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DELTA-9-TETRAHYDROCANNABINOL (THC) DECREASES THE NUMBER OF HIGH AND INTERMEDIATE AFFINITY IL-2 RECEPTORS OF THE IL-2 DEPENDENT CELL LINE NKB61A2

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Abstract — Treatment of the cloned NK-cell line (NKB61A2) with the major psychoactive marijuana component, delta-9-tetrahydrocannabinol (THC), for 24 h suppressed IL-2-induced proliferation of these cells in the cytokine concentration range of 0.25 – 10 pM suggesting that the drug inhibits the functional activity of the high affinity IL-2R. The proliferation inhibitory effect of THC was accompanied by a decrease in the number of high and intermediate affinity IL-2 binding sites as measured by equilibrium binding studies. However, the expression of Tac protein on the surface of these cells was increased as determined by flow cytometry analysis. THC was also shown to decrease proliferation and the number of IL-2 binding sites of cells previously pulsed with IL-2 and then treated with the drug in the absence of IL-2. These results suggest that THC inhibits IL-2-induced proliferation by modulating the expression of high affinity IL-2 receptors (α/β) required for cell activation and also suppresses the ongoing process of functional receptor expression and clonal expansion of cells previously activated by IL-2. Because the number of intermediate binding sites is decreased following drug treatment along with an increase in the expression of Tac protein (α chain), the lowering of high affinity sites possibly results from a drug-induced depression of β chain expression.

Delta-9-tetrahydrocannabinol (THC), a psychoactive component of marijuana smoke, is reported to inhibit immune functions of macrophages (Huber, Simmons, McCarthy, Cutting, Laguarda and Pereira, 1975; Lopez-Cepero, Friedman, Klein and Friedman, 1986), lymphocytes (Smith, Harris, Uwaydah and Munson, 1978) and natural killer-(NK)-cells (Patel, Borysenko, Kumar and Millard, 1985; Klein, Newton and Friedman, 1987). IL-2 plays a critical role in signalling lymphocytes to proliferate and become biologically active. The cell-surface receptor for IL-2 (IL-2R) is comprised of at least two chains: the α chain (p55) that binds IL-2 with low affinity ($K_d = 10^{-8}$ M) and the β chain (p75) that binds IL-2 with intermediate affinity ($K_d = 5 \times 10^{-10}$ M). Both chains noncovalently associate on the cell surface to form the high affinity receptor ($K_d = 10^{-11}$ M) which plays an important role in transduction of the proliferation signal resulting from IL-2/IL-2R interaction (Leonard, Depper, Uchiyama, Smith, Waldmann & Greene, 1982; Robb & Greene, 1983; Sharon, Klausner, Cullen, Chizzonite & Leonard, 1986; Tsudo, Kozak, Goldman & Waldman, 1986; Teshigawara, Wang, Kato & Smith, 1987; Robb & Greene, 1987). Other proteins appear to be involved in the binding of IL-2 by the β chain (Tsudo *et al.*, 1990) and activation of cells is associated with an upregulation in the production of α chain protein (Sayar, Ketzinel, Gerez, Silberberg, Reshef and Kaempfer, 1990).

Previously we showed that THC suppresssed lymphocyte proliferation induced by IL-2 (Kawakami, Klein, Newton, Djeu, Specter and Friedman, 1988a) and inhibited IL-2 activation of a cloned cell line (NKB61A2) with NK activity (Kawakami *et al.*, 1988b). However, the mechanisms involved in the THC suppression of IL-2/IL-2R system remain uncertain. In this study, we report

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that THC inhibits high affinity receptor mediated IL-2-induced proliferation of NKB61A2 cells. Furthermore, the suppression is associated with a significant decrease in both high and intermediate affinity binding sites but an apparent increase in the expression of cell-surface α chain.

EXPERIMENTAL PROCEDURES

Marijuana components

Δ^9-Tetrahydrocannabinol (THC) was obtained from the National Institute on Drug Abuse (Rockville, MD) in absolute ethanol. For use, the alcohol was evaporated with nitrogen gas and the THC resuspended in dimethyl sulfoxide (DMSO; Sigma Chemical, St Louis, MO). It was further diluted to the desired concentrations in warm tissue culture medium.

