

Activators of Protein Kinase C and 5-Azacytidine Induce IL-2 Receptor Expression on Human T Lymphocytes

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Resting human T lymphocytes do not express receptors for interleukin-2, but expression is rapidly induced by exposure to PHA. After maximal expression 2-3 days after stimulation, a progressive decline in receptor number is observed. Receptor expression can be augmented by reexposure to PHA. In this study we show that activators of protein kinase C including phorbol diester, phospholipase C, and the diacylglycerol congener diC₈ also increase IL-2 receptor expression. Moreover, 5-azacytidine, which inhibits cytosine methyltransferase, and hydroxyurea, which inhibits ribonucleotide reductase, also increased receptor number. These studies demonstrate that IL-2 receptor expression can be altered in vitro, and that IL-2 receptor number, in combination with IL-2 secretion, may contribute to the regulation of IL-2-dependent immune responses.

Key words: T-lymphocyte activation, protein kinase C and gene regulation, lymphocyte receptor expression, protein kinase C, 5-azacytidine, IL-2:receptor

After recognition of antigen or lectin, the generation of T-lymphocyte immune responses requires the synthesis of interleukin-2 (IL-2, also known as T-cell growth factor), as well as the synthesis of specific membrane receptors for IL-2. The interaction of IL-2 with its receptor governs the amount of T-cell proliferation. We have examined the human T-cell receptor for IL-2 using monoclonal anti-Tac antibody [1], which specifically recognizes this receptor. IL-2 and anti-Tac cross-compete for cell surface binding, but anti-Tac lacks agonist activity [2,3]. Anti-Tac blocks antigen- and lectin-mediated T-cell proliferation, the generation of cytotoxic lymphocytes, and T-cell-dependent, lectin-mediated, B-cell immunoglobulin production [4]. The receptor has been purified and biochemically characterized [2,3,5]. Recently, cDNAs encoding the IL-2 receptor have been isolated and expressed in eukaryotic cells [6]. Anti-Tac has been tritiated to high specific activity using ³H-sodium borohydride-mediated reductive methylation and used as a probe to evaluate cell surface receptor expression on normal [7,8] and leukemic [9] cell populations. Though IL-2 receptors are not present on resting lymphocytes, they appear within 4-6 hr after T-cell

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stimulation with phytohemagglutinin (PHA). Such cells maximally express 20,000–60,000 receptors per cell based on Scatchard analysis [8]. Of interest, receptor appearance is blocked by inhibitors of RNA and protein, but not DNA synthesis, suggesting that the induction of IL-2 receptors in these cells requires *de novo* transcription of the IL-2 receptor gene [8]. When lectin-activated cells are maintained in culture in IL-2-containing media, a progressive decline in the number of receptors expressed is observed [8], suggesting that IL-2 receptor expression may contribute, at least in part, to the regulation of IL-2 dependent immune responses.

In this study, we have evaluated the possibility that IL-2 receptor expression could be manipulated. We have focused on lectin- or antigen-activated cells that have been maintained in culture in IL-2-containing medium and whose receptor number has declined to less than 25% of maximal expression. We demonstrate that 1) receptor expression can be upregulated by reexposure to the initial stimulus, 2) receptor number is augmented by phorbol diester and other activators of protein kinase C including phospholipase C and diacylglycerol, and 3) receptor expression is increased by the nucleoside analogue 5-azacytidine, which has been used in a variety of systems to evaluate the regulatory role of DNA methylation.

MATERIALS AND METHODS

Cells

Human peripheral blood lymphocytes (PBL) were prepared by ficoll-diatrizoate density gradient centrifugation as previously described [4]. Cells were activated with 0.5 $\mu\text{g}/\text{ml}$ E-PHA (Burroughs-Wellcome, Research Triangle Park, NC). After 3 days of incubation, cells were washed and thereafter maintained at $2\text{--}10 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, and antibiotics, as well as 10% delectinated IL-2 (Cellular Products, Buffalo, NY). Mixed lymphocyte reactions were prepared as previously described [4].

