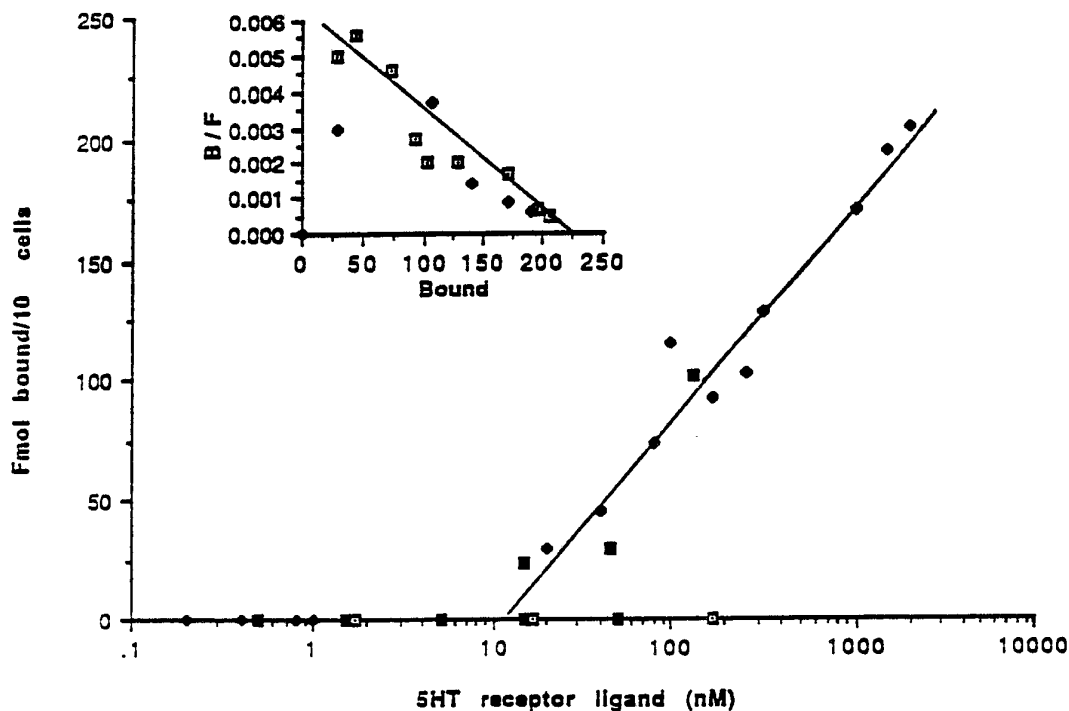




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(54) Title: REGULATION OF T-CELL PROLIFERATION VIA A NOVEL 5HT_{1a} RECEPTOR



(57) Abstract

A method of regulating proliferation of activated T cells exhibiting a 5HT_{1a} receptor by introducing a sufficient amount of agonists or antagonists to either increase or decrease T cell proliferation. The basis for regulating cell proliferation may be (1) via the 5HT receptor, (2) via serotonin synthesis inhibition and/or (3) via serotonin stimulation of CD8⁺ subpopulations of activated T cells.

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TITLE: Regulation of T-Cell Proliferation via a
Novel 5HT1a Receptor

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This is a continuation-in-part application of U.S.
07/578,710, filed September 4, 1990. For purposes of
clarification the novel 5HT2-like receptor has been
10 redesignated as a 5HT1a receptor.

Initial studies demonstrated that a novel 5HT-2-like
receptor was present on transformed Jurkat cells and that
the receptor was present on human T lymphocytes
(activated T-cells). The results of further experiments
15 confirmed that 5HT receptors are present on activated but
not resting T cells, that these 5HT receptors and the 5HT
receptors on Jurkat cells have similar pharmacological
properties, that the 5HT receptors regulate adenylate
cyclase activity and can suppress proliferation of CD4+ T
20 cells and stimulate proliferation of CD8+ T cell
depending upon conditions. The characterization of 5HT
specific receptors on Jurkat cells and activated T cells
by a combination of pharmacological, biochemical and
molecular analyses has confirmed the presence of 5HT
25 receptors and studies link the receptor on Jurkat cells
and activated T cell to the 5HT1a family of receptors,
For Jurkat cells and activated T cells this is based on
the following criteria: binding studies with the 5HT1a
receptor specific agonist (8OHDPAT), studies on signal
30 transduction, northern analysis with specific human 5HT1a
oligonucleotide probes. Resting T cells did not express
5HT1a receptors by these same criteria. These findings
have provided the basis for continuing to explore a
variety of strategies to identify compounds that interact
35 with the receptor or the mechanism of cell proliferation.

The present invention relates to regulation of cell proliferation based on the recognition of a novel serotonin receptor present as a cell surface molecule. The novel receptor is a serotonin receptor linked to the 5 HT2 family. The 5HT2-like receptor is present on neoplastic or tumor cells and activated T-cells, e.g. CD4+ and CD8+.