Cell culture

The cloned NK-cell line, NKB61A2, which is IL-2-dependent, was derived from C57BL/6 mice as previously described (Warner & Dennert, 1982). Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT), L-Glutamine, antibiotics, and 2-mercaptoethanol (5×10^{-5} M) with 20 units/ml human recombinant IL-2 kindly provided by Hoffmann La Roche, Inc. (Nutley, NJ). Cells were harvested and reestablished in fresh growth medium 24 h prior to each experiment.

Proliferation assay

NKB61A2 cells (1×10^5/ml) were added in 0.1 ml volumes to individual wells of 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA). The IL-2 and other test substances were added in 0.05 ml volumes to the cultures. The cultures were incubated at 37°C for 20 h in 5% CO_2 and 95% air. Subsequently, the cultures were pulsed for 4 h with 0.5 μCi of ^{3}H-thymidine (2.0 Ci/mmole: ICN, Irving, CA), the cells harvested on glass fiber filters, and the incorporated radioactivity determined by liquid scintillation counting.

IL-2 binding assay

A modification of the procedure described by Robb *et al.* (Robb, Greene and Rusk, 1984) was used. Cells (1×10^6) were incubated for either 24 h with THC plus IL-2 or for 2 h with THC minus IL-2. Control cultures containing IL-2, DMSO or medium only were also included. Cells were then washed 3× with warm medium to remove cell-bound IL-2 and THC and resuspended in medium. Next, serial dilutions of radioiodinated human recombinant IL-2 (New England Nuclear Corp., Boston, MA) were added, at concentrations ranging from 1 to 200 pM, to 1×10^6 cells in a total volume of 0.5 ml in either the presence or absence of a 400-fold molar excess of unlabelled human rIL-2 at 4°C for 2 h. The cultures were shaken gently every 30 min during incubation. In order to separate cell bound counts from soluble counts, the suspensions were then layered over a 0.2 ml cushion of 25% lymphocyte-M (Cedarlane Laboratories Ltd, Ontario, Canada) in 0.4 ml eppendorf tubes and centrifuged at 14,000 revs/min for 1 min. These preparations were frozen to −70°C and the tips of the tubes containing the cell pellets were cut off. The radioactivity present in the pellet and in the supernatant was counted in a gamma counter. Specific binding was calculated by subtracting nonspecific binding (tubes containing a 400-fold molar excess of unlabelled IL-2) from the total binding. The calculated values for the number of binding sites per cell (capacity) and dissociation constants (K_d) were derived by Scatchard analysis of binding data by using the Enzfitter Computer Program (Elsevier Biosoft, Cambridge, U.K.). All binding samples were done in duplicate.

Flow cytometry

NKB61A2 cells (1×10^6/ml) were incubated for 2, 4, 8 and 24 h with medium alone, DMSO or THC (5 and 10 μg/ml). Cells were washed with cold PBS and viability determined by trypan blue. Cell preparations with viabilities greater than 95% were then treated with a FITC-labelled, rat anti-mouse Tac antibody (clone AMT13; Boehringer Mannheim, Indiannapolis, IN), incubated for 30 min at 4°C, washed and fixed in 1% paraformaldehyde, and analyzed with a Becton – Dickinson, FACScan, five parameter flow cytometer. In some experiments, cells were double-stained with both anti-Tac and phycoerythrin-labelled anti-CD2 (Pharmingen, San Diego, CA) and analyzed by two-color fluorescence.

Statistics

Statistical significance of the drug data was determined by Student's *t*-test using a two-tailed test of significance (Woolf, 1968). A P value of less than 0.05 was considered to be significant.

RESULTS

THC suppresses high affinity receptor mediated proliferation

Our previous data showed that THC suppressed the proliferation of NKB61A2 cells. To explore the

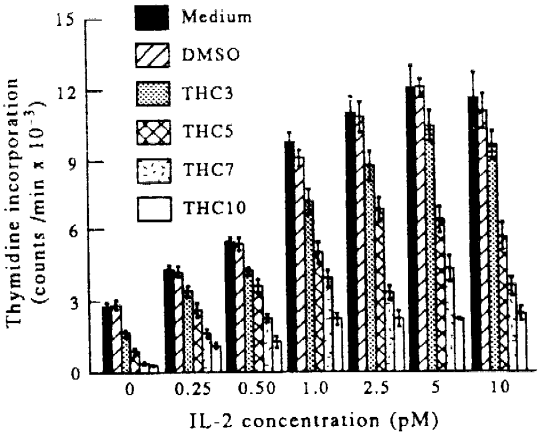

Fig. 1. Suppression of IL-2-induced ^{3}H-thymidine incorporation of NKB61A2 cells by THC. Cells were treated with either culture medium only, DMSO or THC ranging from 3 to 10 μg/ml in the presence of increasing concentrations of IL-2 (except for the 0 group) for 20 h and then pulsed with ^{3}H thymidine for 4 h. Data expressed as mean ± S.E.M. of 4 experiments.

