mechanisms that regulate T-lymphocyte mitosis have permitted an analysis of the variables that determine the progression of T cell proliferation. It was shown that the initiation of the T cell cycle progresses by immunostimulatory signals such as antigens, mitogen, or T cell specific monoclonal antibodies, whereas the transition from G_1 into the replicative phase of the cell

cycle is mediated by IL2 alone (60-62). IL2 is released by T cells in response to two signals provided by the antigen signal plus the accessory cell signal (4). The first signal is antigen presented in the context of proteins of the major histocompatibility complex (MHC), and the second signal is provided by the accessory cell and/or IL1 (63). Certain lectins, PHA, Con A, and monoclonal antibodies to T cells can substitute for antigen signals, whereas phorbol ester TPA can substitute for the accessory cell signal in mitogen-induced proliferation (64). Once released, IL2 promotes the proliferation of any IL2 receptor positive T cells regardless of its antigenic specificity. Thus the immune specificity of T cell clonal expression is guaranteed by the restriction of IL2 receptor expression only to antigen activated clones.

The growth characteristic of activated T cells has been found to correlate with IL2 and IL2 receptor expression. From studies employing monoclonal antibodies to the IL2 receptor (anti-Tac antibody), it was found that IL2 receptor appears asynchronously. That is, receptor density in any given population of activated T cells is heterogeneous and follows normal distribution (61). Since a critical threshold of occupied IL2 receptor density must be reached before commitment to DNA synthesis occurs (61), the IL2 concentration together with the density of IL2 receptor determines the cell cycle progression of a given lymphocyte into S phase. Therefore, at low IL2 concentration, the percentage of occupied receptor sites will be low and only the small fraction of cells with high receptor density numbers will pass the threshold necessary for commitment to division. On the other hand, at high IL2 concentration when the IL2 receptors are nearly saturated, even cells with low receptor density will reach the threshold (65). It follows from this analysis that the

heterogeneity of IL2 receptor expression leads to the asynchronous entry of activated T cells into S phase and that the transient expression of IL2 and IL2 receptor provides an overall control over the magnitude of the T cell proliferative response. The above model stresses the central linkage whereby external stimuli direct the magnitude or extent of T cell clonal proliferation by means of the IL2-hormone-receptor system. The mechanism underlying the transient expression of IL2 receptor and IL2 and the role of the antigen or mitogen in regulating this phenomenon remain obscure.

2) Signal Transduction in T-cell Activation

Activation signals delivered to T cells by mitogens or antigens must be transferred across the membrane of these cells. Recent analysis of membrane signal transduction has revealed that mitogens like PHA and Con A induce increases in lymphocyte membrane phosphatidylinositol turnover, within minutes of binding, which lead to transient elevation of two products, diacylglycerol (DAG) and inositoltriphosphate (IP3) (66-67). The latter may induce a signal by mobilizing internal calcium and diacylglycerol binds to protein kinase C and increases its affinity for calcium ions, rendering this enzyme active (68). Phorbol esters, like TPA, because of their structural similarity to DAG, bind and directly activate protein kinase C (69).

Transmembrane fluxes of calcium, leading to increased free cytosolic calcium concentrations $[Ca^{2+}]_i$, have been demonstrated following binding of mitogen (70) or anti-T_3 antibodies to lymphocytes (71) and have been implicated in the initiation of cell proliferation, whereas the effect of TPA on lymphocyte proliferation has been shown to occur in the absence of extracellular Ca^{2+} or detectable changes in free cytosolic calcium

concentration (68,70). It has been argued that the activation of cellular responses via kinase C activation is separate from and synergistic to those activated via an increase in cytosolic calcium (68). This synergy has been shown in several cellular responses including IL2 production and lymphocyte proliferation. Thus addition of TPA with PHA or calcium ionophore to T-lymphocyte cultures leads to the production of synergistic levels of IL2 mRNA (72), IL2 (73, 74) and lymphocyte proliferation (64).

It has been shown that the induction of IL2 receptor expression and IL2 production may involve different activation signals; some mitogens or antigens may activate both, whereas others may activate only one. Thus an increase in cytosolic Ca^{2+} is not required for expression of the IL2 receptor; in contrast, IL2 production requires an increase in cytosolic Ca^{2+} and does not occur in the absence of extracellular free calcium (75).

Paper 1

Functional properties of the 50 kd protein associated with the E-receptor on human T lymphocytes: Suppression of IL2 production by anti-p50 monoclonal antibodies[1]

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ABBREVIATIONS

IL2	interleukin 2
MLC	mixed lymphocyte culture reaction
E-receptor	human lymphocyte receptor for sheep erythrocyte
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
TPA	12-O-tetradecanoyl-phorbol-13-acetate

ABSTRACT

Monoclonal antibody 9.6 is specific for a 50 kd T-cell surface protein (p50) associated with the sheep erythrocyte (E) receptor on human T lymphocytes. This antibody interferes with many T-cell functions. We have examined the effect of antibody 9.6 on lymphocyte proliferation and interleukin-2 (IL2) production triggered by mitogens, soluble antigens, and alloantigens to elucidate the mechanism(s) of its immunosuppressive action. At concentrations as low as 50 ng/ml, 9.6 suppressed lymphocyte proliferation and the elaboration of IL2 by T-cells stimulated by PHA, alloantigens, or low concentrations of the phorbol ester TPA ($\leq$1 ng/ml). Furthermore, in cultures stimulated by a combination of PHA plus TPA, 9.6 did not inhibit the acquisition of IL2 receptors but inhibited proliferation and IL2 production. Immunoaffinity purified IL2 completely restored lymphocyte proliferation in cultures inhibited by 9.6.

Studies of kinetics of inhibition by 9.6 showed that this antibody inhibited lymphocyte proliferation induced by PHA, alloantigen, and PPD even when added at 24, 48, or 72 hr, respectively, after the initiation of these cultures, suggesting that 9.6 does not block lectin binding or antigen recognition by T cells and that it can inhibit lymphocyte proliferation even after cells have undergone one or more rounds of cell division.

A dose response analysis of lymphocyte proliferation induced by PHA or by TPA demonstrated that the degree of inhibition by 9.6 decreased with increasing concentrations of these mitogens. Antibody 9.6 did not inhibit lymphocyte responses induced by optimal concentrations of PHA (50-100 ug/ml, PHA-M) but inhibited proliferation of maximally induced lymphocytes using a synergistic combination of low concentrations of PHA (5 ug/ml, PHA-M) plus TPA (1 ng/ml).

Taken together, these findings indicate that 1) 9.6 inhibits lymphocyte proliferation by affecting IL2 production; 2) 9.6 does not inhibit the acquisition of IL2 receptors induced by a synergistic combination of PHA plus TPA; and 3) p50 molecules may be involved in multiple pathways of T-cell activation.

INTRODUCTION

A number of *in vitro* immune reactions are modified by monoclonal antibodies that define T-cell surface differentiation antigens or receptors, including antibodies to the antigen receptor complex (T3-Ti) (1), antibodies to the IL2 receptor (Tac) (2), and antibodies to the transferrin receptor such as 42/6 (3). Recently, monoclonal antibodies to a 50 kd polypeptide (p50) identical to or closely associated with the receptor for sheep erythrocytes on human T cells (E-receptor) have been developed by several investigators (4-9). Many of these anti-p50 antibodies (9.6, OKT11A, 35.1, LAF2) have been shown to inhibit various lymphocyte functions such as proliferation (8-11), cytotoxic T-cell function (8-9), and T-cell-dependent immunoglobulin production by B cells (10). Little is known, however, about the mechanisms by which anti-p50 antibodies alter these lymphocyte functions. Palacios et al. (10) have previously reported that OKT11A blocks lymphocyte proliferation induced by PHA but not by the phorbol ester TPA. It was concluded that the inhibition of proliferation was secondary to suppression of IL2 production as well as acquisition of responsiveness to IL2. However, this conclusion was only indirectly implied and was not confirmed by experiments using purified IL2 and antibodies to IL2 receptors. In this report we present the results of experiments that were undertaken to accurately examine the effect of antibody 9.6 on IL2 production and IL2 receptor expression. We have examined quantitatively the ability to 9.6 to inhibit lymphocyte responses to various concentrations of PHA and TPA. This study demonstrates that 9.6 affects both pathways of T-cell activation, at least in part by inhibiting IL2 production. The implications of these findings are discussed.

MATERIALS AND METHODS

Isolation of cells. Human mononuclear cells from heparinized peripheral blood (PBMC) were isolated by centrifugation on Ficoll-Hypaque. T-cells were purified from PBMC by removal of adherent cells on a nylon wool column or by rosetting with AET (2-aminoethyl-isothio-uronium bromide hydrobromide)-treated sheep erythrocytes ($SRBC_{AET}$) (4). Normal human thymocytes were prepared from thymus specimens (4) obtained in the course of corrective open heart surgery from children less than 14 years old. After washing 3 times in RPMI, cells were resuspended in RPMI containing penicillin (50 U/ml), streptomycin (100 ug/ml), L-glutamine (2.0 mM), and 10% (v/v) heat inactivated human AB-positive or pooled serum (herienafter designated medium).

Preparation of antibodies for cultures. Antibody 9.6 (IgG2a) was purified from ascites by column chromatography with protein A-Sepharose (Pharmacia) and adjusted to 0.5 mg/ml in PBS (4). Antibodies 10.2 (IgG2a) and 12.1 (IgG2b), which recognize surface molecules distinct from p50 present on all human T-cells (12), were used here in ascites form as controls. Purified anti-Tac antibody which recognizes the IL2 receptor on activated human T lymphocytes (2) (165 ng/ml in phosphate buffered saline) was a gift from Warner C. Greene (NIH, Bethesda, MD).

Sources of interleukin 2. Supernatants containing IL2 activity were derived from the MLA144 gibbon ape cell line as described by Rabin et al. (13). The MLA144 cell line was selected because it elaborates IL2 without requirement for induction with lectin mitogens or phorbol esters. In addition, this cell line has been shown not to produce interferon functional activity (13).

Human interleukin-2 (kindly provided by Dr. Didier Fradelizi, Hospital St. Louis, Paris, France) was derived from PHA-stimulated PBMC and partially

purified using an AcA 54 ultragel filtration chromatography as previously described (14).

Immunoaffinity purified human IL2 was a gift of Richard J. Robb (Glenolden, PA) and was prepared from the JURKAT-cell line following stimulation with PHA and TPA as described previously (15). This material has been shown to be homogeneous as determined by two-dimensional gel analysis, reverse-phase HPLC, and N-terminal amino acid sequencing (16).

IL2 preparations were tested for activity in a bioassay employing the CTLL-15H cell line (2). Unconcentrated MLA144 supernatants generally contained 25-50 U/ml of IL2 activity.

Lymphocyte proliferation assays. For lectin-mitogen responses, various concentrations of phytohemagglutinin (PHA) PHA-M or PHA-P (Difco, Detroit, MI) and/or TPA (Sigma) were added to flat-bottom microculture wells (Costar) containing $2x10^5$ viable cells in medium. For antigen responses, $5x10^5$ responder cells were stimulated with protein purified derivative (PPD) (5 ug/ml). For mixed lymphocyte culture (MLC) responses, $2x10^5$ responder cells were cultured with $2x10^5$ allogeneic irradiated (4000 rads) stimulator PBMC in round-bottom microtiter plates. Cultures stimulated with lectin-mitogen were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days. PPD cultures and mixed lymphocyte cultures were similarly maintained for 5 or 6 days, respectively. Monoclonal antibodies were added to cultures at the following concentrations: 9.6 (50-500 ng/ml of purified antibody or 1:100 dilution of ascites), 10.2 or 12.1 (1:100 dilution), anti-Tac (0.85 ng/ml), and OKT11A (150 ng/ml). The concentration of anti-Tac antibody used here produced maximal inhibition of the PHA-induced proliferative response, whereas OKT11A (150 ng/ml) and 9.6 (50 ng/ml) caused $\geq$50% inhibition, respectively. Cell proliferation was assayed by terminal 8-16 hour ^{3}H-thymidine

incorporation (0.5 uCi of ^{3}H-thymidine, New England Nuclear specific activity 6.7 Ci/mmol). To determine % inhibition the following formula was used:

$$1 - \frac{\text{Culture with 9.6 (cpm) - medium background (cpm)}}{\text{Culture without 9.6 (cpm) - medium background (cpm)}} \times 100$$

Cpm = counts per minute of ^{3}H-Tdr incorporation.

IL2 production. The production of IL2 was induced in the following systems: (a) MLC; PBMC or purified T cells and an equal number of irradiated allogenic PBMC were incubated at 37°C for various periods of time, as indicated in each experiment, in the presence or absence of various concentrations of monoclonal antibodies in a final volume of 1 ml of culture medium. (b) PHA was added at concentration indicated in each experiment to $4x10^{6}$ PBMC and cells were cultured as described above. At the end of the culture periods the tubes were centrifuged (800xG) for 10 min and the supernatants collected and assayed for IL2 activity in the microassay described below.

IL2 microassay. The amount of IL2 activity in supernatants was determined in a microassay based on the IL2-dependent proliferation of HT-2 cells (a murine [BALB/c] cloned cell line originally prepared by Dr. James Watson; this line was kindly provided by Dr. Howard Gray, National Jewish Hospital, Denver, CO). Supernatants were serially diluted in RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum, $2.5x10^{-5}$ M 2-mercaptoethanol and 100 ul/well added to 96-well flat-bottom microculture plates. $3x10^{3}$ of HT-2 or $5x10^{3}$ of CTLL cells in 100 ul aliquots of medium were then added to each microtiter well. After 20 hr of culture at 37°C in an atmosphere of 5% CO_2 in air, each microwell culture was pulsed for an

additional 6 hr with 0.5 uCi of [^{3}H]-TdR, and [^{3}H]-TdR incorporation determined. The IL2 activity of cultures performed was expressed as units per milliliter. One U/ml of IL2 activity corresponded to the dilution that produced half-maximal proliferation of HT-2 or CTLL cells.

Quantitative analysis of IL2 receptor expression

Monoclonal anti-Tac antibody was conjugated with biotin by the succinamide ester method (17) and then used in a quantitative immunofluorescence assay using a fluorescence activated cell sorter (FACS IV, Becton-Dickinson).

For immunofluorescence assays, PBMC were cultured in 17 x 100 mm Falcon tubes (Falcon 2057) at 10^6/ml in complete medium with PHA-M (5 ug/ml) plus TPA (0.5 ng/ml) in the presence or absence of antibody 9.6 ascites (1:100). Cells were removed from cultures after 24 hr, washed 3 times in HBSS, and resuspended at $5x10^6$/ml in staining buffer: Dulbecco's phosphate buffered saline (pH 7.4) containing 2% bovine serum albumin and 0.02% NaN_3. Viabilities of recovered cells were >90% as determined by trypan blue dye exclusion.

Aliquots of 200 ul of cells in staining buffer were reacted with saturating amounts of biotin-conjugated anti-Tac for 30 min on ice, washed 3 times in staining buffer, and reacted for an additional 30 min on ice with the fluorescein-conjugated avidin (Becton-Dickinson). After 3 washes, stained cells were fixed overnight at 4°C in phosphate buffered saline containing 2.5% paraformaldehyde and stored in the dark. On the day of analysis, fixed cells were washed once, resuspended in 0.5 ml of staining buffer, and filtered through nylon mesh.

In all experiments, lymphocyte [^{3}H]-thymidine incorporation was measured in companion microtiter cultures at 72 hr.

Cells stained with various monoclonal antibodies were analyzed using a FACS equipped with a 488 nm argon laser, as previously described (12). Dead cells, erythrocytes, platelets, and most macrophages were excluded from the analysis by appropriate selection of forward and 90° light scatter parameters. Data were displayed as histograms, with the fluorescence intensity displayed on the X-axis and relative cell number on the Y-axis. The fluorescence signal was log-amplified in four decades. At least 10^5 cells were analyzed for each sample.

RESULTS

Antibody 9.6 inhibits the production of IL2 induced by mitogens and by alloantigens

Supernatants recovered from PBMC cultures stimulated for 24 hr with PHA in the presence or absence of 9.6 were assayed for IL2 activity by a microassay as described in Methods. Table I summarizes the results of representative experiments. As shown, antibody 9.6 decreased IL2 levels in PHA-stimulated cultures (PHA-P, 0.2%) from 3.7 to 0.2 U/ml (>90% inhibition). Adding TPA (0.5 ng/ml) to suboptimally stimulated (PHA-M, 5 ug/ml) PBMC cultures generated a higher level of IL2 (15.2 U/ml) that was reduced to 0.13 U/ml in cultures containing antibody 9.6. This inhibition was not due to a lag in the kinetics of IL2 production (data not shown).

We have also examined the effect of 9.6 on IL2 production in MLC. Figure 1 shows a time course analysis of IL2 production. Replicate cultures were harvested daily for 6 consecutive days and assayed for IL2 activity. Unlike PHA cultures, IL2 measured after 24 hr was low and reached peak levels approximately 48 hr after the initiation of culture, thus indicating slower kinetics of IL2 accumulation. Addition of 9.6 at the onset of cultures caused marked inhibition of IL2 activity throughout the 6 days of culture.

9.6 does not inhibit the acquisition of IL2 receptors induced by a synergistic combination of PHA plus TPA

We have examined the effect of 9.6 on the acquisition of IL2 receptors in PBMC cultures maximally stimulated with a combination of PHA (PHA-M, 5 ug/ml) plus TPA (0.5 ng/ml). IL2 receptors were measured with anti-Tac antibody using a quantitative biotin-avidin FACS analysis. Figure 2 shows that after 24 hr 85% of lymphocytes expressed IL2 receptors in induced cultures. Adding

9.6 to these cultures did not inhibit IL2 receptor expression. Parallel cultures showed that 9.6 inhibited lymphocyte proliferation (90%).

Purified IL2 abrogates inhibition of lymphocyte proliferation mediated by antibody 9.6

Since antibody 9.6 was found to inhibit IL2 production, several preparations of IL2 were used to attempt to restore lymphocyte proliferation in PBMC cultures inhibited by 9.6. Figure 3 demonstrates that the JURKAT-derived purified IL2 restored lymphocyte proliferation in 9.6-inhibited cultures in a dose dependent fashion. Adding IL2 (10 U/ml) restored proliferation to a level equal to that of cultures stimulated with PHA alone. However, adding higher concentrations of purified IL2 up to 70 U/ml did not restore proliferation of 9.6-inhibited cultures to levels obtained in cultures stimulated with PHA and 70 U/ml of IL2. Similar results were obtained using MLA144-derived supernatants as a source of IL2 (data not shown).

Figure 4 shows typical results from a representative MLC experiment wherein the restoration of lymphocyte proliferation using partially purified IL2 (10 U/ml PBMC) was similar to that obtained with the JURKAT-derived purified IL2 (10 U/ml) in parallel PHA cultures. When purified IL2 was used to restore 9.6-inhibited MLC cultures similar restoration was observed.

Anti-Tac antibody blocks the ability of IL2-containing supernatants to restore proliferation in PBMC cultures inhibited by 9.6

To further confirm that the ability of IL2 preparations to abrogate inhibition mediated by p50-binding monoclonal antibodies was attributable to IL2, we attempted to block the restoring ability of MLA-144-derived supernatants with the human IL2 receptor-specific antibody, anti-Tac (2). Table II shows the results from three independent experiments in which the 9.6

antibody was used to inhibit PHA-induced proliferation in PBMC cultures. As before, 9.6 suppressed [^{3}H]-thymidine incorporation in these PHA-stimulated cultures and the inclusion of MLA-144-derived supernatants (25% vol:vol) restored proliferation to normal levels. When the anti-Tac monoclonal antibody was added, MLA-144-derived supernatants failed to restore proliferation in inhibited cultures. Comparable results were obtained with JURKAT affinity purified IL2 (data not shown). These results are consistent with the idea that IL2 is the active molecule restoring proliferation in these cultures.

Kinetics of inhibition of lymphocyte proliferation by antibody 9.6 is dependent on the kinetics of IL2 production and proliferation induced by antigen, alloantigen, and mitogen

T-lymphocytes induced by lectin mitogen PHA reach maximum levels of proliferation at an earlier time than T-lymphocytes stimulated by alloantigen or soluble antigen. This hierarchy in proliferation kinetics has been shown to reflect the frequency of responding cells (18) and the magnitude of IL2 production induced by these ligands (2).

To determine the kinetics of inhibition of lymphocyte proliferation by 9.6 antibody, 9.6 was added to PHA, PPD, and MLC cultures at different times after culture initiation, and the proliferative response was measured by [^{3}H]TdR incorporation at the end of each culture. Figure 5 shows results wherein the addition of antibody 9.6 at 0, 4, 18, or 24 hr after initiation of cultures inhibited proliferation induced by suboptimal concentrations of PHA (PHA-M, 5 ug/ml) by 70 to 51%. Figure 6 demonstrates that 9.6 was able to suppress PPD cultures (79%) even when the antibody was added 72 hr after induction. In Figure 7, adding 9.6 at 0-72 hr after initiation of MLC produced 86-42% suppression, respectively. It is evident from these results that the

slower the kinetics of proliferation of the inducing ligand, the later 9.6 could be added to the culture to produce a similar inhibitory effect.