Antibodies, Labeling, and Binding Assay

Anti-Tac was purified using protein A sepharose, and antibody concentration was determined as previously described [2,8]. Purified anti-Tac was tritiated to high specific activity by a modification of the procedure of Tack et al [10] using formaldehyde-mediated reductive methylation with [^3H] NaBH_4 as previously described [8]. The specific activity of the tritiated antibody was 910 Ci/mmol, or 13.6×10^6 dpm/ μg . ^3H -anti-Tac binding was also performed as previously described [8]. Briefly, aliquots of 10^6 viable cells were suspended in binding medium consisting of RPMI 1640 supplemented with 1% bovine serum albumin, 1 mg/ml human immunoglobulin, 25 mM HEPES, and 0.1% NaN_3 . Cells were incubated with 5–15 ng of labeled anti-Tac with varying amounts of unlabeled antibody for 60 min at room temperature. Cell-associated and unbound ligand were separated by passage through gradients of 1 M sucrose, and cells were transferred to vials for liquid scintillation counting. Binding values were corrected for nonspecific binding, determined by incubations in the presence of a 1,000-fold excess of unlabeled anti-Tac.

Restimulation of Previously Activated T Cells

Peripheral blood lymphocytes (PBL) or E-rosette purified T cells were activated for 3 days with E-PHA (0.5 $\mu\text{g}/\text{ml}$), washed, and thereafter maintained in complete

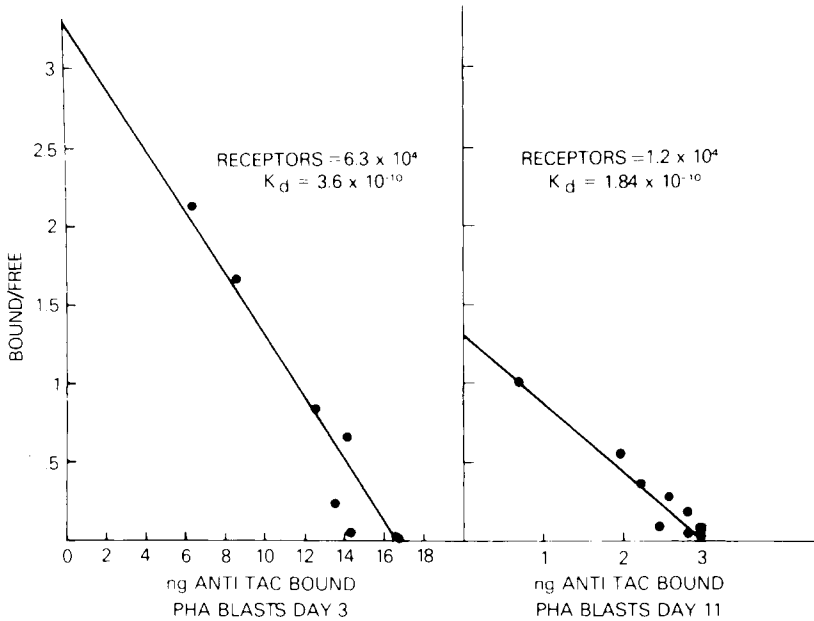


Fig. 1. Scatchard analysis of ^3H -anti-Tac binding to PHA blasts at 3 and 11 days after activation. Left, data are presented for human PBL activated with PHA for 3 days. Right, the same cells depicted on the left were maintained in culture in exponential growth in the presence of IL-2 and binding was performed after a total of 11 days in culture. Similar results were obtained in six additional experiments.