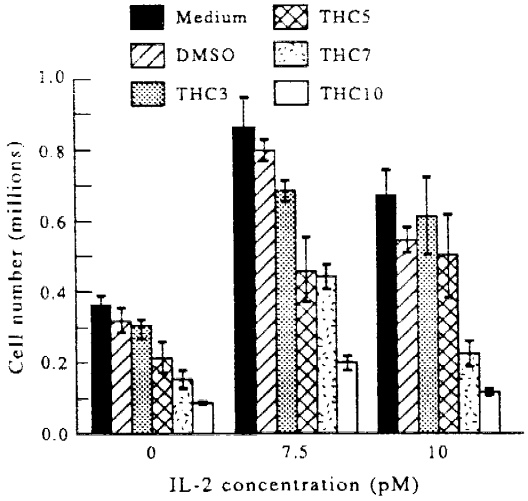

Fig. 2. Suppression of IL-2-induced increase in the number of NKB61A2 cells by THC. Cells (1×10^5), in 1 ml amounts, were incubated in 24-well tissue culture plates with either medium only, DMSO or THC ranging from 3 to 10 μg/ml and increasing concentrations of IL-2 as in Fig. 1. The cells were recovered by centrifugation and counted in a hemacytometer after staining with trypan blue. Data expressed as mean ± S.E.M. of 3 experiments.

involvement of high affinity (10 pM) IL-2R in this effect, we examined cell proliferation in the high affinity range (0.25 – 10 pM) of IL-2 concentrations. Figure 1 (solid bars) shows that cell proliferation, as estimated by the incorporation of ^{3}H-thymidine, increased over a 24 h period with increasing concentrations of IL-2 up to a maximum at 2.5 pM. A slight increase in ^{3}H-thymidine incorporation was noted at 5 and 10 pM IL-2, but this was not significantly ($P>0.05$) different from the 2.5 pM level. Not unexpectedly, the cells incorporated a low level of ^{3}H-thymidine in the absence of IL-2 (0 group) because of continued cell cycling following IL-2 removal (Sayar *et al.*, 1990). These results suggest that NKB61A2 cells express and proliferate through the action of high affinity IL-2R. To test whether THC was suppressing IL-2-induced proliferation mediated through these receptors, proliferation studies were repeated in either the presence or absence of varying amounts of THC or DMSO. Figure 1 shows that THC significantly ($P<0.05$) suppressed proliferation beginning at a concentration of 5 μg/ml, and further that the drug effect occured at all cytokine concentrations tested.

We also examined drug effects on the increase in cell number. Similar to the above results employing ^{3}H-thymidine incorporation, the number of NKB61A2 cells continued to increase at a low level in the absence of IL-2 going from an input number of 10^5 cells/well to approximately 3.5×10^5/well (Fig. 2; 0 group). THC at 5, 7 and 10 μg/ml significantly ($P<0.05$) suppressed this small increase in cell number but not below 10^5 suggesting that cell lysis was not the cause of the decrease in proliferation. Similar results were obtained with cells exposed to IL-2, with THC decreasing the expansion of the cell population but never below the input number. The cell viability was the same as in all cultures (data not shown). Taken together these results suggest that THC inhibits IL-2-induced cell activation and subsequent proliferation in the high affinity range of cytokine concentrations.

Decrease in high and intermediate affinity receptors on NKB61A2 cells

To investigate the effect of THC on the number of cell surface high and intermediate affinity binding sites, NKB61A2 cells were incubated with drug and IL-2 for 24 h as in the previous studies and then tested for the number of high and intermediate IL-2 binding sites using an IL-2 receptor competitive binding assay. Figure 3 shows the binding curves of a representative experiment wherein the cells were