Inhibition of lymphocyte proliferation by antibody 9.6 is dependent on mitogen concentration

To determine whether the ability of 9.6 to inhibit lymphocyte proliferation is dependent on the concentration of the mitogens PHA or TPA, we performed a dose response analysis of lymphocyte proliferation with and without 9.6. Data from a representative experiment are shown in Table III. The degree of inhibition of lymphocyte proliferation by 9.6 was inversely related to the concentration of PHA or of TPA. Antibody 9.6 inhibited lymphocyte proliferation induced by a wide range of PHA concentrations (PHA-M, 2.5-50 ug/ml) by 68-100%. When a supraoptimal concentration of PHA ($\geq$100 ug/ml) was used a decrease in proliferation was observed, and adding 9.6 to these cultures resulted in an enhancement (36%) of proliferation.

Antibody 9.6 inhibited lymphocyte proliferation (98.8%-45%) induced by a rather narrow range of TPA concentrations (0.25-1 ng/ml) that produced a steady increase in the proliferative response. When TPA was added in concentrations equal to or higher than 2 ng/ml, 9.6 did not significantly inhibit lymphocyte proliferation.

Maximal lymphocyte proliferation induced by the synergistic combination of PHA and TPA is inhibited by antibody 9.6 only when low PHA concentrations are used

The phorbol ester TPA has been shown to act synergistically with PHA to induce lymphocyte proliferation. This effect has been attributed to the ability of TPA, when added to cultures containing suboptimal concentrations of PHA, to increase IL2 elaboration to levels equal to or higher than that obtained with optimal concentrations of PHA alone (19,20). To determine whether 9.6 could inhibit lymphocyte proliferation induced by PHA plus TPA,

this antibody was added to PBMC cultures containing suboptimal concentration of PHA (5 ug/ml PHA-M) with increasing amounts of TPA. As shown in Table IV, adding TPA (0.25-0.5 ng/ml) to these PHA cultures induced a lymphocyte proliferative response greater than that generated by cells stimulated with 5 or 25 ug/ml of PHA alone. The peak of lymphocyte proliferation was reached when 1 ng/ml of TPA was added to suboptimally stimulated (5 ug/ml PHA-M) cells. Adding antibody 9.6 to each of these cultures produced a pronounced inhibition of lymphocyte proliferation (98.4-44.0%). The degree of inhibition was inversely related to the amount of TPA added to the cultures.

In contrast, 9.6 did not inhibit lymphocyte proliferation generated by adding 0.5 ng/ml or higher concentrations of TPA to lymphocytes stimulated with relatively high concentrations of PHA (25 ug/ml PHA-M). These results indicate that under conditions that generated maximal lymphocyte proliferation, antibody 9.6 was able to produce nearly complete inhibition of [^{3}H]TdR uptake only when low concentrations of PHA were used. These data suggest a competitive relationship between the signals generated by PHA and those produced by antibody-triggered p50 molecules, and it appears that the extent of T-cell activation is dependent on the balance between these two signals.

DISCUSSION

The present study demonstrates that monoclonal antibody 9.6, which recognizes a 50 kd surface protein associated with the E-receptor, inhibits T-cell proliferation by interfering with the elaboration of IL2 by lymphocytes stimulated with PHA, with PHA plus TPA, or with alloantigens. A time course analysis of IL2 elaboration in MLC showed that the inhibitory effect of 9.6 was maintained throughout the 6-day culture period, suggesting that in this system the concentration of IL2 is the limiting factor.

To test this hypothesis further, lectin-free preparations of IL2, including JURKAT-derived IL2 purified to homogeneity, were used in an effort to restore lymphocyte proliferation inhibited by anti-p50 antibodies (9.6). Data from these experiments (see Figures 3 and 4) demonstrated that all IL2 preparations (used at comparable concentrations) consistently restored lymphocyte proliferation toward normal in cultures inhibited by 9.6. Although a dose dependent restoration was observed, it should be noted that these IL2 preparations typically restored proliferation of 9.6-inhibited PHA cultures to levels equal to or greater than that obtained in PBMC cultures stimulated with PHA alone, but somewhat less than that measured in cultures stimulated with the combination of PHA and increasing concentrations of IL2 (2-70 U/ml).

The incomplete restoration of lymphocyte proliferation by IL2 in these experiments raises the possibility that anti-p50 antibodies also may inhibit the elaboration of other lymphokines that act synergistically with IL2. Experiments performed with the anti-Tac monoclonal antibody directed to IL2 receptors showed that when added at the initiation of cultures, anti-Tac antibody completely blocked the restorative effect of IL2 (see Table II). Thus, antibody 9.6 appears to inhibit lymphocyte proliferation primarily by interfering with IL2 accumulation.

The data reported here also demonstrated that when lymphocytes were maximally induced with a combination of PHA plus TPA, 9.6 inhibited IL2 production and lymphocyte proliferation but did not inhibit the acquisition of IL2 receptors. In a separate study (Reed et al.*) we have shown that 9.6 diminishes the expression of IL2 receptors induced by PHA but not that induced by TPA. This inhibitory effect of 9.6 on IL2 receptor expression, however, was reversible by adding purified IL2. The ability of 9.6 to selectively inhibit IL2 production but not IL2 receptor expression on lymphocytes induced in the presence of TPA suggests that this mitogen induces IL2 receptor expression by a mechanism distinct from that of PHA.

Antibody 9.6 inhibited lymphocyte proliferation in PHA, MLC, and PPD cultures to a comparable level when added 24, 48, and 72 hr respectively after the initiation of these cultures. This kinetics of inhibition appears to depend on the kinetics of proliferation caused by the inducing ligand. Previous studies have shown that the kinetics of lymphocyte proliferation in PHA and PPD cultures depends on the the initial number of responding cells (18). Thus in PPD cultures the delayed maximum proliferation was shown to result from multiple divisions of a few responding cells rather than from a delayed initial response (18). In contrast, in PHA cultures a higher number of initial responding cells led to more rapid kinetics of proliferation. More recently, Depper et al. (2) have shown that the kinetics of IL2 production follows a similar pattern. It was shown that in PHA cultures the accumulation of IL2 was detectable on day 1, whereas in cultures stimulated with soluble antigens the IL2 was detectable only after 72 hr of culture. Taken together, these findings indicate that the kinetics of inhibition by antibody 9.6 can be explained by several but not mutually exclusive alternatives: 1) 9.6 may prevent IL2 concentrations from reaching the critical level necessary to drive

cells into S-phase of cell cycle (21); 2) 9.6 may be able to inhibit activation events after each round of cell division as may occur in antigen stimulated cultures. Thus 9.6 may prevent maintenance of IL2 levels required for subsequent rounds of cell division; 3) the kinetics of inhibition by 9.6 may be attributable to the asynchronous entry of T-cells into cell cycle, as has been shown by Cantrell et al. (21).

Our data on the kinetics of inhibition by 9.6 suggest that this antibody does not interfere with ligand binding or antigen recognition by T cells. Moreover, Fab fragment preparations of 9.6 caused pronounced inhibition of lymphocyte proliferation of PHA and MLC cultures (data not shown), indicating that the effect of 9.6 was not secondary to agglutination of cells.

In an attempt to understand the relationship between signal(s) induced by binding of antibody 9.6 to p50 molecules and the activation signal(s) induced by mitogens, we studied the effect of 9.6 (used above saturation levels) on proliferation induced by various concentrations of two different mitogens, PHA and TPA. The results of this study (see Table III) showed an inverse correlation between the degree of inhibition by antibody 9.6 and the amount of cellular proliferation induced by PHA or TPA in the absence of the antibody. Antibody 9.6 did not inhibit proliferation induced by optimal doses of PHA (50 ug/ml PHA-M). In contrast, this antibody strongly inhibited lymphocyte proliferation and IL2 production induced by a synergistic combination of low concentrations of PHA plus TPA (which produced maximal levels of proliferation).

Taken together, these data suggest that the ability of 9.6 to inhibit lymphocyte proliferation appears to be directly related to the nature and the amount of mitogen used to trigger this response, rather than to the magnitude of IL2 produced. Accordingly, the inverse relationship between the

concentration of the mitogen and the degree of inhibition by 9.6 could be attributable to competition between the molecular signal(s) induced by PHA and those triggered by binding of 9.6 to p50. In support of this hypothesis, we have observed that 9.6 consistently induced an enhancement of lymphocyte proliferation in response to supraoptimal concentrations of mitogens that generated a lymphocyte response below maximal levels. It is likely that this enhancement is secondary to a competition between signals produced by PHA and those induced by antibody-triggered p50 which would lead to a restoration of PHA signals to an optimal level. The exact nature and site of competition remain to be defined.

It has recently been argued that the activation of cellular responses via kinase C pathway is separate from and synergistic to those activated via an increase in intracellular Ca^{2+} (22,23). TPA has a diacylglycerol-like structure and therefore can directly activate kinase C without detectable cellular mobilization of Ca^{2+} (23). Thus, the ability of antibody 9.6 to inhibit lymphocyte activation induced by TPA and/or PHA suggests that this antibody interferes with biochemical events that are common to both of these pathways, namely kinase C activation and Ca^{2+} mobilization. Whether these events are occurring at the membranel level or at a distal site in the activation cascade remains to be determined. Our finding that 9.6 inhibits lymphocyte proliferation induced by TPA conflicts with previous findings by Palacios et al. (10), who reported that OKT11A inhibited lymphocyte proliferation induced by the Ca^{2+} ionophore A23187 but not by TPA. Thus, the proposed mechanism that the OKT11A inhibits lymphocyte response by interfering specifically with Ca^{2+} influx after ligand stimulation remains to be demonstrated.

The interpretation we offer, that the 50 kd protein regulates lymphocyte proliferation through the IL2 pathway of T cell activation, is strengthened by the observation of Meuer et al. (24) showing that culturing T cells with a combination of two monoclonal antibodies (designated $T11_2$ and $T11_3$) that recognize two unique determinants on the p50 molecule leads to T-cell activation with subsequent IL2 receptor expression, IL2 production, and lymphocyte proliferation in the absence of antigen or antigen-presenting cells. Since the $T11_3$ epitope was shown by these investigators to be expressed only after T-cell activation, it may be that the $T11_3$ antibody detects a neo-epitope on the p50 molecule which represents an "active" site necessary for T-cells to proceed to subsequent steps of activation that lead ultimately to the elaboration of IL2. In contrast, the epitopes associated with the (E-) receptor binding sites such as those defined by 9.6, OKT11A, and LFA2 may represent "allosteric" sites that, when triggered by antibody or by a natural ligand, can inhibit the expression and/or function of active p50 sites such as $T11_3$.

The immunologic relevance of the present study resides in the understanding it provides regarding the mechanism whereby the 50 kd protein controls the magnitude of mitogen and antigen induced T-cell activation through an IL2-dependent pathway. Reports of serum factors occurring in patients with various diseases which block E-rosette formation and lymphocyte responses to mitogens (25-28) argue that this "allosteric site" may be of physiologic importance. Though the exact biochemical events mediated by the p50 molecule remain unknown, it is likely that p50 regulates, or is part of, a common pathway of T cell activation triggered by lectin mitogens, by phorbol esters, and by specific antigens.

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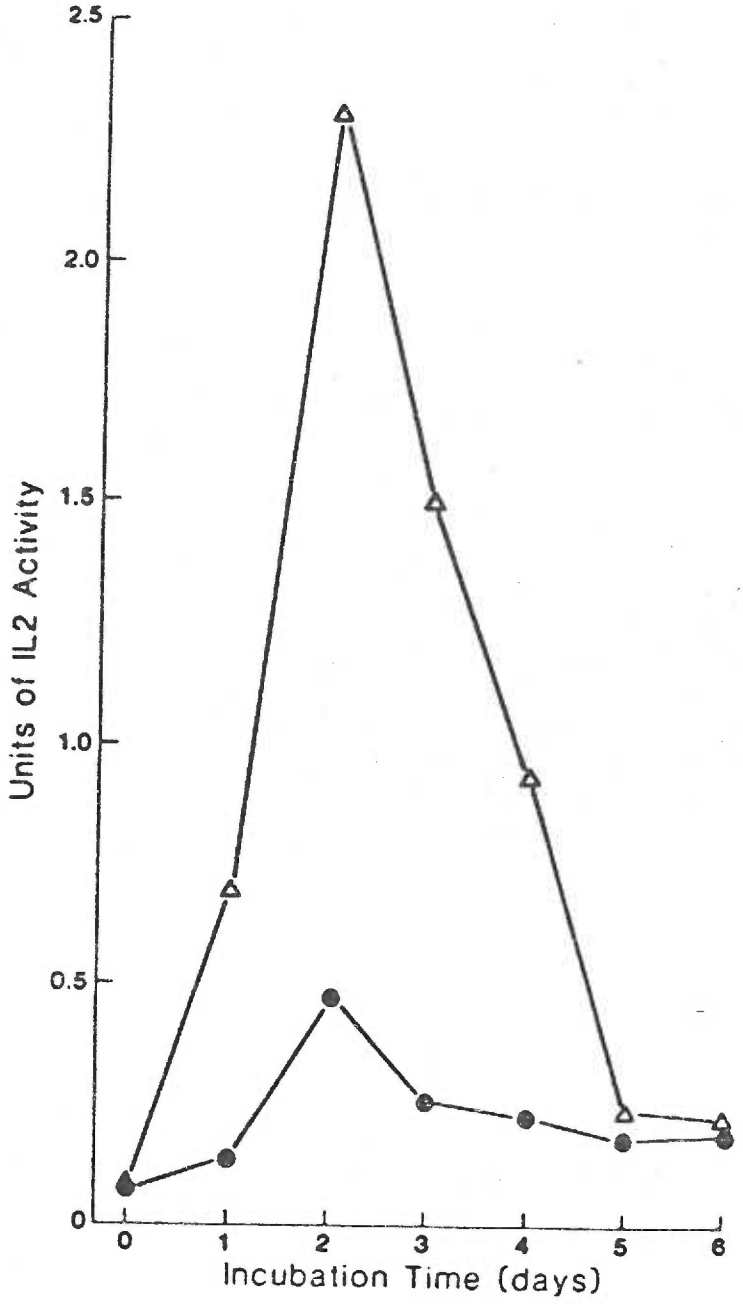

FIGURE 1

Time course of IL2 elaboration in MLC in the presence of 9.6. 10^6 T cells (purified by E-rosetting) were incubated with an equal number of irradiated allogenic PBMC in 1 ml medium containing 10% heat-inactivated human AB serum with (●) or without (△) 9.6 added at the initiation of the culture. Supernatants were collected at the indicated days, and IL2 activity is determined and calculated (U/ml) as described in Methods section.

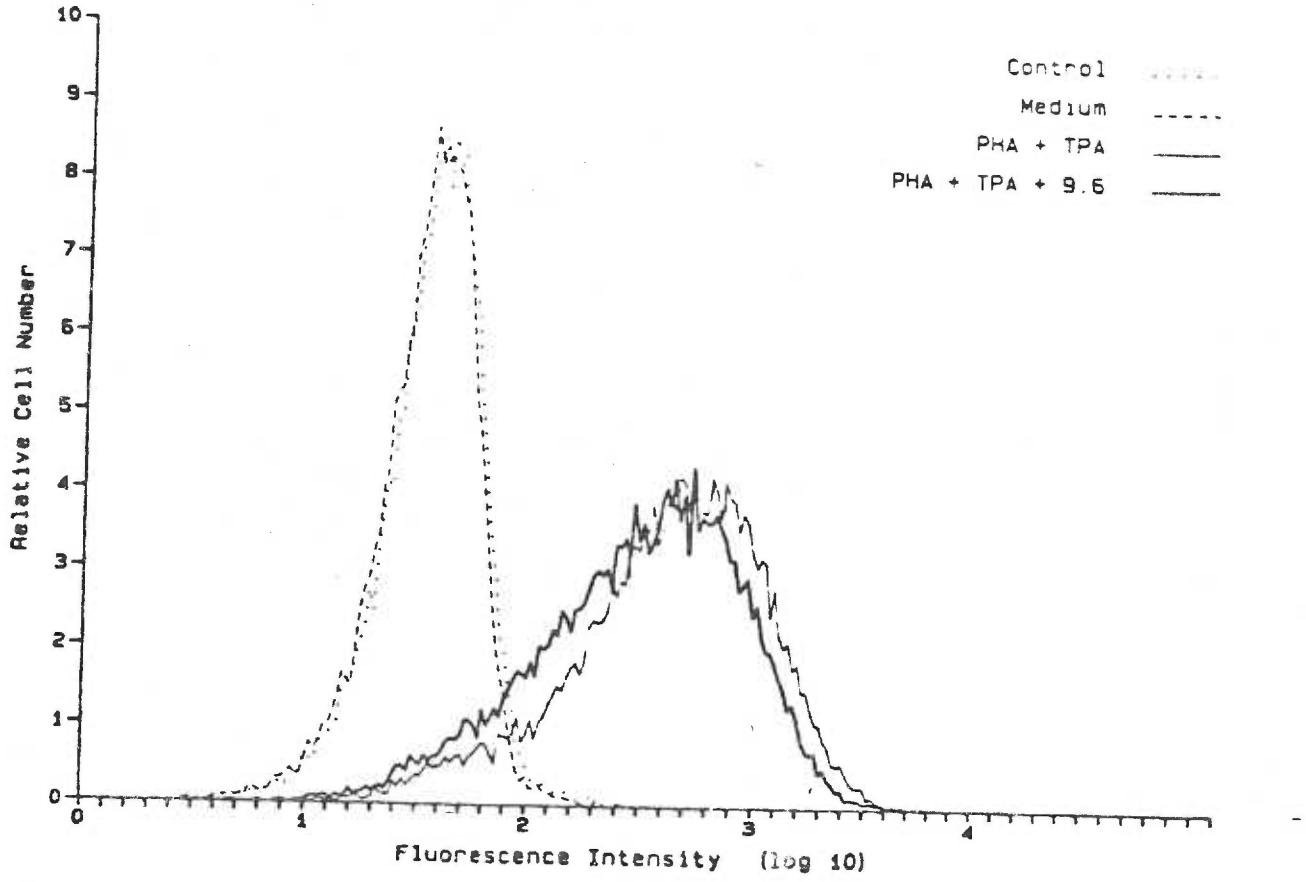

Figure 2

Antibody 9.6 does not inhibit acquisition of IL2 receptors induced by a combination of PHA plus TPA. PBMC were cultured for 24 hr in medium alone or in medium containing PHA-M (5 ug/ml) plus TPA (0.5 ng/ml). Cells were then labeled with biotin-conjugated anti-Tac antibody in an indirect immunofluorescence assay employing FITC-avidin and analyzed by FACS. Nonspecific binding was determined with an irrelevant control antibody. Data are reported as relative cell number (ordinate) vs. fluorescence intensity in $\log_{10}$ units (abscissa) for $\geq 10^4$ cells analyzed.

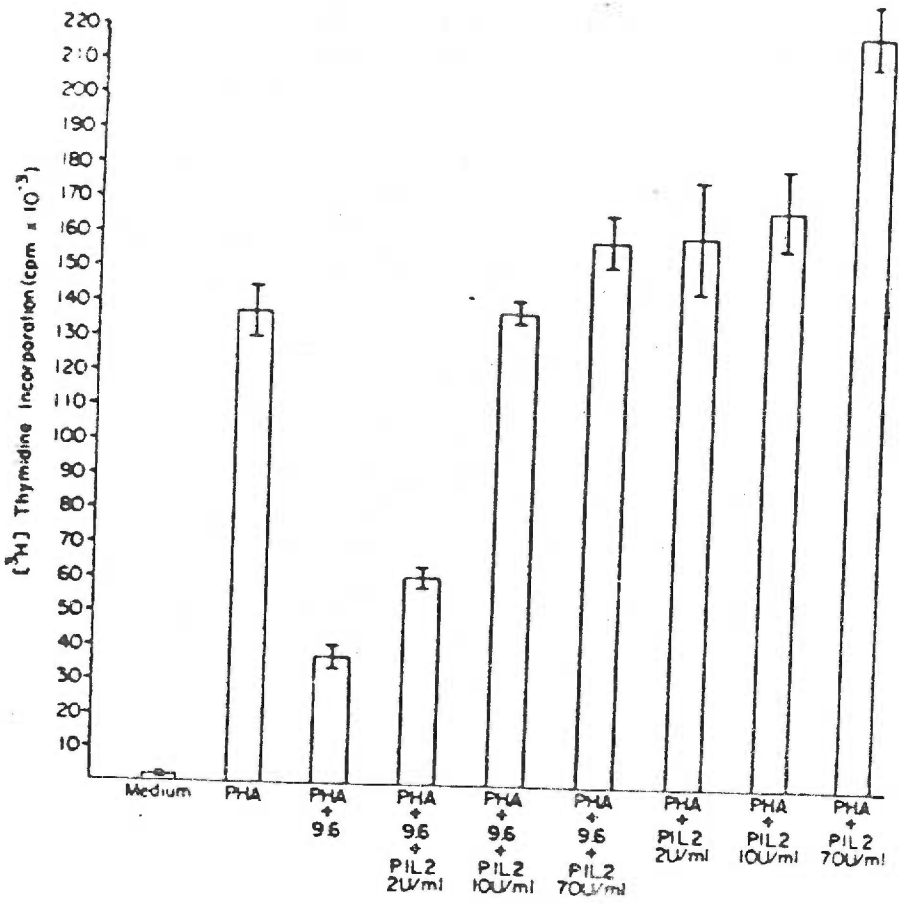

Figure 3

Purified IL2 overcomes 9.6-mediated inhibition of lymphocyte proliferation induced by PHA in a dose-dependent fashion. $2x10^5$ PBMC were cultured for 72 hr in medium with PHA-M (5 ug/ml). Immunoaffinity purified JURKAT-derived IL2 (P.IL2), 2-70 U/ml, was added to various cultures as shown. Data are $[^3H]$-thymidine incorporation (mean cpm ± standard deviation of triplicate cultures) during the last 8 hr of culture.