culture medium supplemented with 10% IL-2. At various intervals, cells were restimulated with E-PHA (0.5 $\mu\text{g}/\text{ml}$), phorbol myristate acetate (PMA, 50 ng/ml), phospholipase C (*C. perfringens*: Sigma, St. Louis, MO; 0.1 U/ml), 1–2.5% final concentration interleukin-1 (Genzyme), or 1–9 μM 5-azacytidine (Sigma). Cells were also stimulated with *sn*-1,2 dioctanoyl glycerol (diC_8), the generous gift of Drs. B. Ganong and R. Bell at Duke University. Cells were suspended ($10\text{--}20 \times 10^6$) in 1 ml of medium and diC_8 was added at a concentration of 200 μM . After 60 min at room temperature, cells were diluted to 10^6 cells/ml for the remainder of the incubation.

RESULTS

The Number of Receptors for IL-2 Varies With Time After Cell Activation and Can Be Upregulated by Stimulating Lectin or Antigen

After activation with the mitogenic lectin PHA, peripheral blood lymphocytes or purified T-cell populations maximally express 20,000–65,000 receptors for IL-2 per cell at 48–72 hr after activation [8]. In the experiment depicted in Figure 1, PBL stimulated by PHA for 3 days bound about 16 ng of anti-Tac per 10^6 cells, corresponding to about 63,000 molecules of antibody bound per cell. An aliquot of these cells was maintained in IL-2-containing medium and binding reexamined 11 days after stimulation (Fig. 1, right panel). These cells now bound only 3 ng of anti-Tac per 10^6 cells, representing about 12,000 molecules of anti-Tac bound per cell. A separate

TABLE I. IL-2 Receptor Number Declines With Time on PHA-Activated T Cells Maintained in IL-2-Containing Media*

Days after activation	Receptors/cell
1	7,800
3	20,800
5	14,650
7	13,370
10	11,240
13	5,340

*E-rosette-purified T lymphocytes were stimulated with E-PHA (0.5 $\mu\text{g/ml}$). After 3 days, cells were maintained in IL-2-containing media without removal of PHA. Receptor number was determined by Scatchard analysis of the binding of ^3H -anti-Tac to aliquots of 10^6 cells.

experiment evaluating the kinetics of receptor expression and decline is shown in Table I and Figure 3. In multiple experiments, this decline in IL-2 receptor number was seen whether PBL or isolated T-cell populations were examined and was progressive with time in culture such that by 3 weeks into culture cells expressed 2–10% of the receptors displayed initially (data not shown). While cytofluorographic analysis of such cell populations demonstrated significant heterogeneity in receptor expression, a majority of cells continued to express receptors [8]. Of interest, cells maintained in IL-2-containing media also demonstrated a progressive decline in proliferation with time, paralleling the decline in receptor number [data not shown, 11].

The decline in receptor number, in concert with decreased proliferation, suggested that IL-2 receptor expression could contribute to the regulation of IL-2-dependent immune responses. To determine whether receptor expression could be manipulated, we restimulated lectin-activated lymphoblasts with PHA for 24 hr and found that receptor number increased three to tenfold (Table II and Fig. 3). Cytofluorographic analysis demonstrated that the receptor expression on the entire population of cells had increased, rather than a subpopulation of cells being reactivated (not shown). To ascertain whether or not IL-2 receptor number could be altered on antigen-stimulated cells, T cells activated in allogeneic mixed lymphocyte reactions and maintained in IL-2-containing media were restimulated with the same irradiated non-T cells. As is shown in Table II, receptor number increased by about threefold within 24 hr of stimulation.