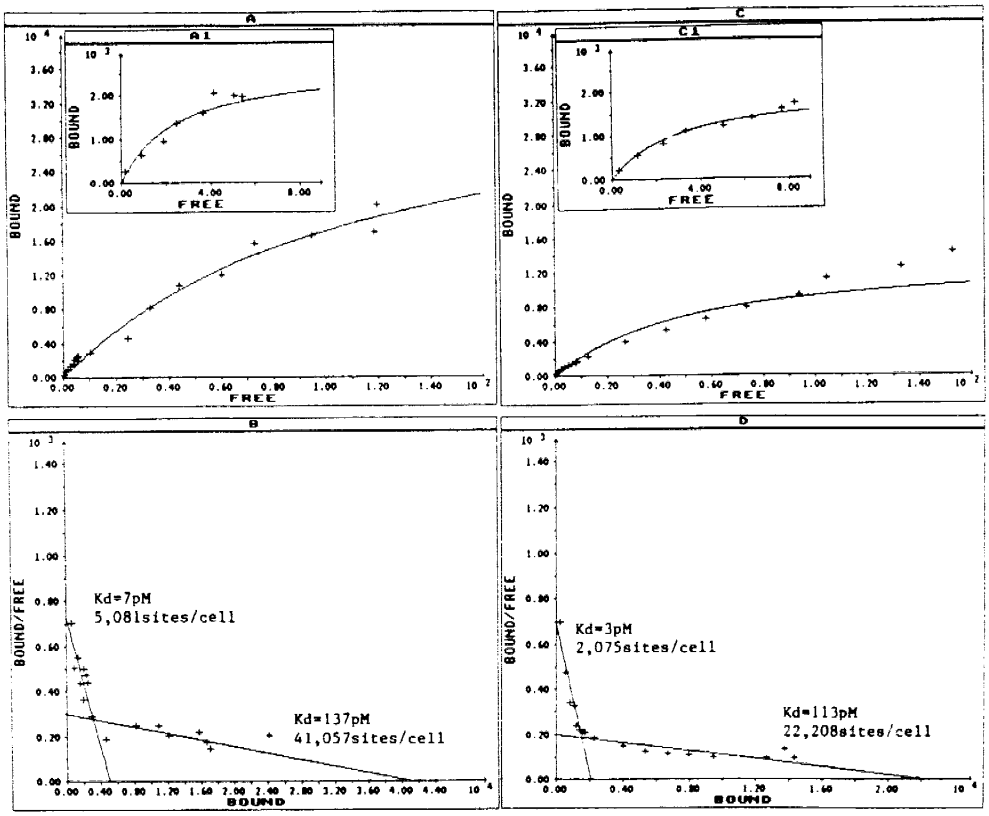

Fig. 3. Suppression of high and intermediate IL-2 receptors on NKB61A2 cells by THC. Cells were incubated with IL-2 and either DMSO (panels A and B) or 10 μg/ml THC (panels C and D) for 24 h. Following incubation, the cells were washed, divided into aliquots, and incubated for 2 h at 4°C in varying concentrations of ^{125}I-labelled and unlabelled IL-2 (see Experimental Procedures). Saturation binding curves are depicted in panel A (DMSO) and panel C (THC 10 μg/ml). Detailed curves of the high affinity binding ranges of A and C are shown in insets A1 and C1. Scatchard plots are shown in panel B (DMSO) and panel D (THC 10 μg/ml). These results are representative of 3 experiments.

Table 1. THC pretreatment decreases the number of high and intermediate affinity IL-2 receptors of NKB61A2 cells*

	High affinity		Intermediate affinity	
Treatment	No. of sites	K_d(pM)	No. of sites	K_d(pM)
2 h incubation				
DMSO	6541 ± 362	10.2 ± 2.4	66,495 ± 2585	118.4 ± 17.3
THC (5 μg/ml)	6286 ± 119	9.3 ± 1.3	63,367 ± 4810	118.8 ± 8.2
THC (10 μg/ml)	2798 ± 53†	3.1 ± 0.4	26,894 ± 489†	110.3 ± 11.1
24 h incubation				
DMSO	5710 ± 598	11.3 ± 1.3	43,263 ± 2375	146.6 ± 39.8
THC (5 μg/ml)	4720 ± 361	7.2 ± 1.5	39,862 ± 1181	144.1 ± 6.6
THC (10 μg/ml)	2125 ± 50†	3.2 ± 0.2	†27,029 ± 4821†	148.2 ± 35.3

*The number of binding sites per cell (capacity) and dissociation constant (K_d) were derived from Scatchard analysis. Each number represents the mean ± S.E.M. of 3 experiments.
†Significantly different from DMSO group. $P<0.01$.