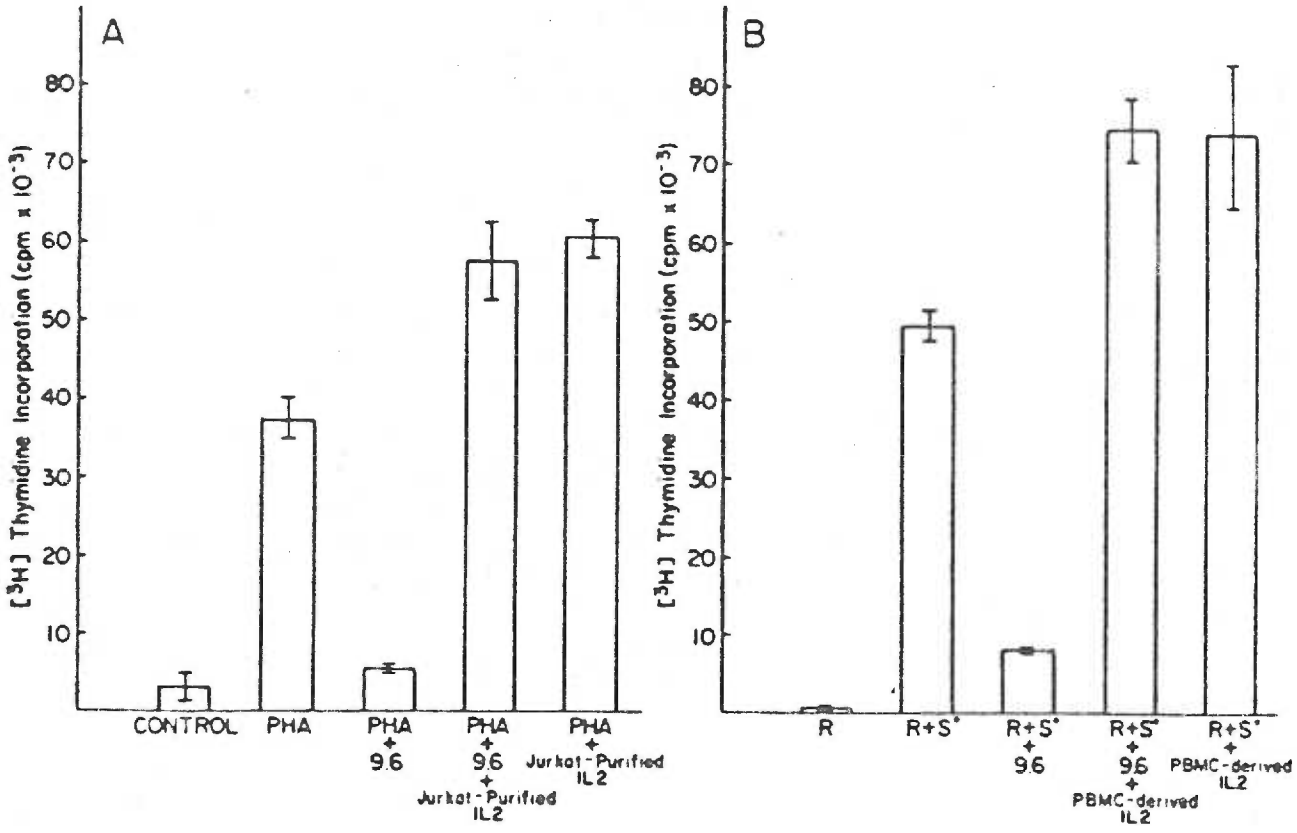

Figure 4.

Purified IL2 overcomes 9.6-mediated inhibition of lymphocyte proliferation induced by PHA or alloantigens. (A) PBMC ($2x10^5$) cultured for 72 hr in medium with PHA-M (5 ug/ml), immunoaffinity purified JURKAT-derived IL2 (10 U/ml), or various combinations of these reagents as shown. Data are [^{3}H]-thymidine incorporation (mean cpm ± standard deviation of triplicate cultures) during the last 8 hr of culture. (B) $2x10^5$ E-rosette purified responder T cells (R) were cultured with $2x10^5$ irradiated allogenic PBMC (S*) in 200 ul medium with 9.6, IL2 (partially purified PBMC-derived, 10 U/ml), or various combinations of these reagents, as shown, added at the onset of culture. Data are [^{3}H]-thymidine incorporation (mean cpm ± standard deviation of triplicate) during the last 16 hr of 6-day culture.

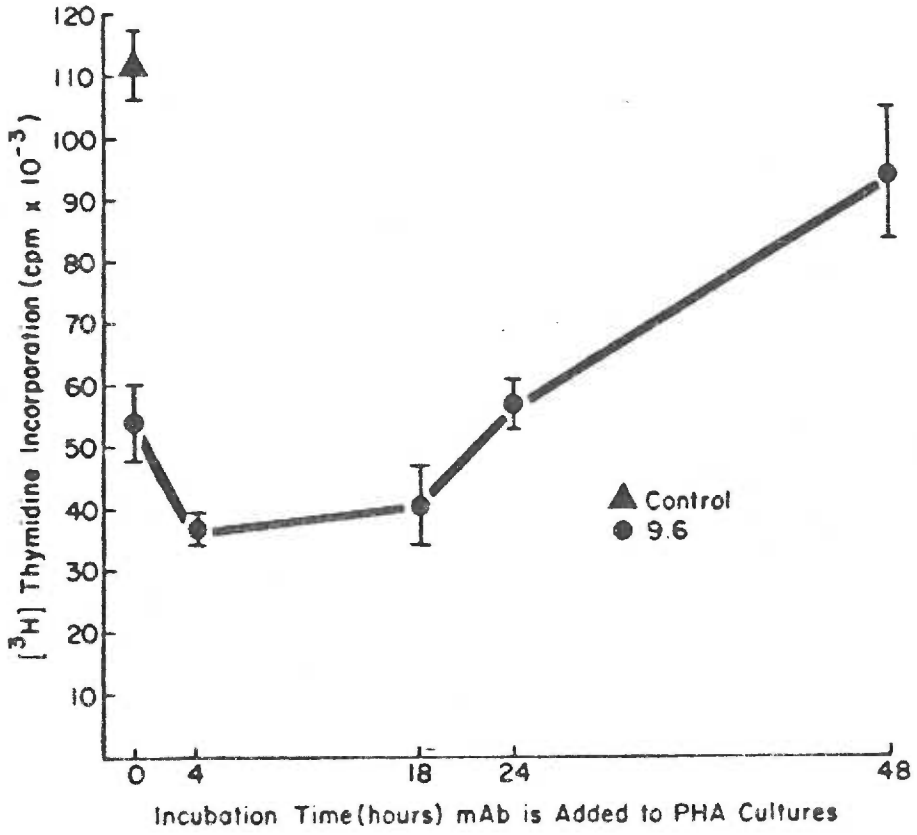

Figure 5

Kinetics of inhibition of mitogen-induced lymphocyte proliferation by monoclonal (mAb) antibody 9.6. PBMC ($2x10^5$) were incubated in medium with PHA-M (50 ug/ml), with (●) or without (▲) 9.6 added at the indicated time. Data are [^{3}H]-thymidine incorporation (mean cpm ± SD of triplicate cultures) during the last 8 hr of 72 hr cultures.

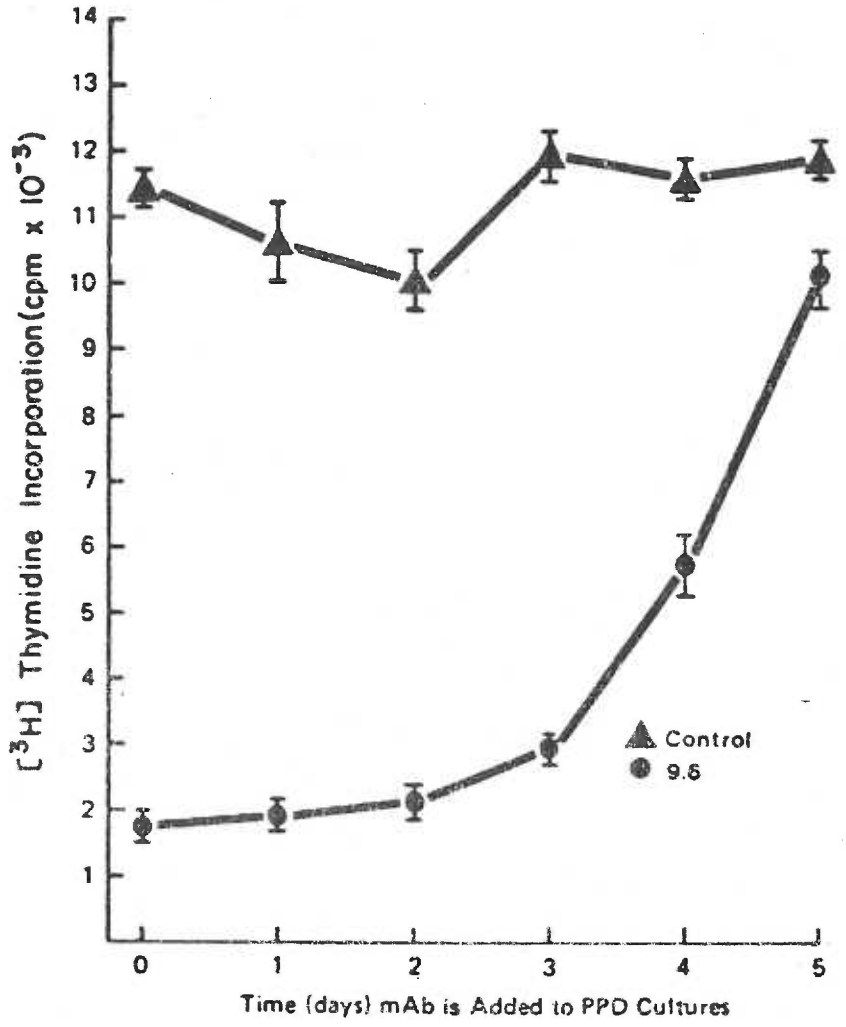

Figure 6

Kinetics of inhibition of antigen-induced (PPD) T cell proliferation by monoclonal antibody (mAb) 9.6. $5x10^5$ nylon wool purified T cells were cultured with protein purified derivative (PPD) (5 ug/ml) with (●) or without (▲) 9.6 added at the indicated time. Data are $[^3H]$-thymidine incorporation (mean cpm ± SD of triplicate cultures) during the last 16 hr of 6-day cultures.

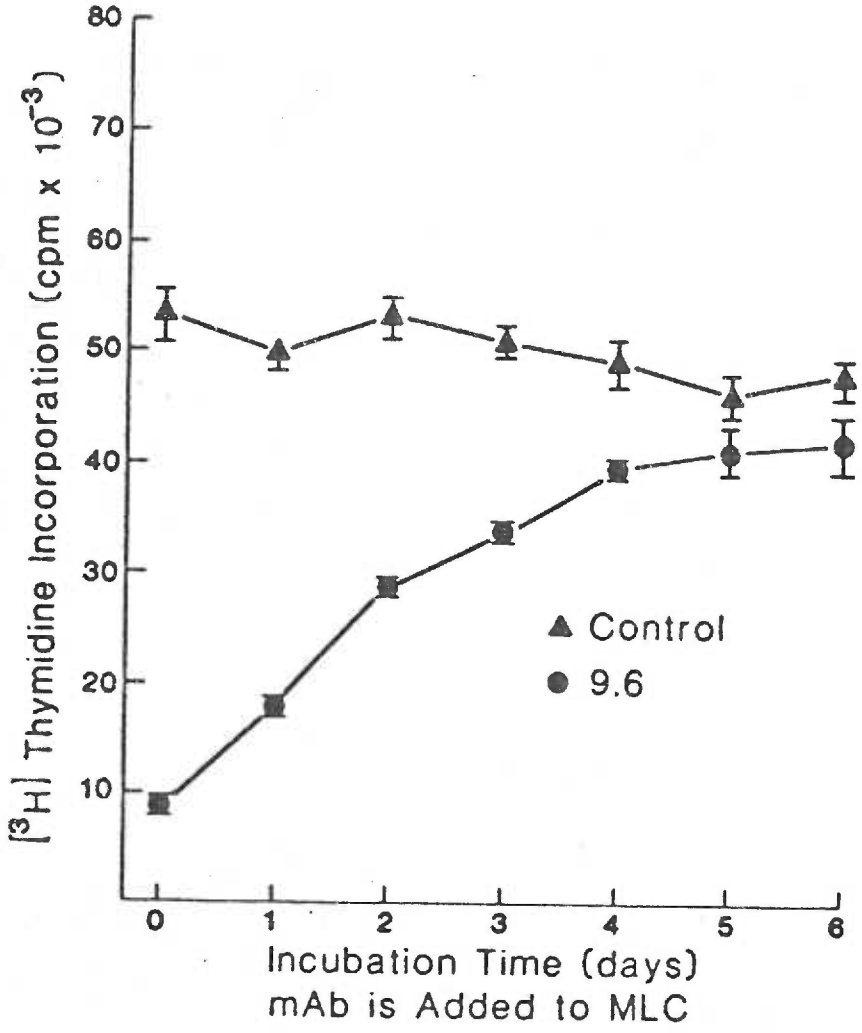

Figure 7

Kinetics of inhibition of alloantigen induced lymphocyte proliferation by monoclonal antibody (mAb) 9.6. $2x10^5$ T cells (purified by E-rosetting) were cultured with $2x10^5$ irradiated allogenic PBMC in medium. On the indicated days 9.6 (●) or control antibody 12.1 (▲) was added. Data are presented as $[^3H]$-thymdine incorporation (mean cpm ± SD of triplicate culture) during the last 16 hr of 6-day cultures.

TABLE I

9.6 inhibits IL2 production by PBMC induced by mitogen

Supernatant source*		
Mitogens	9.6 antibody added	IL2 activity (U/mL)
PHA-P (PBMC)	---	3.7
	+	0.20
PHA-M (PBMC)	---	<.1
	+	<.1
PHA-M + TPA (PBMC)	---	15.2
	+	0.13

* $4x10^6$/ml PBMC were cultured with 0.2% PHA-P (Difco) or PHA-M (5ug/ml), TPA (1 ng/ml), 9.6 antibody, or various combinations of these reagents. Supernatants were harvested 24 hr later and assayed for IL2 activity.

TABLE II

Anti-Tac antibody abrogates the ability of IL2-containing supernatants to restore lymphocyte proliferation inhibited by 9.6

Culture conditions*				^{3}H-thymdine incorporation†		
PHA	9.6	IL2	Tac	Expt. 1	Expt. 2	Expt. 3
-	-	-	-	439 ± 49	2495 ± 1054	3173 ± 1987
+	-	-	-	122661 ± 26246	67780 ± 2333	37261 ± 2883
+	+	-	-	50014 ± 2692	5888 ± 2454	5631 ± 498
+	+	+	-	92500 ± 6531	90032 ± 7657	33766 ± 4825
+	+	+	+	23400 ± 5088	15978 ± 1745	4114 ± 1204
+	-	+	+	94431 ± 13018	77643 ± 1928	18934 ± 900
+	-	-	+	57339 ± 4680	n.d.††	14866 ± 1249
-	-	+	-	23717 ± 6452	n.d.	6452 ± 646
-	-	+	+	1261 ± 322	n.d.	n.d.

* PBMC (10^6/ml) in medium with PHA-M (10 ug/ml), 9.6 antibody (5.0 ug/ml), IL2-containing MLA144 supernatants (25% vol:vol), anti-Tac antibody (0.85 ng/ml), or various combinations of these reagents were incubated for 72 hr.

† Data represent [^{3}H]-thymidine incorporation (mean cpm of triplicate cultures ± standard deviation) during the last 8 hr of culture.

†† n.d. = not done.

TABLE III

Inhibition of lymphocyte proliferation by 9.6
is dependent on the mitogen concentration

Culture conditions[a]		(^{3}H)-TdR (mean cpm) ± S.D.[b]		% inhibition[c]
PHA-M (ug/ml)		Control	Antibody 9.6	
	0.0	(974 ± 369)	1056 ± 237	-0.0
	2.50	3258 ± 811	928 ± 276	-100
	5.0	19023 ± 3032	1314 ± 450	-98.2
	10	48694 ± 4007	10962 ± 1449	-79.1
	25	125876 ± 1653	37243 ± 632	-71.0
	50	164067 ± 30729	52197 ± 9138	-68.2
	100	117157 ± 24480	159589 ± 14333	+36.0
TPA (ng/ml)	.25	3230 ± 451	1284 ± 589	-98.8
	.5	15544 ± 1874	3840 ± 2615	-62.8
	1.0	40434 ± 1511	22413 ± 447	-45.7
	2.0	50758 ± 5742	40907 ± 1989	-19.8
	4.0	54246 ± 7416	51265 ± 3214	-5.6
	10	63112 ± 4750	62280 ± 8414	-1.4
	25	73970 ± 3225	74888 ± 3878	-0.0
	50	75823 ± 1045	74767 ± 2719	-1.5

[a]PBMC ($1x10^6$ cells/ml) were cultured with increasing amounts of PHA-M (Difco) with or without antibody 9.6 in a microtiter culture plate for 72 hr.

[b]Data represent [^{3}H]-thymidine incorporation (mean cpm of triplicate cultures ± standard deviation) during the last 8 hr of culture.

[c]Data are calculated as described in Methods; (-) indicates inhibition, (+) indicates enhancement.

TABLE IV

Maximal lymphocyte proliferation induced by the synergistic effect of PHA and TPA is inhibited by antibody 9.6 only when appropriate concentration of PHA is used.

Culture conditions[a]		(^{3}H)-TdR (mean cpm) ± S.D.)[b]		% inhibition[c]
PHA 5 ug/ml		Control[a]	Antibody 9.6	
+ TPA (ng/ml)	0.0	19023 ± 3032	1314 ± 450	-98.2
	0.25	130943 ± 8273	3136 ± 910	-98.4
	0.5	197684 ± 7294	25739 ± 4413	-87.5
	1.0	201600 ± 7780	61602 ± 1103	-69.7
	2.0	201200 ± 1200	90508 ± 7731	-63.3
	4.0	179192 ± 8767	82300 ± 9030	-64.4
	10	183312 ± 3074	79450 ± 1699	-57.0
	25	152545 ± 18119	86016 ± 8170	-44.0
	50	205916 ± 5460	99793 ± 3669	-51.8
PHA 25 ug/ml				
+ TPA (ng/ml)	0.0	125876 ± 1653	37243 ± 632	-71.0
	0.25	171665 ± 2544	124330 ± 21171	-27.5
	0.5	143550 ± 5715	165400 ± 16418	+15
	1.0	159797 ± 3775	181166 ± 21326	+13

[a]PBMC ($1x10^6$/ml) were cultured with PHA-M (either 5 ug or 25 ug) and various amounts of TPA, as shown. Antibody 9.6 was added in ascites form at 1:100 final dilution to parallel cultures.

[b]Data are [^{3}H]-TdR incorporation during the last 8 hr of 72 hr of culture (mean ± S.D. of triplicate cultures).

[c]Data are calculated as described in Methods (-) indicates inhibition, (+) indicates enhancement.

Paper 2

Suppression of Interleukin 2 Receptor Acquisition by Monoclonal Antibodies Recognizing the 50-kd Protein Associated with the Sheep Erythrocyte Receptor on Human T-Lymphocytes[a]

Suppression of Interleukin 2 Receptor Acquisition by Monoclonal Antibodies Recognizing the 50-kd Protein Associated with the Sheep Erythrocyte Receptor on Human T-Lymphocytes[a]

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Footnotes

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[c]To whom requests for reprints may be addressed.

Abbreviations used in this paper

PHA	phytohemagglutinin
TPA	12-O-tetradecanoyl-phorbol-13-acetate
IL1	interleukin 1
IL2	interleukin 2
PBMC	peripheral blood mononuclear cells
MEM	minimal essential medium
HBSS	Hank's balanced salt solution
PBS	phosphate buffered saline
U/ml	half-maximal units per milliliter
ΔFL	relative mean fluorescence intensity

ABSTRACT

Monoclonal antibodies OKT11A, 9.6., and 35.1 recognize epitopes on a 50000-dalton surface molecule (p50) identical to or closely associated with the sheep erythrocyte receptor (E-receptor) on human T-lymphocytes. These three antibodies were investigated for ability to inhibit T-cell proliferation and interleukin 2 (IL2) receptor acquisition (determined with anti-Tac antibody in an immunofluorescence assay) induced by the lectin mitogen phytohemagglutinin (PHA) or by the phorbol ester 12-0-tetradecanoyl-phorbol-13 acetate (TPA). OKT11A, 9.6., and 35.1 were found to suppress [^{3}H]-thymidine incorporation and IL2 receptor acquisition stimulated by PHA but not by TPA. This inhibition was not attributable to a lag in kinetics but was sustained throughout 4-5 days of culture.