Stimulators of Protein Kinase C Increase IL-2 Receptor Expression

Phorbol diesters have been shown to induce IL-2 receptor expression in the acute lymphocytic leukemia line JURKAT [12], which otherwise does not express receptors. When alloantigen- or lectin-activated T cells were incubated with PMA, 10–50 ng/ml, receptor number increased to the same (or a greater) extent as obtained with reexposure to the initial stimulus (Table II and see below). PMA could increase receptor expression indirectly by inducing IL-1 secretion [13] from residual adherent cells, or by directly activating protein kinase C in these cells [14], or by other

TABLE II. Reexposure to Antigen or Lectin Upregulates IL-2 Receptor Number

Cells ^a	Receptors/cell
MLR-T cells ^a	37,700
+ allo non-T	118,000
+ PMA	121,600
PHA lymphoblasts ^b	5,300
+ PHA	24,900

^aMLR-T cells were generated by incubating purified T cells with allogeneic, irradiated non-T cells. After 7 days, cells were maintained in IL-2-containing media. After 12 days in culture, cells were restimulated with allo-non-T cells or incubated with phorbol myristate acetate (50 ng/ml).

^bPurified T cells were activated with PHA for 3 days and then maintained in IL-2 containing media for 12 days followed by restimulation with PHA for 24 hr. Similar results have been obtained in two additional experiments with antigen-stimulated cells, and in eight additional experiments with lectin-activated cells.

TABLE III. PMA and Phospholipase C Increase IL-2 Receptor Expression*

Activator	Receptors/ cell
Media	6,100
PHA	17,300
PMA	31,600
Phospholipase C	22,000
IL-1	8,200

*PBL were activated with PHA, washed after 3 days, and thereafter maintained in IL-2-containing media. After a total of 7 days, cells were incubated in the indicated reagents, and ³H-anti-Tac binding was measured 3 days later. Reagent concentrations were PHA (.5 µg/ml), PMA (50 ng/ml), phospholipase C (.1 U/ml), partially purified IL-1 (1% final concentration). Similar results have been obtained in two additional experiments.

mechanisms not as yet defined. To evaluate these possibilities, we examined the effects of PMA, IL-1, and phospholipase C on IL-2 receptor expression on PHA-activated PBL maintained in IL-2-containing media. As is shown in Table III, lymphoblasts in culture for 10 days displayed approximately 6,100 receptors. Reexposure to PHA, or exposure to PMA, respectively, increased receptor number three- and fivefold. In contrast, IL-1 did not increase expression of IL-2 receptors at concentrations that supported proliferation of purified T cells activated with antigen. Phospholipase C, which can mimic the endogenous activation of protein kinase C by

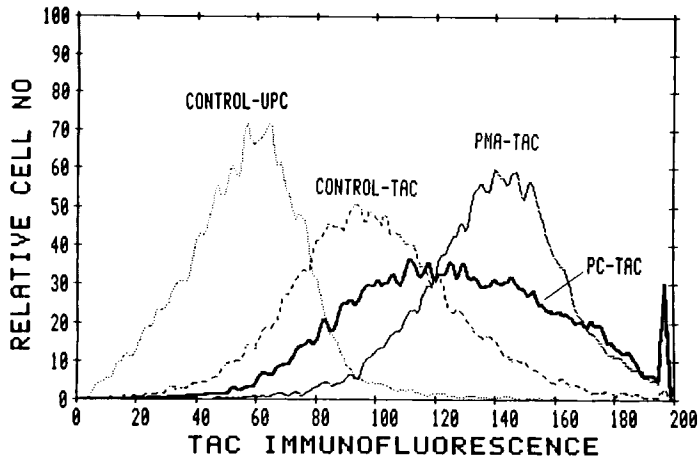


Fig. 2. PMA and phospholipase C increase IL-2 receptor expression. PHA-activated cells maintained in culture in IL-2-containing medium were stimulated with PMA (50 ng/ml) or phospholipase C (PC, 0.1 U/ml), and examined cytofluorographically 24 hr later. Cells were stained with either anti-Tac or a control monoclonal antibody of the same IgG subclass (UPC-10). Fluorescence is depicted on a log scale, with 17 channels representing a doubling of fluorescence.

cleaving phosphoinositol bisphosphate into inositol 1,4,5 triphosphate and diacylglycerol, increased receptor expression about fourfold. These experiments support the hypothesis that PMA augments IL-2 receptor expression via activation of protein kinase C.