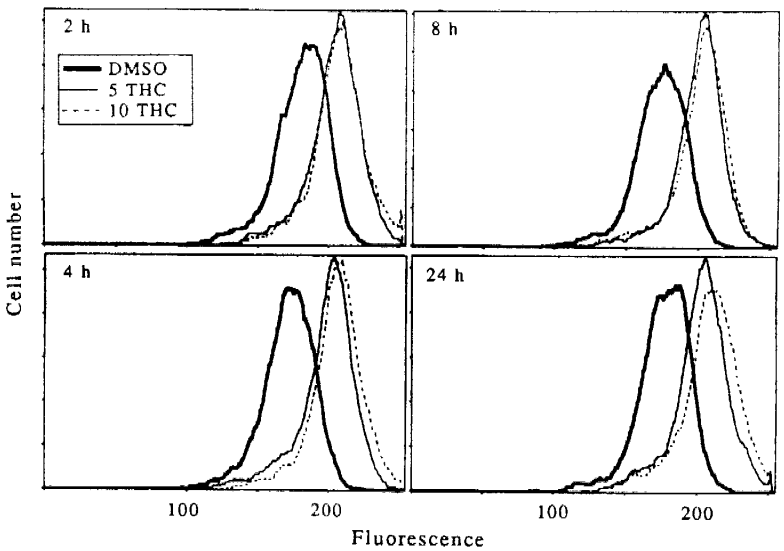

Fig. 4. THC treatment increases the cell-surface density of IL-2α epitopes. NKB61A2 cells were incubated for 2, 4, 8, and 24 h with either medium only, DMSO or THC (5 or 10 μg/ml) and recombinant IL-2. At the times indicated, cells were harvested and stained with trypan blue to determine viability. If the cells were greater than 95% viable, they were stained with antibody to IL-2α (anti-Tac), and analyzed by flow cytometry to determine the surface density of IL-2 receptor α. The relative fluorescent intensity of medium only treated cells (data not shown) was no different from the DMSO-treated cells. These results are representative of 3 experiments.

treated with either DMSO and IL-2 (panels A and B) or THC and IL-2 (panels C and D). With IL-2 concentrations ranging from 1 to 200 pM, two classes of IL-2 receptors were detected on the NKB61A2 cells; a high affinity receptor (K_d = 7 pM, 5081 sites/cell) and intermediate affinity receptor (K_d = 137 pM, 41,057 sites/cell). The 7 pM high affinity K_d is consistent with the proliferation data observed in Fig. 1 wherein maximal proliferation was observed between 2.5 and 10 pM IL-2. Exposure of the cells to THC (panels C and D) caused a significant decrease in the number of cell-surface high and intermediate affinity IL-2 binding sites relative to the DMSO-treated cells. The K_ds, however, stayed relatively constant following drug treatment. Table 1 shows the number of binding sites and K_d data obtained from combining 3 experiments. After 24 h of drug treatment, THC at 5 μg/ml, had a slight effect on the number of sites but, at 10 μg/ml, significantly ($P<0.01$) reduced the number of high affinity sites greater than 50%. Table 1 also shows binding data of cells treated with THC for 2 h in the absence of IL-2. This was done because we had observed (see Fig. 1) that the drug decreased proliferation in the absence of IL-2. The results show that the number of binding sites is higher in cells incubated for 2 h in the absence of IL-2 (compare DMSO samples of 2 and 24 h groups) probably because of less receptor modulation (Tsudo, Karasuyama, Kitamura, Nagasaka, Tanaka & Miyasaka, 1989). Table 1 also shows that the number of binding sites is reduced by the drug suggesting that THC can exert its effect on unoccupied receptors.

Effect of THC on expression of Tac protein on the surface of NKB61A2 cells

The above results suggest that THC treatment decreases cell proliferation by decreasing the expression of IL-2R α/β heterodimers (high affinity) and β chain (intermediate affinity). To gain information on the low affinity IL-2 binding moiety, we examined the effect of THC on surface expression of Tac protein by flow cytometry. NKB61A2 cells were incubated with IL-2 and drugs, and at 2, 4, 8, and 24 h samples were removed and prepared for flow cytometry analysis. As seen in Fig. 4, THC treatment shifted the peak fluorescence to the right suggesting the Tac protein expression on the cells was enhanced. This enhancement was observed as early as 2 h following incubation with THC 5 μg/ml. In some experiments, the cells were analyzed for, in addition to Tac, the surface density of CD2. Under these conditions, no shift of the

fluorescent peak was observed for anti-CD2 suggesting that drug treatment had no effect on the surface density of this epitope (data not shown).