Because OKT11A and 9.6 have been reported to suppress lectin mitogen-induced IL2 production, we attempted to overcome inhibition of proliferation with exogenous IL2 (MLA144 supernatants or immunoaffinity purified human IL2). Adding IL2 at the initiation of culture abrogated the suppressive effect of all three anti-p50 antibodies on proliferation and on the acquisition of IL2 receptors, raising the possibility that IL2 may up-regulate expression of its cellular receptor on human T-lymphocytes.

These data, together with previous reports, indicate that OKT11A, 9.6, and 35.1 suppress lectin mitogen induced T-cell proliferation by impairing both IL2 elaboration and IL2 receptor acquisition, and suggest that IL2 may be capable, at least under some conditions, of increasing expression of IL2 receptors on human T-lymphocytes.

INTRODUCTION

Since the discovery that human lymphocytes of thymic origin form spontaneous rosettes with sheep red blood cells (1,2,3),the sheep erythrocyte receptor (E-receptor) has remained an enigma. The finding that mature T-lymphocytes stimulated with interleukin 1 (4), alloantigens (5), or lectin mitogens (6-8) form stable E-rosettes at 37°C and express 3-6 times more E-receptors per cell than do unstimulated cells (9,10) has suggested that this cell-surface molecule might be involved in T-cell "activation" events (4-11).

Recently, monoclonal antibodies recognizing a 50000-dalton polypeptide molecule (p50) identical to or closely associated with the E-receptor have been developed by several independent groups of investigators (12-17). Many of these anti-p50 antibodies (OKT11A, 9.6, 35.1, LFA-2) have been shown to inhibit T-lymphocyte proliferation in an antigen-nonspecific fashion (16-18), supporting the notion that the E-receptor is important for T-cell responses. At least two of these antibodies, 9.6 and 35.1, recognize distinct epitopes on the E-receptor (16).

Attempts to elucidate the suppressive mechanism(s) of anti-p50 antibodies have revealed that OKT11A and 9.6 inhibit the proliferation of T-lymphocytes induced by the lectin mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) but not by the phorbol ester TPA (18,19). In addition, these anti-p50 antibodies suppress the elaboration of interleukin 2 (IL2) stimulated by lectin mitogens (19 and unpublished observations). The effect of these monoclonal antibodies on TPA-induced IL2 production has not been reported.

In contrast to the inhibitory action of OKT11A and 9.6 on IL2 production, the effect of anti-p50 antibodies on the expression of receptors for IL2 remains poorly defined. Palacios and Martinez-Maza (19) have suggested that OKT11A may inhibit the acquisition of IL2 receptors on PHA-stimulated human

T-cells, but these studies relied on the indirect method of measuring responsivity to IL2 by proliferation. We therefore employed the anti-Tac monoclonal antibody, which recognizes the IL2 receptor on activated human T-lymphocytes (20,24), to determine directly the effects of three anti-p50 monoclonal antibodies (OKT11A, 9.6, and 35.1) on the PHA-induced acquisition of IL2 receptors by human T-cells.

MATERIALS AND METHODS

Isolation of cells

Human peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized venous blood obtained from healthy volunteers (25). After washing 3 times in Hank's balanced salt solution (HBSS), PBMC were resuspended in minimal essential medium (MEM) containing penicillin (50 U/ml), streptomycin (100 ug/ml), L-glutamine (2.0 mM), and 10% (vol:vol) heat-inactivated, pooled human sera (hereinafter designated complete medium). Viability of isolated PBMC was routinely 99% as determined by trypan blue dye exclusion.

Preparation of antibodies for cultures

OKT11A (IgG2a), obtained from Ortho Diagnostics (13), was dialyzed against Dulbecco's phosphate buffered saline (Gibco) overnight at 4°C to remove Sodium Azide and sterilized by Millipore filtration (0.20 um; Nalgene). Antibody 9.6 (IgG2b) was purified from ascites by column chromatography with protein A-Sepharose (Pharmacia) and adjusted to 5 mg/ml in MEM (12). Antibody 12.1 (IgG2a), which recognizes a surface molecule distinct from p50 present on all human T-cells (26), used here as a control, and antibody 35.1 (IgG2a) were used in ascites form (16). Purified anti-Tac antibody (165 ng/ml) in phosphate buffered saline was a gift from Warner C. Greene (NIH, Bethesda, Maryland).

Sources of interleukin 2

Supernatants containing IL2 activity were derived from the MLA144 gibbon ape cell line as described by Rabin et al. (27). Briefly, 10^7 MLA144 cells in 25 ml of complete medium were cultured for 3 days at 37°C in 5% CO_2 and air. Supernatants of these cultures were recovered following centrifugation (400xG) for 10 min, filtered through sterile 0.20 um Millipore filters (Nalgene), and

stored at -20°C. Prior to use, these supernatants were thawed, ultracentrifuged at 100,000xG for 2 hr to remove GaLV (gibbon ape leukemia virus), and refiltered.

Immunoaffinity-purified human IL2 was a gift of Richard J. Robb (Glenolden, Pa.) and was prepared from the JURKAT cell line following stimulation with PHA and TPA as described previously (28). This material is homogeneous as determined by two-dimensional gel analysis, reverse-phase HPLC, and N-terminal amino acid sequencing, and is free of mitogens based on radiolabeled tracer analysis (29).

IL2 preparations derived from MLA144 and from JURKAT were tested for activity in a bioassay employing the CTLL-15H cell line (30). One U/ml of IL2 activity corresponded to the reciprocal of the dilution that produced half-maximal proliferation of the CTLL-15H cells. Unconcentrated MLA144 supernatants generally contained 25-50 U/ml of IL2 activity.

Lymphocyte proliferation assay

PBMC were resuspended at 10^6 cells/ml in complete medium and 200 ul added to 96-well flat-bottom microtiter plates (Linbro, Costar). PHA-M (Difco) was added to cultures at concentrations that produced just optimal proliferation, generally 20-25 ug/ml. TPA (Chemical Carcinogenesis, Inc.) was prepared as a stock solution in ethanol and used at a final concentration of 10 ng/ml. Monoclonal antibodies added to cultures in excess were 9.6 (500 ng/ml), 35.1 (1:250 dilution),and 12.1 (1:100 dilution). Antibody anti-Tac was added at a final concentration of 0.85 ng/ml, which produced maximal inhibition of the PHA-induced proliferative response. OKT11A was employed at concentrations (150 ng/ml) causing $\geq$50% inhibition. MLA144 supernatants were used at 25% (vol:vol) unless otherwise specified.

Cultures were incubated at 37°C in 5% CO_2 and air for various times. Eight hours before termination of cultures, each well was pulsed with 0.5 uCi of [^{3}H]-thymidine (New England Nuclear; specific activity 6.7 Ci/mmol), harvested onto fiberglass filters using a multichannel automated cell harvester (Brandel, model M12V), and counted in a automated liquid scintillation counter (Intertechnique SL4000). Mean cpm (± standard deviation) of triplicate cultures was determined and the percentage of the control response was calculated by dividing the mean cpm for the test response by the mean cpm obtained for the control response and multiplying by 100.

Biotin conjugation of antibodies

Monoclonal antibodies anti-Tac and R3-367 were conjugated with biotin by the succinimide ester method (31). Briefly, antibodies purified by protein-A affinity chromatography were dialyzed overnight at 4°C against 0.1 M $NaHCO_3$ (pH 8.4) and adjusted to approximately 1.0 mg/ml. Biotin succinimide ester (Biosearch, San Rafael, Ca.) was dissolved in DMSO (Sigma) to 1.0 mg/ml, immediately before use, and 120 ul added per ml of protein, while stirring on a vortex mixer. After standing at room temperature for 4 hr, the reaction mixture was dialyzed at 4°C against phosphate buffered saline containing 0.02% Sodium Azide.

Monoclonal antibody R3-367 (IgG2a), used as a control for nonspecific binding in immunofluorescence assays, is a heteroclytic antibody that recognizes the 3-nitro-4-hydroxyphenyl (NP) hapten (32) and was kindly provided by Carol Cowing (University of Pennsylvania, Philadelphia, Pa.).

Immunofluorescence

For immunofluorescence assays, PBMC were bulk-cultured in 25 cm^2 flasks (Falcon 3013) at 10^6/ml in complete medium with PHA, TPA, MLA144-derived supernatants, anti-p50 antibodies (OKT11A, 9.6, 35.1), control antibody

(12.1), or various combinations of these reagents. Cells were removed from cultures at appropriate times, washed 3 times in HBSS, and resuspended at $5x10^6$/ml in staining buffer: Dulbecco's phosphate buffered saline (pH 7.4) containing 2% bovine serum albumin and 0.02% Sodium Azide. Recovery of cells from these cultures was $\geq$80%, with $\geq$90% viability, as determined by trypan blue dye exclusion.

Aliquots of 200 ul of cells in staining buffer were reacted with saturating amounts of primary monoclonal antibodies (anti-Tac, R3-367, OKT11A or biotin-conjugated anti-Tac, R3-367) for 30 min on ice, washed 3 times in staining buffer, and reacted for an additional 30 min on ice with the appropriate secondary reagent, either fluorescein-conjugated goat anti-mouse IgG antiserum (TAGO) or fluorescein-conjugated avidin (Becton-Dickinson). After 3 washes, stained cells were fixed overnight at 4°C in phosphate buffered saline containing 2.5% paraformaldehyde and stored in the dark. On the day of analysis, fixed cells were washed once, resuspended in 0.5 ml of staining buffer, and filtered through nylon mesh.

In all experiments, lymphocyte [^{3}H]-thymidine incorporation was measured in companion microtiter cultures at 72 hr.

Flow cytometry

Cells stained with various monoclonal antibodies were analyzed using a fluorescence-activated cell sorter (FACS IV, Becton-Dickinson) equipped with a 488 nm argon laser. Dead cells, erythrocytes, platelets, and most macrophages were excluded from the analysis by appropriate selection of forward and 90° light scatter parameters. Data were displayed as histograms, with fluorescence intensity displayed on the X-axis and relative cell number on the Y-axis. The fluorescence signal was log-amplified in four decades with the photomultiplier setting selected so that the autofluorescence histogram of

unstained cells was symmetrically bell-shaped in distribution from the origin. The FACS was standardized daily with fixed avian erythrocytes so that fluorescence intensities obtained on different days could be compared. At least 10^5 cells were analyzed for each sample.

The mean log fluorescence intensity for each sample was determined and converted into linear relative fluorescence units (Δ FL) by the formula: $FL = 10^{(E - C)/D}$, where E is the mean log fluorescence intensity of the experimental antibody sample, C is the mean log fluorescence intensity of the control antibody sample (either unconjugated R3-367 plus FITC-goat anti-mouse IgG antiserum or biotin-conjugated R3-367 plus FITC-avidin), and D is 50 channels per decade.

To determine the percentage of positive cells, a fluorescence threshold was set on the appropriate control antibody histogram so that $\leq 5\%$ of cells surpassed this threshold. The threshold point was then used to determine the percentage of positive cells for the experimental antibody histogram. The percentage of T-cells (OKT11+) staining positively with biotin-conjugated anti-Tac antibody was calculated by the formula:

$$\%\ \text{Tac+ T-cells} = \frac{\%\ (\text{biotin Tac:avidin+}) - \%\ (\text{biotin R3-367 avidin+})}{\%\ (\text{OKT11A:goat anti-mouse+}) - (\text{R3-367:goat anti-mouse+})} \times 100$$

The cellular populations analyzed were generally $\geq 85\%$ OKT11+.

Mitomycin C treatment of PBMC

In some experiments, PBMC were treated with mitomycin C, before culturing, to inhibit cell division (33). PBMC (10^7/ml) in HBSS containing 2.5 ug/ml mitomycin C (Sigma) and 10% heat-inactivated human sera were incubated for 45 min in a 37°C shaker bath. The cells were pelleted by centrifugation at 400xG for 10 min and the supernatant was discarded. PBMC were then washed 3 times in HBSS, with 5 min incubations at room temperature between washes. Mitomycin C-treated PBMC were cultured at 10^6 viable cells/ml in complete medium as described above.

RESULTS

OKT11A, 9.6, and 35.1 antibodies inhibit acquisition of IL2 receptors induced by PHA but not by TPA

We employed the anti-Tac antibody (20-24) in a biotin-avidin immunofluorescence assay to determine the effect of OKT11A, 9.6., and 35.1 on the expression of IL2 receptors induced by PHA. As shown in Figure 1, all three anti-p50 antibodies diminished the PHA-induced acquisition of IL2 receptors. OKT11A, 9.6., 35.1, and 12.1 also inhibited [^{3}H]-thymidine incorporation in companion microtiter cultures by 50%, 94%, 89%, and 0%, respectively, in this particular experiment. Though shown only for PBMC analyzed after 48 hr of culture (Figure 1), similar results were obtained at 24 and 72 hr.

Culturing PBMC with a control antibody, 12.1 (IgG2a), which recognizes a surface molecule present on all human T-lymphocytes (26), caused no decrease in the levels of IL2 receptors (Tac) displayed on PHA-activated cells (see Figure 1D). Furthermore, antibodies OKT11A, 9.6, 35.1, and 12.1 did not interfere with binding of the anti-Tac antibody to T-cells, since staining cells with combinations of anti-Tac and these other antibodies produced an additive increase in the levels of fluorescence measured in this FACS assay (data not shown).

The inhibitory activity of OKT11A, 9.6, and 35.1 antibodies in PHA-stimulated cultures was not attributable to cytotoxicity as determined by trypan blue dye exclusion.

Inhibition of PHA-induced acquisition of IL2 receptors is not attributable to a lag in kinetics

To investigate whether OKT11A, 9.6, and 35.1 caused a sustained diminution in IL2 receptor expression on PHA-activated T-cells, we examined

the ability of PBMC cultured with PHA and these p50-binding antibodies to bind anti-Tac at various times after initiation of culture. Figure 2 shows an experiment conducted with the 9.6 antibody, wherein replicate cultures were analyzed by the FACS method at 0, 24, 48, 72, and 96 hr. The results are presented as the percentage of T-cells (OKT11A+) staining positively with the anti-Tac antibody (Figure 2A) and as the relative mean fluorescence intensity , Δ FL, for staining with anti-Tac (Figure 2B). Asshown, 9.6 inhibited the induction of IL2 receptor acquisition by PHA throughout the culture period. This impairment in IL2 receptor acquisition was attributable both to a decrease in the percentage of Tac+ T-cells (determined by an arbitrary threshold set with the biotin-conjugated R3-367 control monoclonal antibody) and to a decrease in the average number of anti-Tac antibody molecules binding per cell as reflected by the relative mean fluorescence intensity. Culturing PBMC with the combination of PHA and the 12.1 antibody resulted in no impairment of IL2 receptor acquisition in these experiments (not shown).

In contrast to PBMC cultures stimulated with PHA, 9.6 did not inhibit the acquisition of IL2 receptors induced by (>2.0 ng/ml) TPA (see Figure 2). Though in this particular experiment there was a slight decrease in the levels of IL2 receptors detected on the cells taken from cultures containing TPA and 9.6 antibody relative to cultures with TPA alone, this was not a consistent finding, and in some cases the levels of IL2 receptors were slightly higher when a p50-binding antibody (OKT11A, 9.6, 35.1) was included in TPA-stimulated PBMC cultures.

OKT11A, 9.6, and 35.1 also did not inhibit proliferation induced by TPA. The failure of these antibodies to decrease proliferation in PBMC cultures stimulated with TPA, however, is attributable to the high

concentrations of TPA (10 ng/ml) used in these experiments, since antibody 9.6 inhibited lymphocyte proliferation when TPA was used at suboptimal concentrations of <2.0 as shown in table III, page 51.

Supernatants with IL2 activity overcome the inhibition of PHA-induced [^{3}H]-thymidine incorporation mediated by OKT11A, 9.6, and 35.1

Because OKT11A and 9.6 have been demonstrated to inhibit IL2 production (19 and unpublished observations), we asked whether the addition of exogenous IL2 could restore proliferation in PHA cultures suppressed by OKT11A, 9.6, and 35.1 monoclonal antibodies. To answer this question, we employed supernatants derived from the gibbon ape lymphosarcoma cell line MLA144 (27). This cell line elaborates large quantities of an IL2 molecule that is functionally and biochemically indistinguishable from human IL2, without requirement for induction with lectin mitogens or phorbol esters (27). In addition, MLA144-derived supernatants have been shown not to contain interferon activity (27).

Figure 3 shows the combined results of two independent experiments wherein MLA144-derived supernatants (5, 15, 25% vol:vol) were added at the initiation of PBMC cultures. The IL2 activity of the supernatants used in these two experiments was approximately 30 U/ml as determined by a bioassay employing the "IL2-addicted" cell line CTLL-15H (30). As shown, MLA144-derived supernatants overcame, in a dose-dependent fashion, the OKT11A-mediated suppression of PBMC proliferation induced by PHA.

Although in these two experiments (see Figure 3) MLA144-derived supernatants (25% vol:vol) completely restored [^{3}H]-thymidine incorporation in OKT11A-containing cultures to levels commensurate with that observed in PBMC cultures containing PHA and IL2, this was not always the case. More typically, IL2-containing MLA144 supernatants (25% vol:vol) augmented

proliferation of OKT11A-inhibited cultures to levels equal to or greater than that obtained in PBMC cultures stimulated with PHA alone, but somewhat less than that measured in cultures stimulated with the combination of PHA and IL2 (see Figure 5).

When anti-Tac (0.85 ng/ml) was added, MLA144-derived supernatants failed to restore proliferation in inhibited cultures (Figure 3).These results are consistent with the idea that IL2 is the active molecule in MLA144-derived supernatants. Though not shown here, we have repeated these experiments using purified human IL2 and have obtained comparable results with equivalent amounts of IL2 activity (determined by the CTLL-15H bioassay).

Supernatants containing IL2 activity restore IL2 receptor expression in cultures inhibited by OKT11A, 9.6, and 35.1

The finding that IL2 abrogated suppression mediated by OKT11A was somewhat surprising, given that T-lymphocytes inhibited by these antibodies express diminished levels of receptors for IL2 (see Figure 1). We therefore examined the ability of T-cells to acquire IL2 receptors when cultured in the presence of IL2, PHA, p50-binding monoclonal antibodies, or various combinations of these reagents.

Figure 4 shows representative FACS profiles obtained after 72 hr of culture with the antibodies OKT11A, 9.6, and 12.1. As before, culturing PBMC with OKT11A or 9.6 impaired the PHA-induced acquisition of receptors for IL2. The control antibody 12.1 had no inhibitory activity. When MLA144-derived supernatants (25% vol:vol) were included at the initiation of cultures containing PHA and either OKT11A or 9.6, expression of IL2 receptors reached levels equal to or greater than that observed for PBMC cultures stimulated with PHA alone (see Figure 4). The combination of PHA and IL2 (MLA144 supernatant) always produced an increase over PHA alone in both the

levels of IL2 receptors expressed (see Figure 4) and in the amount of [^{3}H]-thymidine incorporation (see Figure 3) observed in PBMC cultures. Though not shown here, experiments conducted with 35.1 yielded comparable results.

The abrogation by IL2 (MLA144 supernatants) of the inhibition of IL2 receptor acquisition mediated by these three anti-p50 antibodies was due both to an increase in the percentage of cells staining positively with anti-Tac and to the average number of anti-Tac molecules bound per cell, as demonstrated in Figure 5. As shown, the inclusion of MLA144-derived supernatants (25% vol:vol) in PHA cultures containing OKT11A, 9.6, or 35.1 resulted in normal acquisition of IL2 receptors at 48 hr of culture, compared to cultures containing PHA alone. Culturing PBMC with IL2 alone produced only a slight increase in IL2 receptor expression. The combination of PHA and IL2 led to greater IL2 receptor acquisition than that obtained in PBMC cultures containing only PHA or those containing PHA, IL2, and an anti-p50 antibody (see Figure 5). When these experiments were repeated with purified human IL2, nearly identical results were obtained (not shown).

Similar experiments were performed at 24 hr, before cell division or DNA synthesis occurred (34). As shown in Figure 6, adding IL2 (MLA-144 supernatants) to PBMC cultures containing PHA and OKT11A resulted in augmentation of IL2 receptors to levels commensurate with that obtained at 24 hr in cultures stimulated with PHA alone. Measuring [^{3}H]-thymidine incorporation at 48 hr in parallel microtiter cultures verified that OKT11A inhibited proliferation induced by PHA and that IL2 abrogated this inhibition (see Figure 6).

To determine more definitely whether the IL2-induced augmentation of IL2 receptor expression on human T-cells was independent of cellular proliferation, we treated PBMC with mitomycin C before culturing in the

presence of PHA-M (15 ug/ml), IL2-containing supernatants (25% vol:vol), or combinations of these reagents. A suboptimal concentration of PHA was used so that the effects of added IL2 would be more pronounced.

Table I shows the results of one such experiment. At 24 hr, PBMC treated and not treated with mitomycin C incorporated little [^{3}H]-thymidine. Note, however, that at this suboptimal concentration of PHA, about 25% of the cells in these cultures became Tac-positive within 24 hr and that when IL2 was included, the frequency of cells reaching threshold fluorescence increased to approximately 40% (Table I). In addition, the average number of anti-Tac molecules bound per cell also increased when IL2 was added, determined by the relative mean fluorescence intensity, Δ FL. At 48 hr, these observations regarding IL2 receptor expression were generally unchanged, except that DNA synthesis was actively underway in the untreated PBMC cultures, whereas [^{3}H]-thymidine incorporation in cultures containing the mitomycin C-treated cells was reduced markedly.