Because phospholipase C incubation was associated with significant toxicity, we examined cells treated with PMA or phospholipase C cytofluorographically to exclude binding to a significant number of dead cells, and to evaluate whether each of these treatments augmented receptor number on the entire cell population or only on a subset of cells. As can be seen in Figure 2, both PMA and phospholipase C increased receptor display on the entire population of cells.

To more directly examine protein kinase C activation, we incubated cells with the diacylglycerol congener *sn*-1,2 dioctanoyl glycerol (diC_8). In preliminary experiments, diC_8 (100–200 μM) increased receptor expression three- to fivefold after 24–48 hr of incubation. For example, in one experiment control cells bound 4,600 molecules of anti-Tac per cell. After a 48 hr incubation in diC_8 , cells bound 14,100 molecules of anti-Tac per cell.

Effect of 5-Azacytidine on IL-2 Receptor Expression

The nucleoside analogue 5-azacytidine has been used to evaluate the regulatory role of DNA methylation in a wide variety of systems [15–18]. Specifically, methylation of cytosine residues has been correlated with transcriptionally inactive DNA, and hypomethylation has been associated with increased transcriptional activity. Moreover, 5-azacytidine, by inhibiting cytosine methyltransferase, has resulted in DNA hypomethylation and increased gene transcription [15–18]. We postulated that the degree of DNA methylation could be associated with transcriptional activation of the

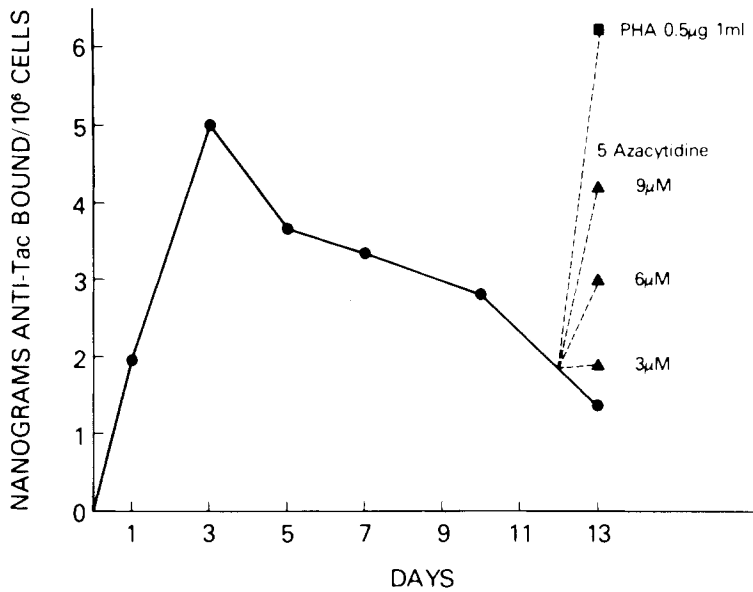


Fig. 3. Decline in IL-2 receptor expression with time in culture and effect of restimulation with PHA or 5-azacytidine. Lymphocytes were stimulated with PHA and maintained in IL-2-containing medium after 3 days in culture. ^3H -anti-Tac binding was measured on aliquots of these cells at the indicated times. On day 12, some cells were incubated with PHA, or the indicated concentrations of 5 azacytidine, and binding was measured 24 hr later.

IL-2 receptor gene. Accordingly, we incubated both freshly isolated PBL and previously lectin-activated cells maintained in IL-2 media with various concentrations of 5-azacytidine. This agent had no obvious effect on freshly isolated PBL. In contrast, in previously activated cells, exposure to 5-azacytidine produced an increase in the number of IL-2 receptors. Figure 3 depicts the number of receptors on E-rosette purified T cells at various times after activation with PHA. On day 12 after activation, cells were incubated with PHA, or 5-azacytidine (3, 6, and 9 μM), and binding was assayed 24 hr later. Control cells displayed 5,300 receptors per cell, whereas increasing concentrations of 5-azacytidine were associated with step-wise increases in receptor number, to 17,000 per cell at 9 μM .