DISCUSSION

THC suppresses lymphocyte proliferation but the molecular mechanisms of this effect are poorly understood. Early reports suggested that the drug either inhibited macromolecular transport (Desoize, Leger & Nahas, 1979) or the activity of several membrane enzymes (Greenberg & Mellors, 1978; Sadler, Sharom & Mellors, 1984). However, the ways in which these changes compromised lymphocyte proliferation were not elucidated. Because of the importance of IL-2/IL-2R interaction in lymphocyte proliferation, several years ago we examined the effect of THC treatment on the proliferation of the IL-2-dependent cell line, NKB61A2. We observed that the drug suppressed the enhanced cytolytic function and proliferation of these cells in response to IL-2 stimulation (Kawakami *et al.*, 1988b). We hypothesized that THC inhibited the binding of IL-2 to the IL-2R and/or inhibited molecular events following IL-2 binding.

Because of the relatively high concentrations of IL-2 used in our initial study (Kawakami *et al.*, 1988b) and the complexity of IL-2R system, it was important to establish that NKB61A2 cells utilized high affinity receptors for proliferation and, therefore, that THC was interfering with proliferation mediated through these receptors. Figure 1 suggests that NKB61A2 cells have an effective display of high affinity IL-2R because maximum proliferation was observed at IL-2 concentrations below 10 pM. Furthermore, the THC effect on these cells appears to be mediated at least partially through these receptors because the drug suppressed proliferation in this IL-2 concentration range. This is the first report, to our knowledge, suggesting that this cell line possesses functional high affinity IL-2R receptors rather than intermediate or low affinity receptors demonstrated on either other cell lines (Robb & Green, 1987) or freshly isolated lymphocytes (Aribia *et al.*, 1989). These results also indicate that THC treatment might be affecting IL-2/IL-2R interaction at the level of the high affinity receptor. Accordingly, we next examined the drug effects on the number of high and intermediate affinity binding sites using a receptor binding assay in the IL-2 range of $1-200$ pM. Under these conditions, drug treatment in the presence of IL-2 was noted to significantly suppress the number of high and intermediate affinity binding sites. This finding, along with previous reports establishing that these two receptor types are important for proliferation (Robb & Greene, 1987; Tsudo *et al.*, 1990), supports the conclusion that a portion of the THC effect on cell proliferation is related to a drug-induced decrease in the functional expression of these receptors.

During lymphocyte activation, IL-2Rα and IL-2Rβ chains associate with each other to form the high affinity (IL-2Rα/β) complex needed for cell activation (Robb & Greene, 1987; Tsudo *et al.*, 1990). In addition, IL-2Rα chain protein synthesis is increased (Aribia *et al.*, 1989; Sayar *et al.*, 1990). It is possible, therefore, that THC inhibits the expression of functional receptors by either blocking IL-2 binding to functional receptors or inhibiting the production and cell surface expression of either α or β chains. Of these possibilities, direct competitive blocking by THC was not likely because the cells were washed extensively to remove bound THC. However, decreased production of at least the β chain was suggested by the finding that the number of intermediate affinity binding sites was decreased (Table 1). Regarding α chain expression, we examined the cell surface expression of the Tac epitope following treatment with THC and IL-2. Figure 4 shows that even though the number of high affinity sites is reduced by THC treatment, the surface density of Tac actually increases suggesting that the drug is suppressing the expression of the α/β high affinity complex by inhibiting β chain production rather than α chain production and expression. We are currently examining by Northern blotting and immunoprecipitation drug effects on IL-2Rα and β cellular mRNA and protein levels in order to more precisely distinguish among these possibilities. It is also possible that THC is augmenting the shedding of the α/β complexes or the β chains from the cell surface and we are currently also examining this possibility.

Another finding of interest involves THC suppression of functional IL-2R expression and proliferation in the absence of exogenous IL-2. This suggests that the drug can inhibit molecular events which sustain cell cycling and proliferation subsequent to the signal delivered by IL-2 binding. These molecular events involve signal transduction mechanisms resulting in the activation and repression of a variety of genes (Mills, Girard, Grinstein & Gelfand, 1988). Our results show that THC can negatively impact these events including maintaining the expression of functional high

affinity IL-2R. In summary, it appears that THC treatment suppresses IL-2-induced lymphocyte proliferation by reducing the capacity of the cell to display functional high affinity receptors and also by inhibiting mechanisms of signal transduction and gene expression responsible for continued cell cycling and proliferation following the initial IL-2 activation.

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