PBMC cultured with IL2 (MLA144 supernatant) alone exhibited a small increase in IL2 receptor expression above background (see Table I), perhaps reflecting stimulation of lymphocytes activated <u>in vivo</u>. This small increase in proliferation and IL2 receptor expression in response to IL2 alone occurred even when purified IL2 was used (data not shown).

DISCUSSION

The results reported here, taken together with previous work by us and by others (16-19), demonstrate that the inhibitory anti-p50 antibodies OKT11A, 9.6, and 35.1 suppress human T-lymphocyte proliferation induced by PHA (but not by TPA) through at least two mechanisms: 1) the diminished elaboration of IL2, and 2) the decreased acquisition of receptors for IL2. At least two of the p50-binding monoclonal antibodies in this study (9.6 and 35.1) are known to recognize distinct epitopes on or closely associated with the E-receptor based on competitive antibody binding assays, sequential immunoprecipitations, E-rosette blocking, and lysostripping (cocapping) experiments (16). It was therefore of interest that the 35.1 antibody exhibited functional characteristics similar to 9.6 and OKT11A with regard to inhibition of PHA-induced IL2 receptor expression and failure to inhibit TPA-mediated proliferation, since 35.1 binds to the p50 molecule but does not block E-rosetting (16).

Several trivial explanations for inhibition of lymphocyte proliferation by p50-binding antibodies have been excluded. For instance, the inhibitory activity of OKT11A, 9.6, and 35.1 monoclonal antibodies was not due to a generalized suppression of cellular metabolism since TPA-induced proliferation was not inhibited by these anti-p50 antibodies and since IL2 abrogates their suppressive effects. Nor is it likely that these p50-binding antibodies inhibited by blocking the binding of PHA to cells because OKT11A also suppresses proliferation induced by pokeweed mitogen (18,35), OKT3 antibody (19), and the calcium ionophore A23187 (19); and all three of the anti-p50 antibodies studied here inhibit proliferation induced by antigens, alloantigens, and concanavalin A (16,19). Also, OKT11A and 9.6 antibodies

inhibit optimally even when added 4 hr after initiation of PHA cultures (19 and unpublished observations).

The inhibition of PHA-induced IL2 receptor acquisition mediated by OKT11A, 9.6, and 35.1 was attributable both to a decrease in the percentage of cells expressing IL2 receptors and to a decrease in the average density of IL2 receptors expressed per cell (see Figures 1, 2, 4, 5, and 6). Despite the fact that the IL2 receptor on human T-lymphocytes possesses a similar molecular weight of 47000-53000 daltons (22), it is unlikely that anti-p50 antibodies directly bind to and block IL2 receptors or sterically prevent the interaction of IL2 (or anti-Tac) with IL2 receptors because: 1) anti-p50 antibodies fail to inhibit TPA-induced IL2 receptor expression (Figure 2); 2) OKT11A has been demonstrated not to impair proliferation of IL2-dependent continuous T-cell lines (19); and 3) staining PHA-activated T-cells with the combination of anti-Tac antibody and an anti-p50 antibody results in an additive increase in the levels of fluorescence measured by FACS assay (unpublished observations).

The amount of IL2 required to abrogate suppression mediated by OKT11A, 9.6, and 35.1 (5-10 U/ml) is probably not above physiological concentrations, despite the fact that we generally detect less than 1 U/ml of IL2 activity in supernatants recovered from PBMC cultures stimulated with PHA (unpublished observations), given that the levels of IL2 measured in culture supernatants reflect the balance between production and utilization of IL2. It may also be that such high levels of IL2 are attainable locally in the microenvironment of in vivo immune responses.

The ability of excess, exogenous IL2 to abrogate the inhibition of IL2 receptor acquisition by anti-p50 antibodies (Figures 4, 5, and 6) and to augment IL2 receptor expression in PHA-stimulated cultures (Figures 4, 5, and

6, and Table I) is probably not attributable to the proliferation of Tac-positive cells, since we have observed here (Figure 6) and in previous experiments (35) that IL2 both abrogates the OKT11A-mediated inhibition of IL2 receptor acquisition and elevates the PHA-induced expression of IL2 receptors even at 24 hr, before the first round of cell division (34). The additional experiments reported here (Table I), wherein PBMC were treated with mitomycin C before culturing, support this notion.

The IL2-mediated increase in IL2 receptor expression seen in these experiments raises the intriguing possibility that IL2 may up-regulate expression of its own cellular receptor on human T-lymphocytes. There is precedence from studies with lymphocytes and from other types of cells for receptor-ligand interactions leading to increased expression of the receptor. Incubation of lymphocytes with large quantities of IgE, for example, apparently results in an up-regulation of Fc receptors specific for IgE from low, barely detectable levels to high densities (36). Similarly, binding of prolactin to its target tissues (37) and of insulin to chondrosarcoma cells (38) produces an up-regulation of prolactin and insulin cell-surface receptors, respectively.

That the increase in IL2 receptor expression mediated by IL2 is specific and does not merely reflect a general increase in membrane antigen synthesis prior to cell division is indicated by the observations 1) that levels of OKT3 antigen are reduced on T-cells following activation (39 and unpublished observations); and 2) that in our experiments, levels of the 12.1 antigen were unchanged on T-cells following stimulation either with PHA alone or with the combination of PHA plus IL2 (not shown).

If IL2 is required for maximal expression of IL2 receptors, then the failure of T-cells inhibited by OKT11A, 9.6, and 35.1 to acquire IL2 receptors

could be related, at least in part, to the suppressive effects of anti-p50 antibodies on IL2 production. It should be noted, however, that several examples have been reported wherein human T-cells were shown to express normal levels of IL2 receptors despite producing little or no IL2 (39-42).

Previously, we have observed that IL1 augments the acquisition of IL2 receptors on T-cells induced by PHA, and have suggested that IL1 may be necessary for optimal expression of IL2 receptors (35). Given the results reported here, showing that IL2 increases IL2 receptor expression on PHA-stimulated T-cells (Table I), it may be that IL1 augments IL2 receptor expression indirectly by increasing IL2 production.

The data reported here do not prove definitively that IL2 directly augments IL2 receptor expression on anti-p50 antibody-inhibited T-cells, as opposed to increasing IL2 receptor expression on the fraction of T-cells that escaped inhibition by OKT11A, 9.6,and 35.1 in these cultures. Given that these p50-binding antibodies often inhibited proliferation by >90%, however, this latter possibility is improbable (see Figure 5). Nevertheless, our findings demonstrate that in characterizing suppressors of lymphocyte proliferation, it is important to quantitate both IL2 production and IL2 receptor expression.

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Table I
IL2 augments IL2 receptor acquisition on mitomycin C-treated PBMC stimulated with PHA.

	Treatment with	Culture conditions§		FACS analysis		^{3}H-TdR incorporation
Time*	mitomycin C†	PHA	IL2	(% Tac+)**	(Δ FL)††	(cpm)#
24 hr	-	-	-	2	1.2	217 ± 114
	-	-	+	5	1.4	2709 ± 267
	-	+	-	27	2.4	1293 ± 297
	-	+	+	43	3.5	1760 ± 267
	+	+	-	26	2.5	406 ± 180
	+	+	+	39	3.4	412 ± 305
48 hr	-	-	-	1	1.3	590 ± 295
	-	-	+	7	1.5	2482 ± 1060
	-	+	-	27	3.6	48283 ± 4037
	-	+	+	44	5.6	62581 ± 2531
	+	+	-	37	3.3	9166 ± 185
	+	+	+	53	4.4	11527 ± 1074

Table I Footnotes

* PBMC were cultured for either 24 or 48 hr before FACS analysis.

† In some cases PBMC were treated with mitomycin C, as described, before culture.

§ PBMC were cultured at 10^6/ml in MEM supplemented with antibiotics and 10% heat-inactivated, pooled human sera with suboptimal concentrations of PHA-M (15 ug/ml), IL2-containing supernatants derived from MLA144 (6-8 Units/ml), or combinations of these reagents.

** The percentage of OKT11A+ cells that stained positively with anti-Tac antibody was determined in an indirect immunofluorescence assay with FACS analysis, as described. The % Tac+ cells before culture was 3%, which was subtracted from the data.

†† Relative mean fluorescence intensity for cultured PBMC labeled with anti-Tac antibody in an indirect immunofluorescence assay employing FITC-goat anti-mouse antiserum was determined as described in the Methods section.

Incorporation of [^{3}H]-thymidine was determined in replicate microtiter cultures at the indicated times. Microtiter wells were pulsed with 0.5 uCi of [^{3}H]-thymidine 8 hr before termination of culture. Data are expressed as mean cpm ± standard deviation for triplicate cultures.

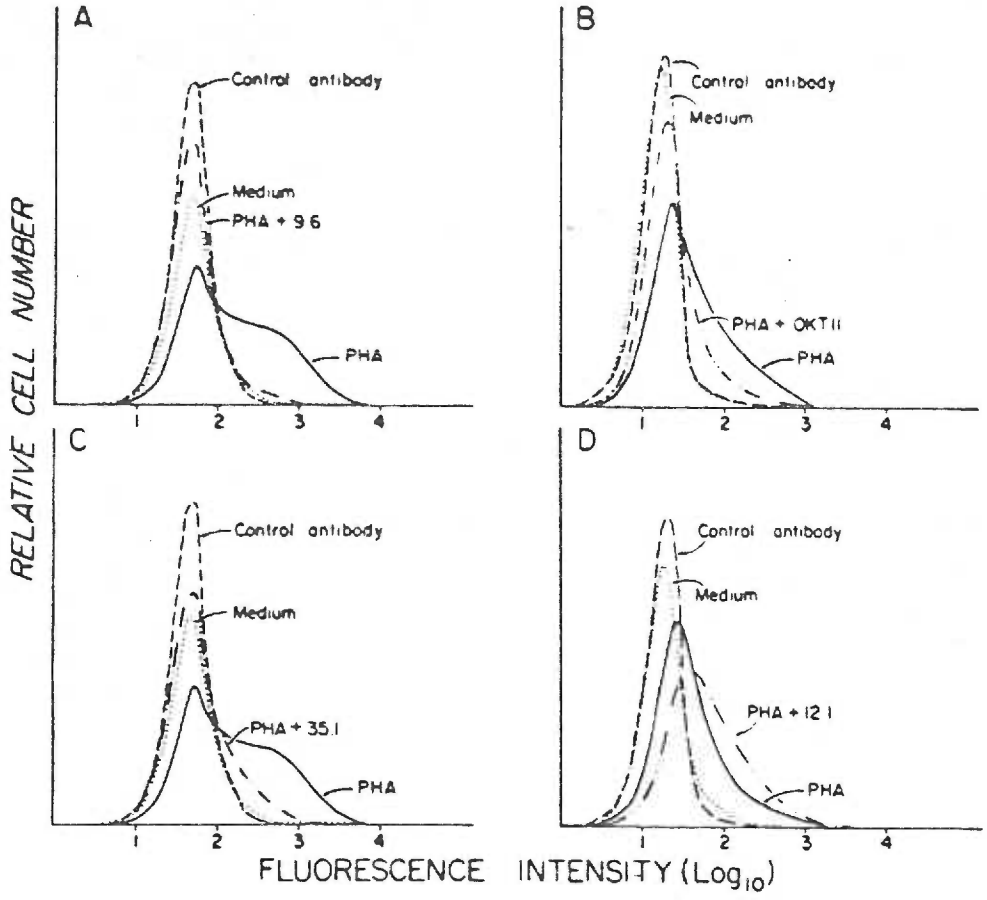

Figure 1

OKT11A, 9.6, and 35.1 antibodies inhibit acquisition of IL2 receptors induced by PHA. PBMC were cultured for 48 hr in medium alone (•••), in PHA (▬▬), or in PHA plus one of the following monoclonal antibodies (▬••): 9.6 (A), OKT11A (B), 35.1 (C), 12.1 (D). Cells were then labeled with biotin-conjugated anti-Tac antibody in an indirect immunofluorescence assay employing FITC-avidin and analyzed by FACS. Nonspecific binding was determined with an irrelevant control antibody (▬ ▬). Data are reported as relative cell number (ordinate) vs. fluorescence intensity in $\log_{10}$ units (abscissa) for $\geq 10^4$ cells analyzed. A and C represent data obtained in the same experiment; B and D represent data obtained in a separate experiment.

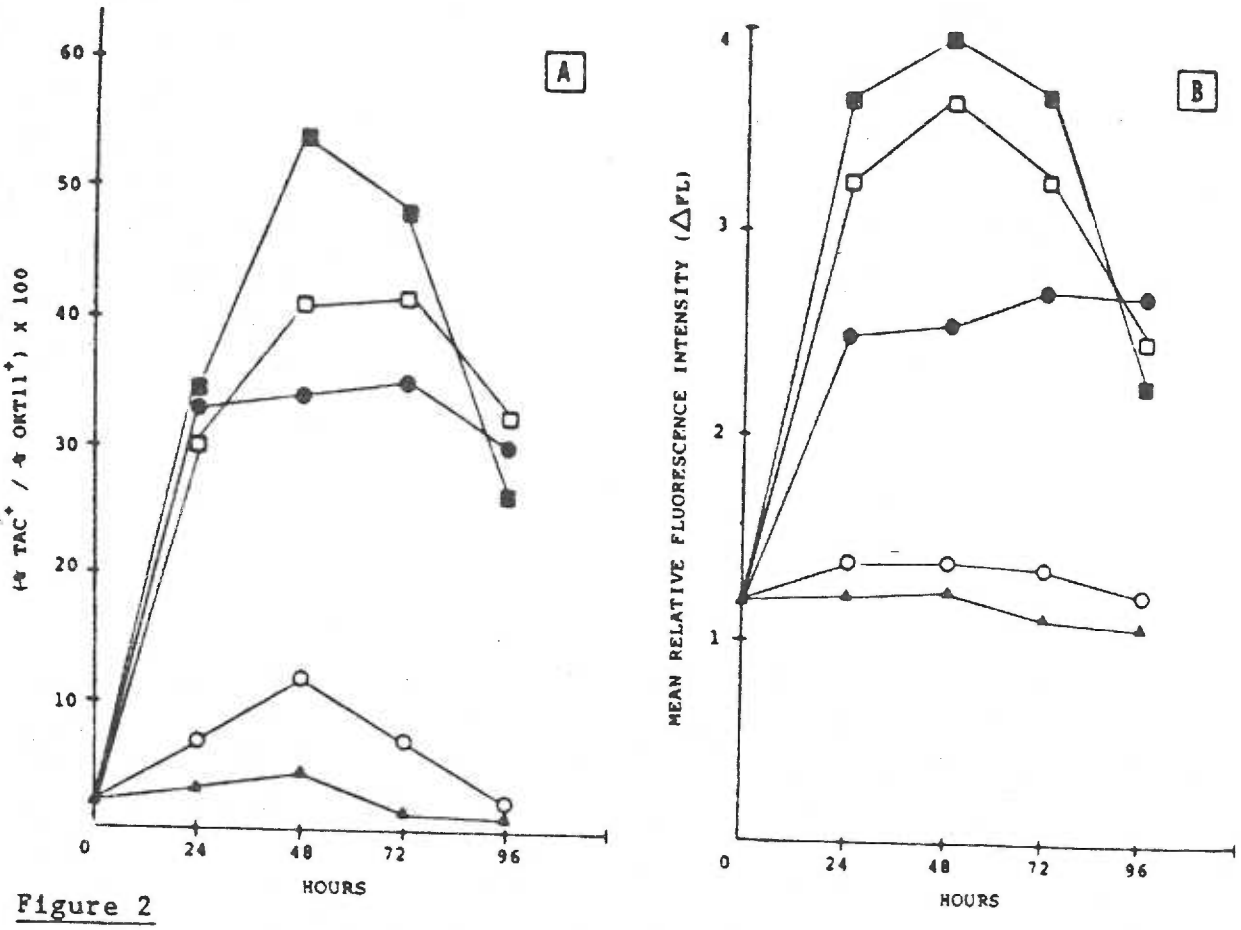

Figure 2

Inhibition of PHA-induced acquisition of IL2 receptors is not attributable to a lag in kinetics. PBMC were cultured for 0, 24, 48, 72, or 96 hr with medium alone (▲), PHA (●), PHA plus 9.6 (○), TPA (■), or TPA plus 9.6 (□). Cells were then labeled with biotin-conjugated anti-Tac antibody in an indirect immunofluorescence assay employing FITC-avidin and analyzed by FACS. Data are reported in (A) as the percentage of Tac-positive cells divided by OKT11A-positive cells, and in (B) as the relative mean fluorescence intensity, △FL, as described in the Methods section.

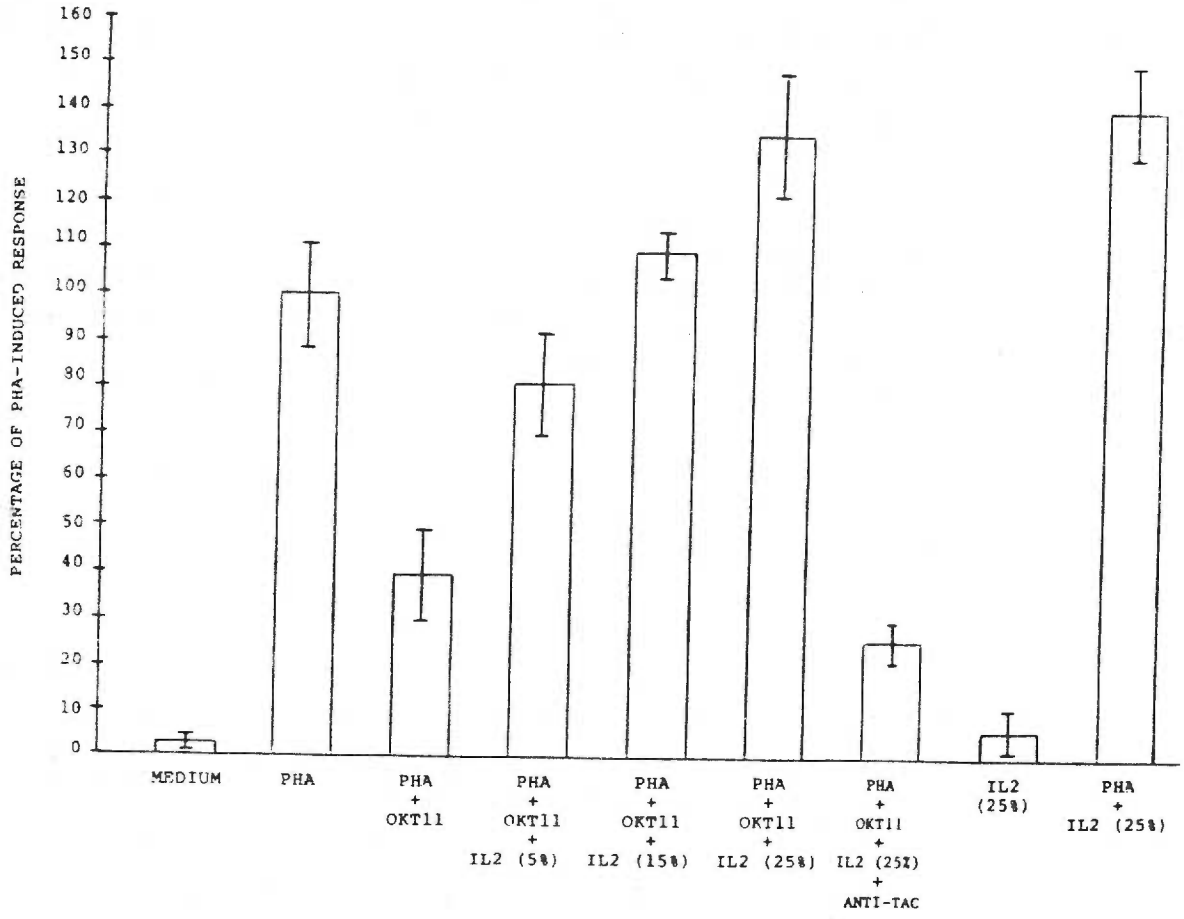

Figure 3

IL2-containing supernatants overcome OKT11A-mediated inhibition. PBMC were cultured with PHA-M (25 ug/ml), OKT11A antibody (150 ng/ml), MLA144 supernatants (5, 15, or 25% vol:vol), anti-Tac antibody (0.85 ng/ml), or various combinations of these reagents. [^{3}H]-thymidine incorporation was determined after 72 hr of culture. Data represent mean cpm ± standard deviation for 2 independent experiments and are expressed as a percentage of the PHA-induced responses, which were 90143 and 76766 cpm.