Hypomethylation should be associated with increased gene expression for several cell generations. We therefore examined the effects of 5-azacytidine over a 2 week period of incubation. Cells were activated with PHA for 3 days, washed, and then recultured in IL-2-containing media alone or media containing 7 μM 5-azacytidine (Table IV). Control cells maximally expressed 30,000 receptors per cell, and the receptor number gradually declined to 7,900 at 13 days. In contrast, 5-azacytidine-treated cells continued to increase the number of receptors expressed to about 90,000 per cell at 3 days. Thereafter, the number of receptors continued to fall, to about 22,000 after 13 days in 5-azacytidine.

5-Azacytidine may have multiple effects on cell metabolism in addition to hypomethylation of cytosine residues. Some investigators have attributed some of

TABLE IV. Time Course of 5-Azacytidine Effect on PHA-Activated PBL*

Day of incubation	Media alone	5-Azacytidine
0	28,500	
3	30,000	88,500
5	22,400	47,300
13	7,900	21,600

*PBL were activated with PHA for 3 days, washed, and incubated in 5-azacytidine (7 μ M) in the presence of IL-2 or in IL-2-containing medium alone. 3 H-anti-Tac binding was measured on the days indicated. Similar results have been obtained in one additional experiment.

these effects to inhibition of DNA synthesis, with arrest of cells in the S phase of the cell cycle [19]. We were concerned that 5-azacytidine-treated cells demonstrated decreased proliferation compared to control cells. In preliminary experiments, we have evaluated the effects of hydroxyurea, an inhibitor of ribonucleotide reductase and thus DNA synthesis [20] on IL-2 receptor expression. Treatment with hydroxyurea (33 μ M for 72 hr) was associated with a fourfold increase in IL-2 receptor number in cells stimulated with PHA and then maintained in IL-2-containing media for 2 weeks prior to the experiment. Thus, it is clear that hydroxyurea-mediated inhibition of DNA synthesis is associated with an increase in IL-2 receptor expression. Further experiments will be required to determine whether or not such cells are truly arrested in S phase and whether or not 5-azacytidine also increases IL-2 receptor expression by the same mechanism.

DISCUSSION

IL-2 receptors are not present on resting lymphocytes but appear rapidly after lectin or antigen activation [1,8]. We [8] and others [11] have shown that the number of receptors expressed declines as such cells are maintained in culture in IL-2-containing medium. Moreover, receptor expression is magnified by reexposure to the initial stimulating antigen or lectin. Changes in expression of IL-2 receptors in relation to lectin exposure [11] and increases in receptor number on antigen-specific cloned T cells with reexposure to antigen [21,22] have also recently been described in other laboratories. These findings are consistent with the notion that the number of IL-2 receptors expressed contributes to the regulation of IL-2-mediated immune responses in addition to the amount of IL-2 secreted.

In this study, we have explored strategies to alter IL-2 receptor expression using monoclonal anti-Tac as a probe to measure IL-2 receptor number and distribution on lectin-activated cells maintained in IL-2-containing medium. The finding that phorbol diesters increased receptor expression on such cells was consistent with the de novo induction of IL-2 receptors by PMA on JURKAT acute lymphocytic leukemia cells [12]. However, it was possible that PMA could be acting indirectly via induction of IL-1 secretion from macrophages or directly via protein kinase C activation. Whereas IL-1 had no effect on IL-2 receptor expression, phospholipase C and the diacylgly-

cerol congener diC₈ both augmented receptor display. Much attention has focused on protein kinase C since its discovery in 1977 [23] as a phospholipid-dependent, calcium-activated, noncyclic nucleotide-dependent kinase of wide cellular distribution mediating phosphorylation of serine and threonine residues in multiple proteins [14, 24]. The demonstration of direct activation of protein kinase C by phorbol diesters [25] led to a search for endogenous activators. Such endogenous compounds are produced by the action of phospholipase C on phosphoinositol bisphosphate, after an appropriate ligand-receptor interaction. This cleavage yields diacylglycerol, which binds directly to protein kinase C, and inositol 1,4,5-triphosphate, which results in mobilization of intracellular calcium stores and direct protein kinase C activation [26, 27]. Thus, the demonstration that PMA, phospholipase C, and diC₈ all result in an augmentation in IL-2 receptor expression strongly supports a link between protein kinase C activation and IL-2 receptor expression.

With regard to gene activation, we have also evaluated the effects of 5-azacytidine on IL-2 receptor expression. Several investigators have demonstrated that incorporation of this nucleoside analogue into DNA inhibits cytosine methyltransferase and have been able to correlate induction of gene expression with the resulting hypomethylated state [15–18]. Such correlations have been most clearly demonstrated in systems evaluating cellular differentiation. We considered the possibility that the induction of IL-2 receptor gene activation in resting T lymphocytes could be, at least in part, regulated by such a mechanism. However, incubation of freshly isolated IL-2 receptor-negative resting lymphocytes with 5-azacytidine did not result in receptor display.

In contrast, cells that had already been activated with lectin were susceptible to 5-azacytidine treatment. PBL stimulated with PHA maximally express IL-2 receptors 48–72 hr later, after which a progressive decline in receptor number is observed. When such cells were exposed to 5-azacytidine after 48 hr of PHA stimulation, receptor number continued to increase up to 6 days after initial activation, with about a threefold increase in number of receptors expressed. Thereafter, a decline was observed. When cells were treated 2–3 weeks after PHA activation, at a time when IL-2 receptor expression had declined, 5-azacytidine again resulted in an increase in receptor number, albeit transiently. These effects were consistent with an effect of 5-azacytidine on DNA methylation. However, were one to view regulation of immune expansion and subsequent contraction as regulated by the degree of DNA methylation one would have to hypothesize a bidirectional effect, which would be without precedent. Moreover, although T-cell activation and IL-2 receptor expression are physiologically correlated with a burst of cell proliferation, 5-azacytidine-treated cells often demonstrate decreased proliferation despite maintenance in IL-2 containing medium. Some investigators have suggested that the effects of 5-azacytidine, at least in the induction of hemoglobin production [28], might be due to arrest of treated cells in the S phase of the cell cycle. To evaluate this possibility, we treated cells with hydroxyurea and found that IL-2 receptor expression was also augmented. It is thus clear that treatment with both 5-azacytidine and hydroxyurea can increase IL-2 receptor expression in previously activated lymphoblasts. We are currently actively investigating whether both agents do so by similar or different modes of action.

In summary, we have shown that IL-2 receptor expression varies with time on normal T lymphocytes activated by lectin or antigen, that receptor number appears to correlate with proliferative response to IL-2, and that expression can be magnified by

reexposure to the initial stimulus. We further present two additional strategies for induction of receptor expression. First, PMA and agents that may more closely mimic the endogenous activation of protein kinase C, phospholipase C, and diC₈ result in increased expression of IL-2 receptors. These findings raise a number of important experimental queries: Do antigen and lectin activation also result in activation of protein kinase C? What are the substrates for protein kinase C-mediated phosphorylation in the lymphocyte? What are the mechanisms by which the IL-2 receptor gene is induced following protein kinase C activation? Second, an augmentation in IL-2 receptor expression occurs following treatment with 5-azacytidine or hydroxyurea. Tools are currently available to evaluate the mechanisms of action of each of these agents by taking advantage of specific IL-2 receptor cDNA probes to evaluate gene expression at the transcriptional level.

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