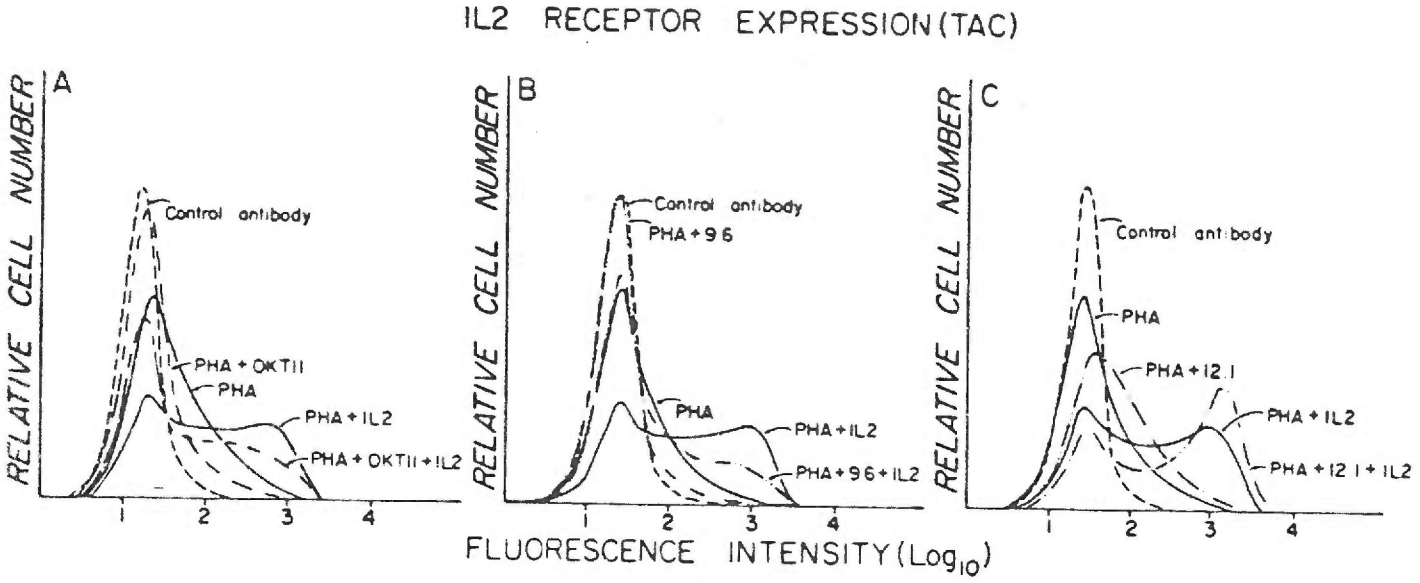

Figure 4

Supernatants containing IL2 activity augment IL2 receptor expression in cultures inhibited by OKT11A and 9.6. PBMC were cultured for 72 hr in complete medium with PHA, IL2, OKT11A (A), 9.6 (B), 12.1 (C), or various combinations of these reagents as shown. Cells were then labeled with biotin-conjugated anti-Tac antibody in an indirect immunofluorescence assay and data reported as described for Figure 2. Nonspecific binding was determined with an irrelevant control monoclonal antibody (32).

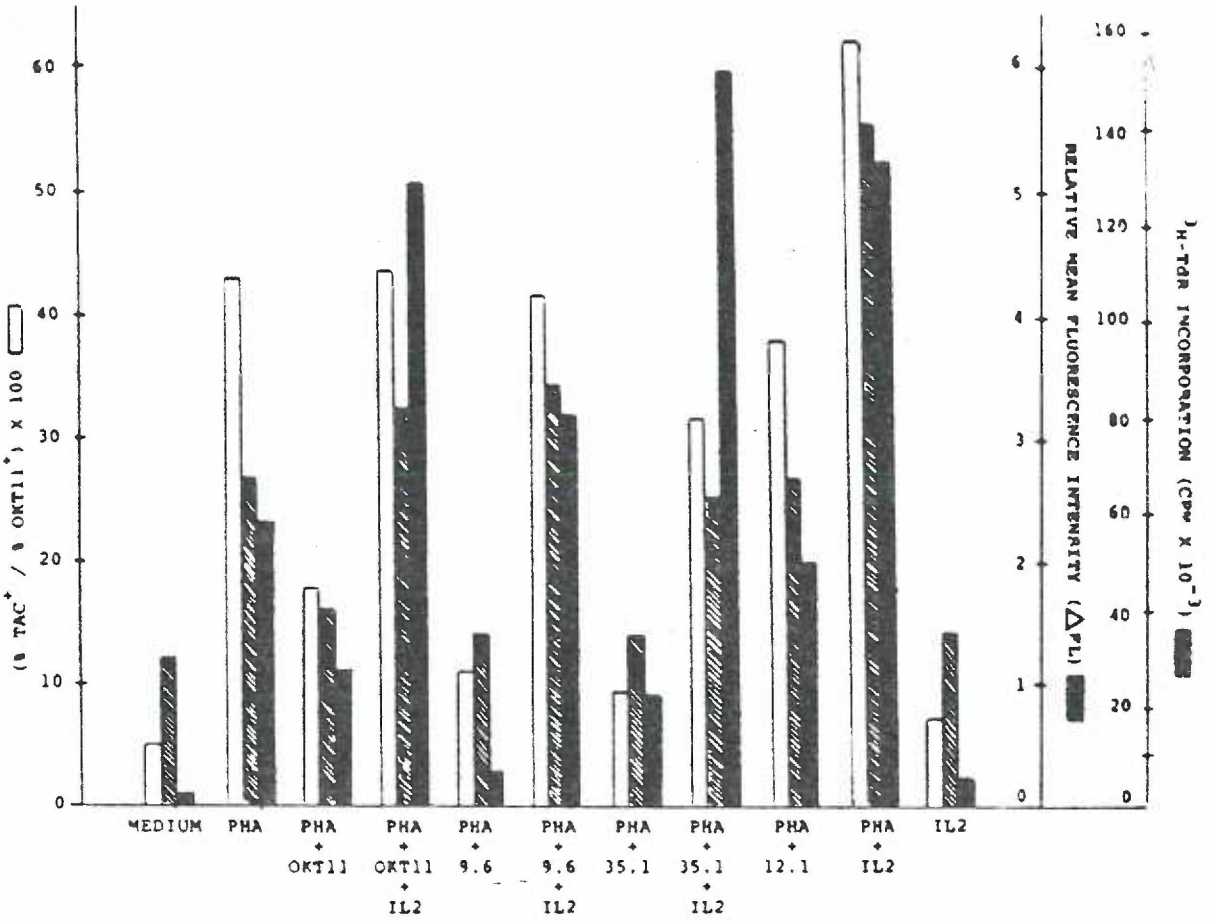

Figure 5

Supernatants containing IL2 activity augment IL2 receptor expression in cultures inhibited by OKT11A, 9.6, and 35.1. PBMC were cultured for 48 hr in complete medium with PHA (25 ug/ml), IL2-containing MLA144 supernatants (25% vol:vol), or various monoclonal antibodies, as shown. Cells were then labeled with biotin-conjugated anti-Tac antibody in an indirect immunofluorescence assay employing FITC-avidin with FACS analysis. Data are reported as the percentage of Tac-positive cells dividied by OKT11A-positive cells (□), and as relative mean fluorescence intensity, △FL (▨), as described in the Methods section. [^{3}H]-thymidine incorporation was assessed in companion microtiter cultures at 72 hr (■). (Mean cpm for triplicate cultures.)

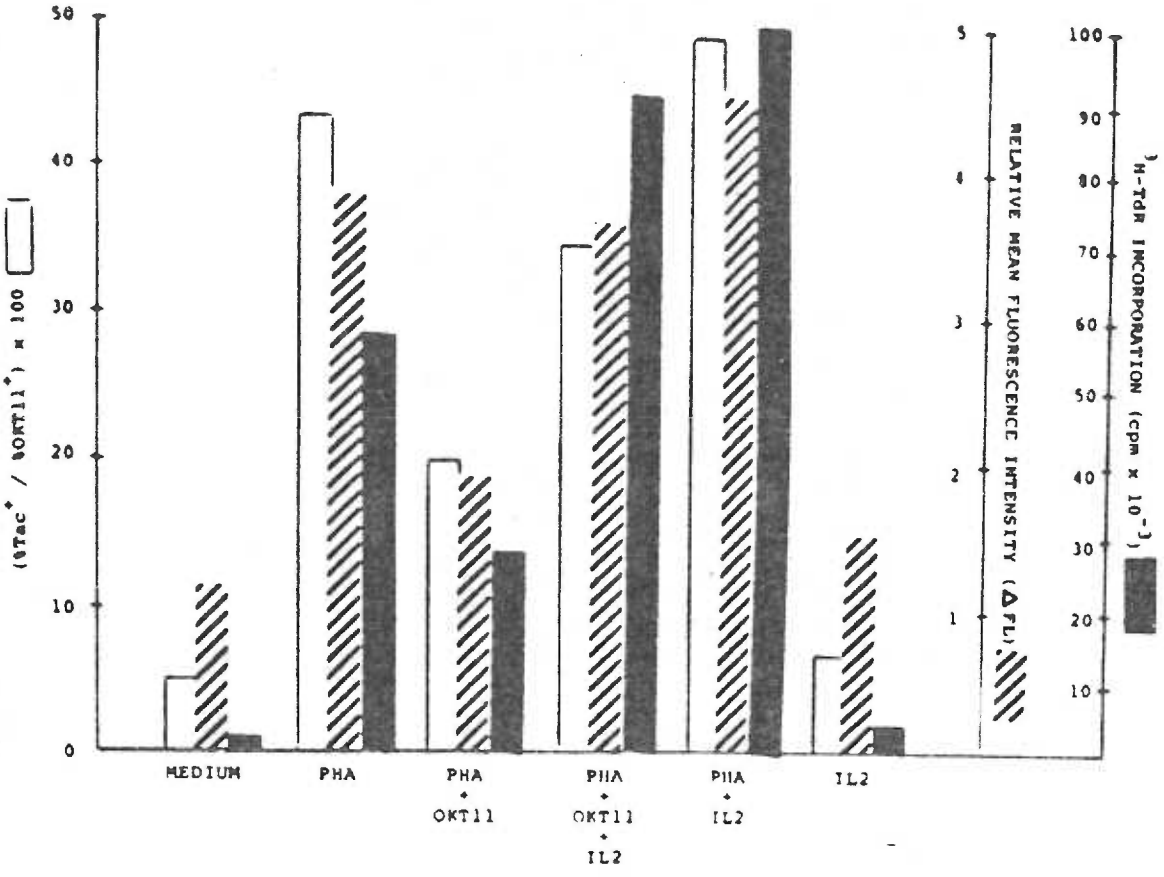

Figure 6

Supernatants containing I12 activity augment IL2 receptor expression in cultures inhibited by OKT11A even at 24 hr. Experiments were conducted as described under Figure 5, except that immunofluorescence assays were performed after 24 hr of culture and [^{3}H]-thymidine incorporation was assessed at 48 hr.

Paper 3

Down Regulation of IL2 mRNA by Antibody to the 50 kd Protein Associated with E Receptors on Human T Lymphocyte[1]

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Footnotes

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[2]Recipient of the Leukemia Society of America's Special Fellow Award.

[3]J.A.K. is an established Investigator of the American Heart Association

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Abbreviations used in this paper

PHA	phytohemagglutinin
TPA	12-O-tetradecanoyl-phorbol-13-acetate
CHX	cycloheximide
DRB	5,6 dichloro-1-β -D-ribofuranasyl benzimidazole
IL1	interleukin 1
IL2	interleukin 2
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
U/ml	units per milliliter
E receptor	human lymphocyte receptor for sheep erythrocytes
kd	kilodalton

ABSTRACT

Recent studies have shown that antibodies to certain epitopes on the 50 kd molecule associated with sheep erythrocyte receptors (E receptor) on human T cells can suppress T cell proliferation and Interleukin 2 (IL2) elaboration. We used a human IL2 cDNA clone to investigate the effect of antibody 9.6 and Cyclosporin A (CsA) on the regulation of IL2 mRNA levels in the cloned human leukemic T cell line Jurkat, J32. Maximal levels of IL2 mRNA were reached 6 hr after induction of Jurkat cells with a combination of mitogen phytohemagglutinin (PHA) and phorbol ester (TPA). Antibody 9.6 added during the first 4 hr after lymphocyte stimulation markedly inhibited IL2 mRNA accumulation induced by a low but synergistic combination of PHA (5 ug/ml) and TPA (1.0 ng/ml). The inhibition by 9.6 was not demonstrable as the concentration of PHA or TPA was increased. In contrast, the ability of CsA to suppress IL2 mRNA accumulation appeared to be independent of PHA or TPA concentration and was minimal if CsA was added 4 hr following stimulation.

IL2 mRNA could be superinduced several fold by addition of cycloheximide 3 hours after induction of J32 with mitogens. Antibody 9.6 did not prevent IL2 mRNA superinduction induced by cycloheximide, whereas CsA as well as transcription inhibitor DRB completely blocked this phenomenon. These findings indicate that signals induced by antibody 9.6 1) regulate IL2 production at a pretranslational level, 2) are operative for an extended period of time overlapping with the early phase of IL2 mRNA accumulation, 3) suppress IL2 gene expression induced by PHA as well as TPA, 4) antibody 9.6 and CsA exert their inhibitory effect by distinct mechanism(s).

INTRODUCTION

The 50 kd surface protein associated with the sheep erythrocyte receptor (E receptor) on human T lymphocytes can serve as an integral component of a T cell activation pathway resulting in interleukin 2 (IL2)-dependent proliferation (1). Several monoclonal antibodies to certain epitopes on the 50 kd structure have been shown to suppress the T cell proliferative response to antigen, mitogens (2-7), and antibodies to T3 (CD3). This suppressive effect is associated with reduction in IL2 production (3,4). The mechanism(s) of this antibody-mediated suppression of IL2 production is poorly understood.

To gain further insight into the mechanisms of the regulatory role of the 50 kd molecule in T cell growth, we have examined the effect of anti-50 kd, monoclonal antibody 9.6 on the regulation of IL2 mRNA accumulation induced by PHA and TPA and compared it to the effect of the immunosuppressive agent Cyclosporin A (CsA).

MATERIALS AND METHODS

Preparation and Stimulation of Jurkat Cells: Jurkat leukemic T cells, subclone 32 (J32) were grown in RPMI containing penicillin (50 u/ml), streptomycin (100 ug/ml), L-glutamine (2 mM) and 10% (vol:vol) heat inactivated fetal calf serum (complete medium) at $0.1-0.5x10^6$ cells/ml.

Prior to each experiment cells were washed once with RPMI and resuspended at $4x10^6$ cells/ml in complete medium. To stimulate Jurkat cells, the following mitogens were added unless otherwise indicated: lectin mitogen PHA-M (Difco Lab, Detroit, MI) 5 ug/ml, Phorbol ester TPA (Chemical for Cancer Research, Eden Praire, MN) 0.75 ng/ml. TPA was stored in a 1 mg/ml stock solution in absolute ethanol and diluted further with complete medium immediately before use. Monoclonal antibodies mouse ascites was added to cultures in saturating amounts (1:100). Where indicated, cycloheximide (CHX) was used at 20 ug/ml, and 5,6 dichloro-1- -D-ribofuranosyl benzimidazole (DRB) at 40 uM (both from Sigma, St. Louis, MO). To determine the effect of CHX on IL2 superinduction in culture supernatant, J32 cells from cultures containing CHX and control cultures were washed three times with complete medium 6 hr after stimulation and were recultured in fresh complete medium containing PHA and TPA. IL2 assay was performed on the 24 hr and 36 hr supernatants of these cultures. Cultures were set up in 15x75 mm plastic tubes (Fisher Scientific, Fairlawn, NJ), and were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for the indicated periods.

Monoclonal antibody: Antibody 9.6 reacts to a 50 kd protein associated with E-receptors on human T cells (4,5). Control antibodies include two anti-T cell antibodies 10.2 (CD5) generously provided by Dr. John Hansen and 12.1 (CD6) (4,7).

RNA Isolation and Northern Blot Analysis: Following culture, Jurkat cells were washed with phosphate-buffered saline (PBS) 3 times and total cellular RNA was isolated by phenol chloroform mixture as described (8), quantified by absorbance at 260 nm, and 10-20 ug samples were denatured at 65°C for 5 min in 50% formamide, 2.2 M formaldehyde. RNA was size-fractionated by electrophoresis through 1% agarose gels containing 1.1 M formaldehyde. RNA was then transferred to nitrocellulose in 20xSSC (1xSSC = 0.15 N NaCl/0.015 M trisodium citrate). Following transfer the nitrocellulose membrane was air dried and baked in a vacuum oven at 80°C for 2 hr. Membranes were prehybridized at 42°C in roller bottles for 16-24 hr in prehybridization solution containing 50% (vol/vol) formamide, 5xSSC, 50 mM sodium phosphate at pH 6.5, denatured salmon sperm DNA at 250 ug/ml and 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. Denatured ^{32}P labeled IL2 cDNA ($5x10^8$dpM/ug) and fresh prehybridization solution containing 10% dextran sulfate was added and the membrane hybridized 28-32 hr at 42°C. The blots were then washed 4 times with 2xSSC/0.1% SDS for 5 min each at room temperature and twice with 0.1xSSC/0.1% SDS for 20 min at 65°C. The damp blots were then exposed at -70°C for 24 hr, or a variable time to achieve desired density, to Kodak XAR-5 film.

Cytoplasmic Dot Blot Hybridizations: Relative levels of IL2 mRNA were determined using a modification of the cytoplasmic dot blot hybridization protocol of White and Bancroft (9). $2-4x10^6$ J32 cells were washed 3 times with cold PBS. The cells were resuspended in 1 ml of PBS and transferred to 1.5 ml microfuge tube (Eppendorf, Westbury, NY) and repelleted at 11,600xg for 15 seconds in a Beckman Microfuge II (Beckman, Palo Alto, CA). Cells were resuspended in 90 ul of ice-cold 10 mM Tris (pH 7.0), 1 mM EDTA, then lysed by adding two 10 ul aliquots of 5% Nonidet P40 (Sigma, St. Louis, MO) with 5 min

of mixing on ice in between. Nuclei were pelleted (11,600xg, 2.5 min), 100 ul of the supernatants were transferred to a sterile 1.5 ml tube containing 60 ul of 20xSSC, plus 40 ul of 37% (w/w) formaldehyde (Fisher No F-79, Fairlawn, NJ). The mixture was then incubated at 60°C for 15 min. To precipitate residual contaminating proteins, cold absolute ethanol was added to 33% final concentration, and the tubes incubated on ice for 5 min, then centrifuged in an Eppendorf Microfuge 5 min at 4°C. Supernatants representing 2.0, 1.5, 0.75, 0.5×10^6 cells were blotted on a BA85 nitrocellulose sheet using a Minifold apparatus (Schleicher and Schuell, Keene, NH) and each well was washed with 200 ul of 15xSSC followed by 200 ul of RNase-free water. The nitrocellulose was washed in RNase free water at room temperature for 5 min 3 times. The membrane was then soaked in freshly made 0.1 M triethanolamine solution containing 0.25% (v/v) acetic anhydride (both from Sigma, St. Louis, MO) at room temperature for 10 min, dried and the membrane was baked (80°C, 90 min) in vacuo. Prehybridization, hybridization and autoradiography were done as described above.

Where indicated, autoradiograms were quantitated on a Shimadzu C5930 TLC densitometer (Kyoto, Japan) with a programmable data recorder which determined the area under symmetrical peaks for each dot or lane. Multiple exposures for each blot were made to be sure that the density of the film and the concentration of RNA have a linear relationship. Initial experiments showed a good linear relationship between the number of cells processed (0.4 to 2.0×10^6 cells) and the density reading for films exposed 18-24 hr. Each experiment was done 3 to 4 times. Each experiment included stimulated and unstimulated J32 cell controls. The densitometer readings for unstimulated cells were subtracted from experimental values to obtain the amount of specific RNA hybridized. The following formula was used to calculate relative IL2 mRNA level (% of stimulated):

$$1 - \frac{\text{Density of experimental cells dot} - \text{Density of unstimulated cells dot}}{\text{Density of the stimulated cells} - \text{Density of unstimulated cells dot}} \times 100$$

cDNA Probes: A pBR322 plasmid containing a 450 bp insert of a human IL2 cDNA clone (11) was the generous gift of Dr. Gerald Crabtree (Stanford University, Stanford, CA). A pBR322 plasmid containing a 1400 bp insert of HLA B7 cDNA clone pDP001 (12) was a gift of Dr. Sherman Weismann (Yale University, New Haven, CT). A pBR322 plasmid containing a 2000 bp insert of chick -actin cDNA clone A1 (13) was a gift of Dr. Don Cleveland (Johns Hopkins University, Baltimore, MD). Plasmid DNA were made radioactive by nick-translation to a specific activity of 1 to 5 x 10^8 [^{32}P] cpm/ug.

IL2 Bioassay: The amount of IL2 activity in supernatants was determined in a microassay based on the IL2-dependent proliferation of CTLL-15H cell line (14). Supernatants were serially diluted in RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum, 2.5x10^{-5} M 2-mercaptoethanol, and 100 ul/well were added to 96-well, flat-bottom microculture plates. 5x10^3 CTLL cells in 100 ul were added to each well. After 20 hr of culture at 37°C in an atmosphere of 5% CO_2 in air, each microwell culture was pulsed for an additional 6 hr with 0.5 uCi [^{3}H]TdR, and [^{3}H]TdR incorporation was determined. The IL2 activity of cultures performed was expressed as units per milliliter; 1 U/ml of IL2 activity corresponded to the dilution that produced half maximal proliferation of CTLL cells. The % suppression of IL2 production by antibody 9.6 was calculated as follows

$$1 - \frac{\text{IL2 U/ml with 9.6}}{\text{IL2 U/ml without 9.6}} \times 100$$

RESULTS

Antibody 9.6 suppresses IL2 mRNA accumulation in J32 cells stimulated with a combination of PHA and TPA.

In preliminary experiments it was found that stimulation of leukemic T cells (Jurkat, subclone 32) with an optimal concentration of PHA-P (20 ug/ml) induced IL2 mRNA levels lower than that obtained with the combination of low concentrations of PHA (PHA-M 5 ug/ml) and TPA (1.0 ng/ml). This synergistic combination was used to determine the time course of IL2 mRNA accumulation in J32 cells employing cytoplasmic dot blot hybridizations. As shown in Figure 1, IL2 mRNA was detectable at 3 hr; large amounts accumulated between 4-6 hr; levels then declined gradually to baseline by 24 hr. These results are similar to those reported by others (15,16).

To study the effect of antibody 9.6 and CsA on IL2 gene expression, we determined IL2 mRNA levels in J32 cells stimulated for 6 hr with the synergistic combination of PHA and TPA in the presence of antibody 9.6 or CsA. Total RNA was isolated from each culture and analyzed by Northern blot hybridization. As shown in Figure 2A, IL2 mRNA was detectable in stimulated (lanes 2 and 3) but not in unstimulated cells (lane 1). Adding antibody 9.6 at the initiation of the culture caused marked reduction of IL2 mRNA levels (lanes 4 and 5), whereas the addition of CsA (1 ug/ml) produced complete inhibition of IL2 mRNA accumulation (lanes 6 and 7). Anti-T cell antibodies 10.2 (CD5) and 12.1 (CD6) reacting with J32 cells used as control for the effects of 9.6 did not inhibit IL2 mRNA accumulation (data not shown). The effects of antibody 9.6 and CsA on the accumulation of IL2 mRNA were relatively selective because levels of HLA mRNA were less affected in stimulated cells recovered from these cultures (Fig. 2B).

Suppression of IL2 mRNA accumulation by antibody 9.6 is dependent on mitogen concentration.

Since antibody 9.6 did not completely inhibit IL2 mRNA accumulation in J32 cells stimulated by the chosen combination of PHA and TPA we examined the ability of antibody 9.6 to suppress IL2 mRNA levels in J32 cells stimulated with a low concentration of PHA (PHA-M 5 ug/ml) and varying amounts of the phorbol ester TPA using a quantitative cytoplasmic dot blot hybridization. As shown in Figure 3, IL2 mRNA levels were dependent on the concentration of TPA. Adding 0.5 ng/ml of TPA produced low but detectable levels of IL2 mRNA. In contrast, increasing the concentration of TPA to 0.75 ng/ml produced a sharp enhancement in IL2 mRNA levels, indicating a critical threshold for TPA concentration. The presence of antibody 9.6 in these cultures caused marked inhibition of IL2 mRNA levels. However, the effect of antibody 9.6 decreased with increasing concentration of TPA. When a higher concentration of TPA ($\geq$1 ng/ml), or when an optimal concentration of PHA-P (20 ug/ml) was used alone, antibody 9.6 failed to inhibit IL2 mRNA accumulation (data not shown). The effect of antibody 9.6 was specific because levels of β-actin mRNA were relatively constant in cells recovered from these cultures (Fig. 3). IL2 levels in the supernatants of these cultures showed a similar dependence on the amounts of TPA added (Table I). A sharp increase of IL2 levels was obtained with 0.75 ng/ml of TPA. Furthermore, the inhibition of IL2 levels in cultures containing antibody 9.6 correlated with the inhibition of IL2 mRNA. In J32 cultures induced with high concentration of TPA (10 ng/ml) or with PHA-P alone (20 ug/ml) antibody 9.6 failed to inhibit IL2 elaboration. Parallel cultures showed a different pattern of inhibition by CsA which completely inhibited IL2 mRNA accumulation in these cells.

Inhibition of IL2 mRNA accumulation by antibody 9.6 and cyclosporin A shows different time courses.

We examined the kinetics of 9.6 and CsA inhibition of IL2 mRNA accumulation in stimulated J32 cells. Antibody 9.6 or CsA was added simultaneously with or at different time intervals after stimulation. IL2 mRNA levels were then measured 6 hr after stimulation using a cytoplasmic dot blot hybridization. As shown in Figure 4, lane A, the addition of antibody 9.6 any time during the first 4 hr after the initiation of the culture produced a pronounced but decreasing level of inhibition (89-64% measured by densitometry), whereas little inhibition was observed when the antibody was added 5 hr after initiation of the cultures (11%) and none if added at 6 hr. In contrast, the inhibitory effect of CsA declined more rapidly and an appreciable inhibition of IL2 mRNA was seen only if CsA was added during the first 3 hr after stimulation (Figure 4, lane B). The above data indicate that antibody 9.6 was still inhibitory even after a moderate amount of cytoplasmic IL2 mRNA had accumulated.

Superinduction of IL2 mRNA by mitogens in the presence of cycloheximide.

The expression of the IL2 gene in stimulated lymphocytes has been shown to be subject to regulation by a cycloheximide (CHX) sensitive repression mechanism (17). To determine if J32 cells exhibit a similar repression mechanism, CHX was added to stimulated J32 cells at the times indicated in Figure 5 (lane A), and cytoplasmic IL2 mRNA levels were determined 6 hr after the initiation of the cultures. CHX at the concentration used in these studies (20 ug/ml) had no effect on J32 cell viability or on their ability to form E-rosettes with sheep erythrocytes nor on the ability of antibody 9.6 to inhibit E-rosette formation. Furthermore, in previous studies (17), it was shown that at this concentration CHX inhibits protein synthesis without

affecting RNA synthesis. The addition of CHX to stimulated Jurkat cell cultures in the first hour after stimulation virtually abolished IL2 mRNA levels Figure 5 (lane A). In contrast, adding CHX 3 hr or later caused marked enhancement in the levels of IL2 mRNA over those seen in cultures which had not been treated with CHX. Maximum enhancement was observed when CHX was added 3 hr after culture initiation. Moreover, the accumulation of IL2 activity measured in the 24 and 36 hr supernatants of parallel cultures (which were washed 6 hr after stimulation and recultured in CHX free medium) showed a proportional augmentation of IL2 (data not shown). CHX alone was unable to induce the synthesis of IL2 mRNA in the absence of mitogen. Thus, CHX does not induce IL2 gene transcription but only enhances IL2 mRNA accumulation induced by mitogens.

These data suggest that IL2 mRNA accumulation and disappearance in mitogen stimulated J32 cells is regulated, at least in part, by de novo cycloheximide sensitive protein synthesis possibly induced by mitogen stimulation.

Effect of antibody 9.6 and CsA on CHX-induced IL2 mRNA superinduction in J32 cells.

To further characterize the effect of 9.6 and CsA on IL2 gene expression we examined their effect on IL2 mRNA superinduction. CHX was added 3 hr after stimulation of J32 cultures containing antibody 9.6 added at 0 hr or 3 hr, and IL2 mRNA levels were determined 6 hr after the initiation of the cultures. Analogous cultures wherein CsA or DRB was added with CHX 3 hr after stimulation were also examined. As shown in Figure 5 (lane B), addition of CHX (3 hr) to stimulated Jurkat cells caused marked enhancement (560% measured by densitometry) in IL2 mRNA level. CHX also caused a substantial increase in IL2 mRNA levels in cultures containing antibody 9.6. This increase was from

9% to 160% when antibody 9.6 was added at the initiation of the cultures and from 36% to 255% when antibody 9.6 was added 3 hr after stimulation. In contrast, CHX failed to cause superinduction of IL2 mRNA levels over levels seen in CHX-free cultures when CsA or a transcription inhibitor (DRB) (18,19) was added 3 hr after the initiation of the cultures. CsA (1 ug/ml) and DRB (40 um) did not affect the viability of the J32 cells or their ability to form E-rosettes in these cultures. The ability of transcription inhibitor DRB to inhibit superinduction suggests that transcription is necessary for superinduction of IL2 mRNA.

DISCUSSION

We and others (3,4,7) have reported that antibodies to a 50 kd protein associated with the sheep erythrocyte receptor on human T lymphocytes suppress IL2 production in stimulated lymphocytes. However, it was not clear at what level(s) of IL2 gene expression these antibodies exert their suppressive effect. Such an effect could be attributed to regulation at pretranslational or posttranslational levels. We have employed a cDNA probe for human IL2 in Northern and cytoplasmic dot blot analysis to study and compare the effect(s) of antibody 9.6 and CsA on the regulation of IL2 mRNA accumulation in cloned leukemic T cell line Jurkat (subclone 32).

The data presented here demonstrated that both antibody 9.6 and CsA markedly suppressed IL2 mRNA accumulation in J32 cells stimulated with PHA and TPA (Fig. 2), indicating that antibody 9.6 and CsA exert their effects on IL2 production, at least in part, at pretranslational levels. The effect of antibody 9.6 was relatively selective since -actin gene expression was not affected by this antibody. Further HLA gene expression was also less affected than IL2 in 9.6-treated cells. The moderate 9.6 and CsA inhibition of HLA gene expression in induced cells may be secondary to inhibition of interferon-γ production. A similar effect of CsA on HLA gene expression in J32 induced cells has been observed by Kronke et al. (16).

The ability of antibody 9.6 to suppress IL2 mRNA accumulation was dependent on the concentration of the mitogen (Fig. 3), whereas the inhibitory effect of CsA was not. These findings suggested a competitive relationship between mitogen induced signals and those induced by the binding of antibody 9.6 to the 50 kd molecule. In contrast, CsA does not appear to suppress IL2 mRNA in a competitive way. Attributing the inhibitory effect of antibody 9.6 to a pretranslational level is further supported by the finding that

inhibition of IL2 mRNA accumulation was accompanied with a proportional decrease in IL2 measured in these culture supernatants (Table I). Moreover the finding that IL2 levels in stimulated J32 culture supernatants are proportional to the levels of IL2 mRNA suggests the absence of detectable regulatory signal effects on IL2 mRNA translation. A similar observation was made in normal lymphocytes (17).

The time course of IL2 mRNA inhibition by antibody 9.6 showed that marked inhibition of IL2 mRNA accumulation could be attained even if antibody 9.6 was added to cultures at the time when moderate amounts of IL2 mRNA have accumulated (4 hr after stimulation). It is noteworthy that in a previous study (4) we showed that antibody 9.6 reduced normal lymphocyte proliferative response to PHA even when the antibody was added 18 hr after induction. IL2 mRNA in these cells was detectable at 20 hr after induction (17 and unpublished observaton), further supporting the kinetics of inhibition obtained with Jurkat cells. These data from two independent experimental conditions suggest that the regulatory signals mediated through the 50 kd molecule are operative during an extended phase of T cell activation overlapping with the beginning of accumulation of IL2 mRNA. In contrast the inhibitory effect of CsA decreased rapidly and became insignificant if CsA was added at 4 hr. Because CsA exerts its effect, at least in part, by inhibiting IL2 gene transcription (16) it is likely that antibody 9.6 affects regulatory events at the posttranscriptional levels.

Several assay systems have been employed to examine the effect of CHX on IL2 gene expression. In our studies the appearance of cytoplasmic IL2 mRNA upon induction of J32 cells with mitogen(s) is prevented by CHX suggesting that cytoplasmic IL2 mRNA accumulation requires prior protein synthesis. A similar finding in human tonsil T lymphocytes has been reported by Efrat et al

(17) who attributed the need for protein synthesis at early hours of induction to the requirement for IL1. Our studies did not include Mϕ or IL1, thus indicating that other intracellular protein synthesis is required for IL2 mRNA accumulation. Recent studies by Kronke et al (20) using in vitro nuclear transcription assays with isolated nuclei from mitogen-stimulated peripheral blood human T cells demonstrated that IL2 gene transcription is not affected by concentrations of CHX that inhibited protein synthesis. Since these studies measured either cytoplasmic or nuclear mRNA accumulation, the differing effect of CHX may reflect the necessity of protein synthesis in the transport or the processing of nuclear mRNA transcript. We can also not exclude differences in the response to CHX using T cells from different origin.

The study of the kinetics of IL2 mRNA accumulation in stimulated Jurkat cells showed a rapid pattern of accumulation of IL2 mRNA followed by rapid shutoff after peaking at 6 hr. Similar kinetics were obtained by Kronke et al. (16) despite the use of a much higher dose of TPA by these investigators. The rapid shutoff of cytoplasmic IL2 mRNA accumulation has been attributed to nontranscriptional regulatory mechanisms (16,17). Here we show that shutoff of IL2 mRNA accumulation in stimulated J32 is regulated by protein(s). Since these studies were performed with a cloned T cell line, the data indicate that the accumulation of IL2 mRNA and its subsequent decline result from intracellular events and occur in the absence of signals from a second cell type. The most likely mechanism of shutoff of IL2 mRNA accumulation is the synthesis of a protein(s) during the early phase of induction by mitogen. Accordingly, the mitogenic signals induce IL2 gene transcription as well as the necessary regulatory proteins to make the IL2 appearance transient, thus providing control against continuous production of IL2 and subsequent abnormal

lymphocyte growth. Comparison of the regulation of the IL2 gene with that of other inducible genes, i.e., γ interferon and c-myc distinguishes the IL2 gene regulation in several aspects. The accumulation of mRNA and c-myc does not require prior protein synthesis; moreover c-myc, unlike IL2 (17 and unpublished observation), could be induced by treatment of resting cells with CHX alone (21). γ interferon can be superinduced by CHX (22) but, unlike IL2 (17), also by DRB (22).

Antibody 9.6 did not prevent CHX from augmenting the accumulation of IL2 mRNA. In contrast, CsA, which was a less effective inhibitor of IL2 mRNA accumulation when added at 3 hr after induction (see Figure 4, lanes A & B), was able to block IL2 mRNA superinduction. The effect of CsA may be attributed to its ability to suppress directly IL2 gene transcription (16). This conclusion is further supported by the finding that the transcription inhibitor DRB (18) also blocked CHX effect. The ability of DRB to inhibit IL2 mRNA superinduction indicates that this phenomenon requires the formation of new IL2 transcripts.

The mechanism of suppression of IL2 mRNA by antibody 9.6 in these studies could, in principle, be attributed to two different possibilities. First, antibody 9.6 specifically causes degradation of IL2 mRNA. This possibility is unlikely since antibody 9.6 did not decrease the levels of IL2 mRNA induced by PHA-P alone which induced lower levels of IL2 mRNA. Moreover, antibody 9.6 did not prevent IL2 mRNA enhancement induced by CHX treatment. Second, antibody 9.6 may suppress IL2 mRNA accumulation by down regulation of the IL2 gene at a transcriptional and/or posttranscriptional level. Thus the emerging picture of this study is that antibody 9.6-induced signals appear to compete with signals necessary for IL2 mRNA accumulation, whereas CsA interfers directly with IL2 gene transcription events. The nature of the signals

regulated by the 50 kd and the site for competition remain to be elucidated. Transmembrane fluxes of calcium, leading to increased free cytosolic calcium concentration ($[Ca^{2+}]i$), have been demonstrated following mitogen binding to lymphocytes and have been implicated in the initiation of cell proliferation (23-24), whereas the effect of TPA on lymphocyte proliferation occurs in the absence of extracellular Ca^{2+} or detectable changes in free cytosolic calcium concentration (24,25). It was recently suggested that mitogen can induce lymphocyte proliferation by Ca^{2+} independent signals (26). In the present study as well as in a previous study (4), we have shown that antibody 9.6 affected lymphocyte response induced by PHA as well as TPA or synergistic combination of the two mitogens. These results suggest that the 50 kd molecule regulates a signal transduction that is not solely attributed to increase in Ca^{2+} flux. Thus it is possible that this molecule also regulates calcium independent signals induced by the combination of PHA and TPA. Activation of human lymphocytes via the 50 kd molecule with mitogenic combination of monoclonal antibodies $T11_2$ and $T11_3$ has been shown to require exogenous Ca^{2+} (27). However, the study of O'Flynn et al (28) demonstrated that antibodies to T11 molecule (OKT11 or LFA-2) blocks the increase in free cytosolic calcium that occurs if lymphocytes were stimulated by PHA but not if stimulated with concanavalin A (Con A) or mitogenic anti-T_3 monoclonal. These findings further support the conclusion that other calcium independent signals are regulated by the 50 kd surface protein.

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Table I

The ability of antibody 9.6 to suppress detectable IL2 levels in supernatants of J32 cells stimulated with PHA and TPA is dependent on TPA concentration

TPA Concentrations[1] (ng/ml)	IL2 U/ml		% Suppression by antibody 9.6
	-9.6	+9.6	
0.1	4.8	1.2	74
0.25	9.6	2.3	76
0.5	16.0	3.5	78
0.75	280	175	37
1.0	289	202	30
10.0	319	296	7
J32 cells + PHA-P (20 ug/ml)	70	64	8

[1] 4×10^6/ml J32 cells were stimulated with PHA (PHA-M, 5 ug/ml) and the indicated concentration of TPA in the presence or absence of of antibody 9.6. Supernatants were harvested 24 hours later and assayed by IL2 microassay as described in Materials and Methods.

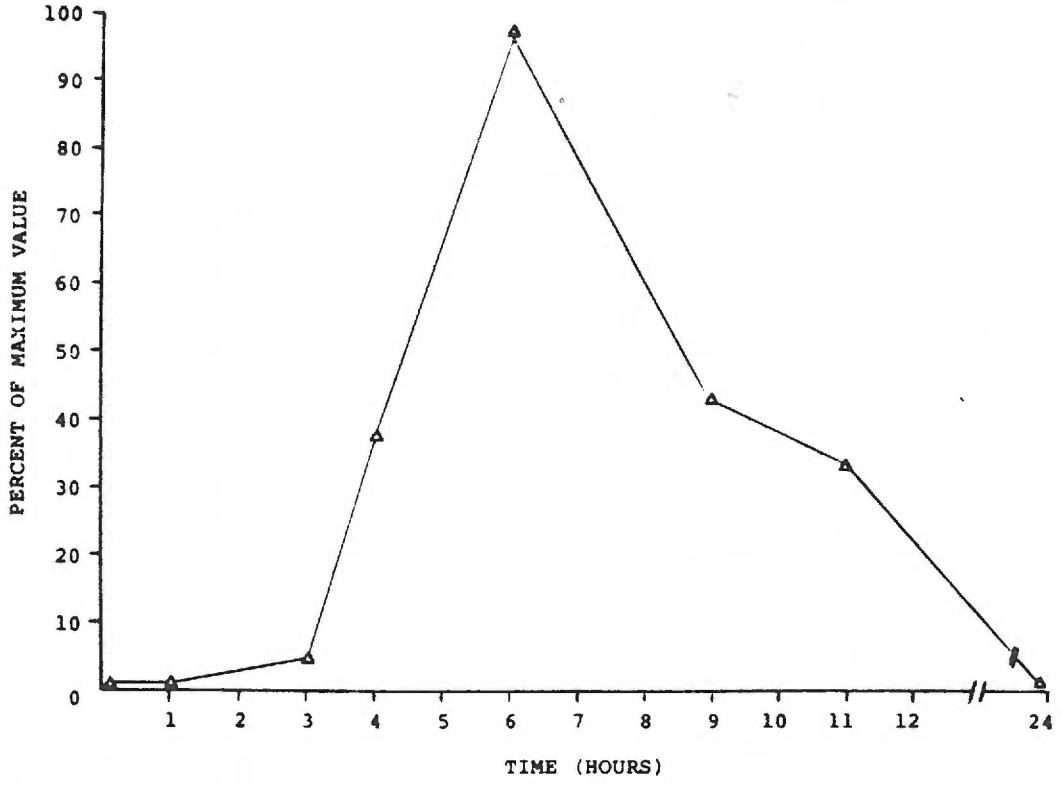

Figure 1. Time course of IL2 mRNA accumulation in J32 cells stimulated with PHA and TPA. $2x10^6$ J32 cells were stimulated with PHA (PHA-M, 5 ug/ml) and TPA (1.0 ng/ml). At the indicated times, cells were harvested and processed for cytoplasmic dot blot hybridization with a ^{32}P labeled cDNA probe for human IL2. Three dilutions were spotted for each time point. Autoradiograms were quantitated by scanning densitometry as described in Material and Methods and relative mRNA levels expressed as percentage of the maximal mRNA level detected (6 hr after stumulation).

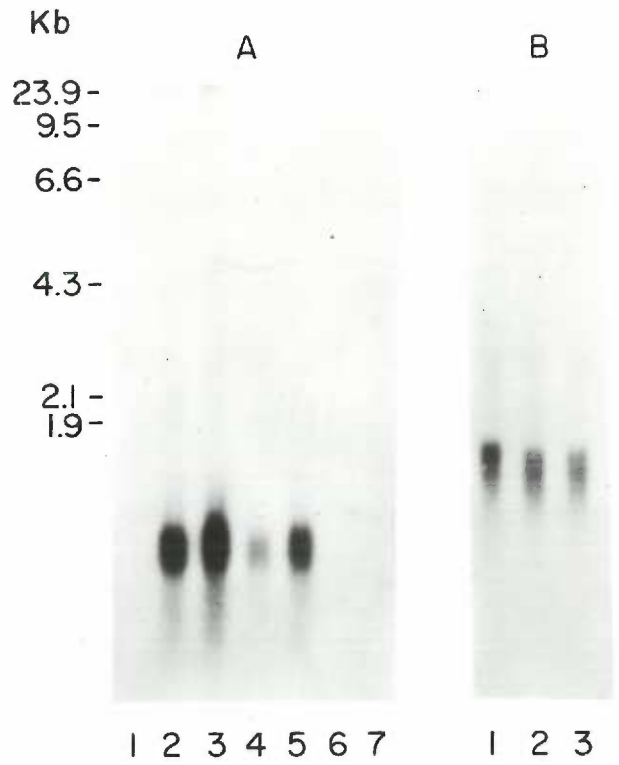

Figure 2. Antibody 9.6 and CsA suppress IL2 mRNA accumulation in J32 cells stimulated with PHA and TPA. J32 cells $4x10^6$ cells/ml were cultured with PHA (PHA-M, 5 ug/ml) and TPA (1.0 ng/ml) with and without antibody 9.6 (1:100) or CsA (1 ug/ml). Six hours after induction, total RNA was isolated and electrophoresed, transferred to nitrocellulose membrane. A. RNA blot was hybridized to a nick translated ^{32}P labeled human IL2 cDNA. Lane 1, 20 ug RNA from unstimulated cells; lanes 2 and 3 are 10 and 20 ug RNA from stimulated J32 cells. Lanes 4 and 5 are 10 and 20 ug RNA from stimulated J32 in the presence of antibody 9.6. Lanes 6 and 7 are 10 and 20 ug RNA from stimulated Jurkat in the presence of CsA. B. RNA blot was hybridized to a nick-translated ^{32}P labeled HLA cDNA. Lane 1, 5 ug RNA from stimulated cells; lanes 2 and 3 are 5 ug RNA from stimulated cells in the presence of antibody 9.6 and CsA respectively.

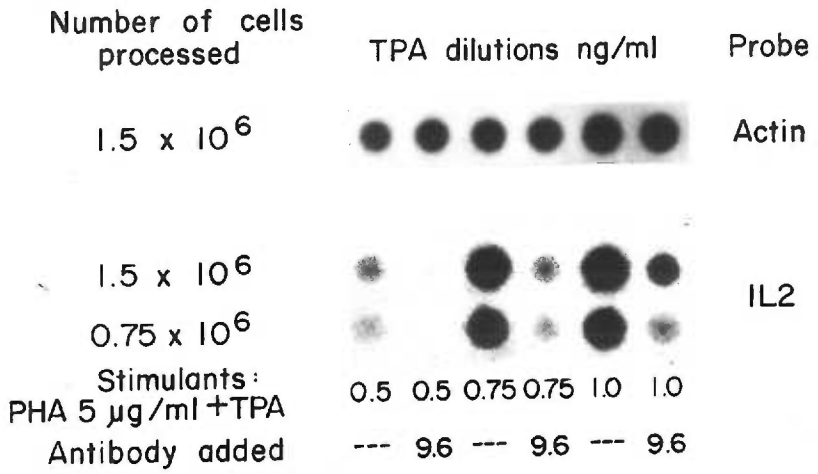

Figure 3. The ability of antibody 9.6 to suppress IL2 mRNA accumulation in J32 cells is dependent on concentrations of TPA added with PHA. J32 cells ($4x10^6$ cells/ml) were cultured with PHA (PHA-M, 5 ug/ml) and increasing concentrations of TPA (0.5 to 1.0 ng/ml) with and without antibody 9.6. Six hours after culture stimulation, cells were processed for cytoplasmic dot blot hybridization with a nick translated ^{32}P labeled cDNA probe for human IL2 or β-actin.

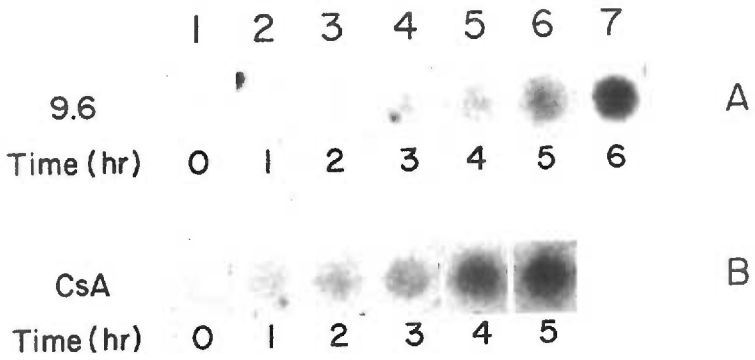

Figure 4. Inhibition of IL2 mRNA accumulation by antibody 9.6 and CsA shows different time courses. Antibody 9.6 (lane A) or CsA (lane B) was added to PHA and TPA stimulated J32 ($2x10^6$) at the indicated time. Six hours after stimulation, cells were harvested and processed for cytoplasmic dot blot hybridization.

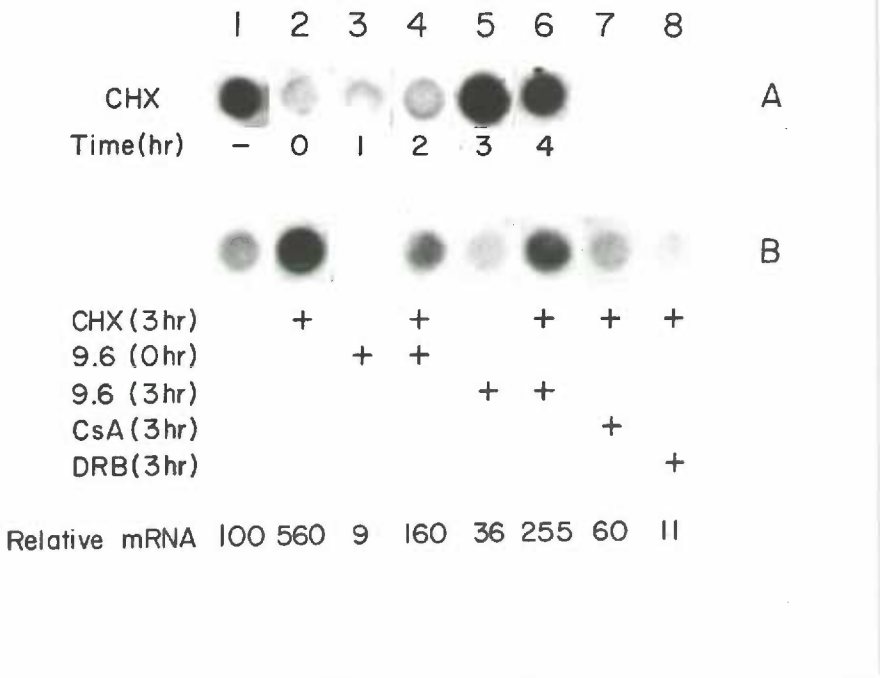

Figure 5. Lane A: Superinduction of IL2 mRNA by mitogens in the presence of CHX. To J32 cell cultures ($2x10^6$) stimulated with PHA and TPA; CHX was added at the indicated times following stimulation. Six hours after stimulation cells were harvested and processed for cytoplasmic dot blot hybridization. Lane B: The effect of antibody 9.6 on IL2 mRNA superinduction by mitogens in the presence of CHX. To PHA and TPA stimulated J32 cells antibody 9.6, CHX (20 ug/ml), CsA (1 ug/ml), DRB (40 uM), or combinations of these agents were added at the indicated time. Six hours hr after stimulation cells were harvested and processed for cytoplasmic dot blot. Relative mRNA is % of IL2 mRNA detectable in inhibitor-free stimulated J32 cells was determined by densitometry as described in Materials and Methods. Lane A and Lane B represent two independent experiments. For Lane B short exposure autoradiogram was selected to demonstrate the relative enhancement in IL2 mRNA.

SUMMARY AND DISCUSSION

A) Summary of the Principal Findings

To determine the mechanism(s) of the functional property of the 50 kd (T11) molecule in human T lymphocyte proliferation, the effect of a monoclonal antibody to the 50 kd protein on proliferation, IL2 production, IL2 receptor acquisition,and IL2 gene expression in stimulated T lymphocytes was examined. The results indicate:

1) Anti-50 kd antibody 9.6 suppresses T lymphocyte proliferation by down regulating the elaboration of IL2 and the acquisition of IL2 receptors by stimulated lymphocytes. Antibody 9.6 suppressed PBMC proliferation induced by antigen, mitogen PHA and the Phorbol ester TPA. Kinetics of inhibition was dependent on the kinetics of IL2 production in these cultures. IL2 purified to molecular homogeneity restored proliferation in PBMC cultures inhibited by antibody 9.6. The ability of antibody 9.6 to suppress proliferation and IL2 production by PBMC induced by PHA and TPA was inversely related to the concentration of these mitogens. In contrast, immunosuppressant cyclosporin A (CsA) inhibited IL2 production induced by a wide range of mitogen concentration.

2) Antibody 9.6 suppressed IL2 production by PBMC induced by PHA or PHA plus TPA. However, this antibody suppressed IL2 receptor acquisition only if it was induced by PHA in the absence of TPA. Purified IL2 restored IL2 receptor expression in cultures inhibited by antibody 9.6 This restoration is attributable to the ability of IL2 to upregulate its own receptor on activated lymphocytes and not due to proliferation of IL2 receptor bearing cells.

3) Antibody 9.6 supression of IL2 elaboration by stimulated J32 cells was secondary to proportional decrease in the accumulation of IL2 mRNA. The

kinetics of inhibition of cytoplasmic IL2 mRNA accumulation showed that antibody 9.6 can block IL2 mRNA accumulation even after IL2 mRNA began to accumulate in the cytoplasm of stimulated J32 cells suggesting that the antibody induced signals are operative during an extended phase of T-cell activation overlapping with the beginning of accumulation of IL2 mRNA. In contrast, the inhibitory effect of CsA decreased rapidly and became insignificant if CsA was added at the beginning of accumulation of IL2 mRNA.

4) Transient expression of IL2 in stmulated J32 cells could be attributed to regulation of IL2 mRNA by CHX-sensitive de novo protein synthesis. Inhibition of protein synthesis a few hours after stimulation of J32 cells lead to superinduction of IL2 mRNA. This phenomenon was inhibitable by transcription inhibitor DRB and by CsA, but not by antibody 9.6. However, 9.6 reduced the absolute amount of IL2 mRNA detectable under superinduction conditions.

B) Discussion of the Main Findings

To determine the regulatory role of the 50 kd surface protein associated with the E-receptor on human T lymphocytes in T cell proliferation, the effect of monoclonal antibody to the 50 kd on proliferation, IL2 production, IL2 receptor acquisition by stimulated PBMC was examined. Our studies revealed that the suppression of T lymphocyte proliferation by anti-50 kd, antibody 9.6 is attributable, at least in part, to its ability to down regulate IL2 production by stimulated lymphocytes. This conclusion is supported by the finding that 9.6 mediated inhibition of lymphocyte proliferation induced by antigen, PHA or PHA plus TPA was accompanied by a marked and sustained reduction of IL2 levels in supernatants of the inhibited cultures. And the finding that IL2 preparation purified to molecular homogeneity added to 9.6-inhibited cultures restored lymphocyte proliferation in a dose dependent

fashion. Moreover the kinetics of inhibition of lymphocyte proliferation by antibody 9.6 was dependent on the kinetics of IL2 accumulation. Thus antibody 9.6 inhibited lymphocyte proliferation in PHA, MLC, and PPD-induced cultures to comparable levels when added 24,48, and 72 hr respectively after initiation of these cultures.

The relationship between antigen/mitogen induced signals and those induced by the binding of antibody to the 50 kd appear to be competitive in nature. This is suggested by finding that ability of antibody 9.6 to suppress lymphocyte proliferation induced by two different mitogens PHA and TPA was inversely related to these ligands concentrations. In contrast to antibody 9.6, CsA inhibited IL2 accumulation in lymphocytes stimulated with a wide range of mitogens , suggesting a distinct, apparently noncompetitive mechanism of action for this agent.

Antibody 9.6 also mediates its inhibitory effect on PHA-induced lymphocyte proliferation by suppressing the levels of the IL2 receptor. This decrease was attributable both to decrease in the percentage of cells expressing IL2 receptors and to a decrease in the average density of IL2 receptors expressed per cell. However, antibody 9.6 did not suppress IL2 receptor acquisition on lymphocytes induced by a combination of PHA and TPA. The ability of antibody 9.6 to selectively inhibit IL2 production, but not IL2 receptor acquisition on lymphocytes induced in the presence of TPA, suggests that TPA induces IL2 receptor expression by a mechanism distinct from that of PHA.

Purified IL2 was able to abbrogate the inhibition of IL2 receptor acqisition by 9.6 in PHA-stimulated cultures. Thus the failure of T cells inhibited by 9.6 to acquire the IL2 receptor could be related, at least in part, to the suppressive effect of 9.6 on IL2 production. Moreover, IL2 augmented IL2 receptor expression in PHA-stimulated cultures. This finding is

probably not attributable to the proliferation of IL2 receptor-bearing cells (Tac^+), because we observed that IL2 abbrogates the 9.6-mediated inhibition of IL2 receptor acquisition and elevates the PHA-induced expression of IL2 receptors even at 24 hrs before the first round of cell division (76). Furthermore, when PBMC were treated with mitomycin C before culturing, similar augmentation in IL2 receptors was observed. The IL2-mediated increase in IL2 receptor expression suported the conclusion that IL2 may up-regulate expression of its own cellular receptor on human T lymphocytes. Several more recent reports support this finding (77,78). Since IL2 alone was not sufficient to induce expression of its own receptor on resting lymphocytes, its effect on activated lymphocytes indicate that IL2 is only able to amplify the effect of submoptimal amounts of stimuli. These findings point to a primary role of mitogen and antigen in IL2 receptor gene induction and regulation.

Antibody 9.6 mediated down regulation of IL2 production is exerted at a pretranslational level of IL2 gene expression. This follows from the finding that suppression of IL2 levels in the supernatants of stimulated J32 cells was accompanied by proportional decreases in IL2 mRNA detectable in the cytoplasm of these lymphocytes. The finding that IL2 levels in stimulated J32 culture supernatants are proportional to the levels of IL2 mRNA suggest the absence of detectable regulatory effects on IL2 mRNA translation. Similar observations were made in stimulated normal human lymphocytes (79). The kinetics of IL2 mRNA inhibition by antibody 9.6 in stimulated J32 cells were in qualitative agreement with the kinetics of proliferation inhibition in PBMC. This follows from the finding that in stimulated J32 cells, antibody 9.6 suppressed IL2 mRNA accumulation even when this antibody was added after 3 hr, the time at which moderate amounts of IL2 mRNA have accumulated. In stimulated PBMC

antibody 9.6 suppressed lymphocyte proliferation even when added 18 hr after induction, the time at which moderate amounts of IL2 mRNA have accumulated (79, and unpublished observation). These data from two independent experimental systems suggest that the regulatory signals mediated through the 50 kd molecule are important for an extended period overlapping with the accumulation phase of IL2 mRNA. In contrast, kinetics of inhibition of IL2 mRNA by cyclosporin A demonstrate that this agent is effective only when added prior to the appearance of detectable cytoplasmic mRNA. The inhibitory effect of CsA is thought to be exerted at the transcriptional level of IL2 gene expression (80). It is likley that antibody 9.6 may exert its effect at the posttranscriptional phase of RNA processing, maturation, and transport to the cytoplasm.

Transient expression of IL2 in stimulated lymphocytes is attributable to regulation of IL2 mRNA by de novo protein synthesis. Accumulation of IL2 mRNA in J32 cells stimulated by PHA and TPA showed a rapid rate of accumulation followed by a rapid shutoff after peaking at 6 hr. Both the appearance and the shutoff of IL2 mRNA require CHX sensitive protein synthesis. Thus adding CHX the first hour after induction of J32 cells prevent IL2 mRNA accumulation, whereas adding CHX 3-4 hr after induction leads to augmented accumulation of I12 mRNA. Qualitatively similar observations have been found in normal lymphocytes stimulated by PHA (79). However, this need for protein synthesis at early hours of induction of IL2 mRNA in normal lymphocytes was attributed to the need for Interleukin 1 (IL1) synthesis (79). Since our cultures did not include IL1 or Mø, other intracellular protein(s) are required for IL2 mRNA accumulation.

It has been suggested that the rapid shutoff of cytoplasmic IL2 mRNA accumulation is attributable to nontranscriptional regulatory mechanisms (79,80). Here we show that shutoff of IL2 mRNA accumulation in stimulated J32

cells is dependent on protein synthesis. Since these studies were performed with a T cell line, the accumulation of IL2 mRNA and its subsequent decline result from intracellular events and occur in the absence of signals from a second cell type. The most likely mechanism of shutoff of IL2 mRNA accumulation is the synthesis of a protein(s) during the early phase of induction by mitogen. Accordingly, the mitogenic signals induce IL2 gene transcription as well as the necessary regulatory proteins to make the IL2 mRNA and IL2 appearance transient. Moreover, the above regulatory model of the transient expression of IL2 stresses the central linkage whereby external stimuli direct the magnitude of T cell clonal expansion by regulating IL2 gene expression.

Antibody 9.6 did not prevent CHX from augmenting the accumulation of IL2 mRNA. In contrast, CsA, which was a less effective inhibitor of IL2 mRNA accumulation when added at 3 hr after induction was able to block CHX-induced IL2 mRNA augmentation. The effect of CsA may be attributed to its ability to suppress directly IL2 gene transcription (80). This conclusion is further supported by the finding that the transcription inhibitor DRB (81) also blocked the CHX effect. The ability of DRB to inhibit IL2 mRNA superinduction indicates that this phenomenon requires continual transcription of the IL2 gene.

The effect of anti-50 kd antibodies appears to be limited to groups of inducible genes that are involved in T cell activation. Since, in addition to the suppressive effect of antibody 9.6 on IL2 mRNA (this report) and IL2 receptor mRNA (unpublished data), antibody OKT11A has been shown to suppress c-myc but not actin mRNA (82). Moreover, antibody 9.6 has been shown to suppress interferon production by stimulated lymphocytes (83).

The mechanism of suppression of IL2 mRNA by antibody 9.6 in these studies could be, in principle, attributed to two different possibilities. First,

antibody 9.6 specifically may cause degradation of IL2 mRNA. This possibility is unlikely since antibody 9.6 did not decrease the levels of IL2 mRNA induced by PHA-P alone which induced low levels of IL2 mRNA. Moreover, antibody 9.6 did not prevent IL2 mRNA enhancement induced by CHX treatment. Second, antibody 9.6 may suppress IL2 mRNA accumulation by down regulation of the IL2 gene at a transcriptional and/or posttranscriptional levels. Thus it appears that antibody 9.6-induced signals suppress signals necessary for IL2 mRNA accumulation, whereas CsA interfers directly with IL2 gene transcription events. The nature of the signals induced by the antibody 9.6 remain to be elucidated. Transmembrane fluxes of calcium, leading to increased free cytosolic calcium concentration ($[Ca^{2+}]i$), have been demonstrated following mitogen binding to lymphocytes and have been implicated in the initiation of cell proliferation (70-71), whereas the effect of TPA on lymphocyte proliferation, thought to be mediated by activation ofprotein kinase C, occurs in the absence of extracellular Ca^{2+} or detectable changes in free cytosolic calcium concentration (68,70,71). It was suggested that cells stimulated with mitogen can induce proliferation by Ca^{2+} independent signals (84). In the present studies, we have shown that antibody 9.6 affected lymphocyte response induced by PHA as well as TPA or synergistic combination of the two mitogens. These results suggest that the 50 kd molecule regulates a signal transduction that is not solely attributed to increase in Ca^{2+} flux. Activation of human lymphocytes via the 50 kd molecule with mitogenic combination of monoclonal antibodies $T11_2$ and $T11_3$ has been shown to require exogenous Ca^{2+} (85). However, other studies (86) demonstrated that antibodies to the T11 molecule (OKT11 or LFA-2) blocks the increase in free cytosolic calcium that occurs if lymphocytes were stimulated by PHA but not if stimulated with concanavalin A (Con A) or mitogenic anti-T_3 monoclonal. These

findings further support the conclusion that other calcium independent signals are regulated by the 50 kd surface protein.

Thus it is possible that this molecule also regulates calcium independent biochemical events that are common to activation pathways of both PHA and TPA. Both of these mitogens have been shown to affect membrane phospholipid turnover (66,87). Moreover it has been shown that activation of protein kinase C is subject to negative or positive cooperativity by various membrane phospholipids (88). It could be speculated then that the 50 kd structure is a molecule (enzyme?) that has a regulatory effect on membranal phospholipid turnover. This hypothesis further suggests that antibodies binding to various epitopes on this molecule have a specific effect with positive or negative cooperativity in the function of this surface protein. Indeed based on the developments of both types of antibodies to the 50 kd two models have been proposed for its function. Palacios et al (17) have suggested that this protein may be a "negative signal receptor". According to this model the binding of OKT11A to the 50 kd mimic the binding of unidentified endogenous suppressor molecules. Recently however, Meuer et al (15) have suggested that the 50 kd represent an alternative pathway of T-cell activation wherein the binding of two specific antibodies, $T11_2$ and $T11_3$, to the 50 kd mimic the binding of unknown stimulatory natural ligands.

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