The present invention also relates to the regulation of cell proliferation based on the recognition that 10 proliferating cells contain/express serotonin. The proliferation of neoplastic or tumor cells and activated T-cells containing serotonin can be decreased by inhibition of serotonin synthesis.

The present invention also relates to the regulation 15 of the proliferation of cell exhibiting the novel 5HT2 receptor by introducing an effective amount of agonists or antagonists to either increase or decrease cell proliferation.

A number of neurotransmitters have been shown either 20 to bind to or to have certain effects on cells of the immune system (for review, see ref. 1). One such neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT), is also a major product of platelets released at sites of inflammation (2-4). Serotonin has been shown to augment 25 IFN induced phagocytosis (5) and suppress IFN induced Ia expression on macrophages (6), to augment NK cell cytotoxicity (7), to affect ion permeability in isolated pre-B lymphocytes (8), and to suppress mitogen stimulated T cell proliferation in in vitro systems (9). 30 Additionally, serotonin and serotonin receptors have been suggested to be required for expression of delayed type hypersensitivity in murine models (10-11).

The functional, pharmacological and molecular properties of serotonin receptors have been characterized 35 in the peripheral and central nervous systems (12). Distinct categories of receptors have been defined which mediate different effects of 5HT(13). Experiments using

5HT antagonists have suggested that T lymphocytes which can transfer delayed type hypersensitivity responses to naive recipients may express 5HT receptors (11). Specific 5HT2 antagonists such as ketanserin or
5 ritanserin will prevent transfer of this response. The purpose of the experiments reported here was to determine if 5HT receptor subtypes could be identified on human T lymphocytes and if 5HT receptors could mediate signal transduction events similar to those previously reported
10 for 5HT receptors in the nervous system. The results show that a human T lymphocyte line (Jurkat) expresses a 5HT2 like-receptor subtype which can mediate phosphatidylinositol hydrolysis and intracellular Ca^{++} mobilization.

15 The activation of resting T lymphocytes is critical to most immune responses since cellular activation allows these cells to exert their regulatory or effector activities. During activation relatively quiescent cells undergo complex changes involving cell differentiation
20 and proliferation. The activation of T lymphocytes is a consequence of ligand-receptor interactions that occur at the interface of the T cell and an antigen-presenting cell. A number of different cell surface molecules on the T lymphocyte and the antigen-presenting cell may
25 participate in the complex cell-cell interaction which occurs during antigen presentation, antigen-induced T lymphocyte activation must involve stimulation of the T cell antigen receptor. Stimulation of the T cell antigen receptor alone is insufficient to induce proliferative
30 responses. Other cell surface molecules expressed on T cells function as accessory molecules. Accessory molecules may function as adhesion molecules, modify the transmembrane signal initiated via the antigen receptor and/or initiate their own transmembrane signaling events.
35 A number of cell surface molecules appear on the surface of the T cell during the events associated with the activation, differentiation, and proliferation of T

cells. T cell proliferation is believed to be regulated primarily through the actions of IL-2 on its specific cell surface receptor. The role of IL-2 includes both autocrine and paracrine effects resulting in the proliferation of other T cells.

Although IL-2 driven T cell proliferation is considered the major mechanism responsible for T cell growth, under some circumstances T cell proliferation occurs independent of IL-2.

In addition to determining that a novel 5HT2-like receptor is present as a cell surface molecule on activated T cells, it has been determined that the novel 5HT2-like can be regulated by inhibiting serotonin synthesis.

The present invention also relates to the regulation of the proliferation of cell exhibiting the novel 5HT2 antagonists to either increase or decrease cell proliferation.

The present invention also relates to regulation of cell proliferation exhibiting the novel 5HT2-like receptor. Said antibodies include a plurality of "types" of antibodies having an epitope or epitopes specific for the 5HT2-like receptor. Such antibody "types" may include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized or human antibodies.

The present invention also relates to the regulation of cell proliferation exhibiting the novel 5HT2-like receptor by generating mimotopes, small peptide ligands, that bind to either the 5HT2-like receptor or to antibodies to the 5HT2-like receptor. Such small peptide ligands would function as agonists and/or antagonists.

Proliferation of normal diploid cells generally requires the presence of a continuous supply of endogenous or exogenous growth factors. Proliferation of tumor cells has generally been found not to require addition of exogenous growth factors. This is due to the ability of tumor cells to produce their own growth

factors, due to changes in growth factor receptors so they are continuously activated or changes in signal transduction elements which result in the continuous activation of tumor cells. Tumor cells may also express
5 unique receptors or produce unique hormones or growth factors which are not usually associated with the parent tissue. Recently it has been shown that expression of 5HT1c or 5HT2 receptors (by gene transfection) in normal diploid murine fibroblasts results in the acquisition of
10 a transformed phenotype in these cells. After introducing cDNA's encoding these receptors, transfected cells express high affinity receptors for 5HT which transduce second messenger signals. These fibroblasts also acquired the ability to form foci in tissue culture,
15 to grow in soft agar and to form tumors in nude mice. This raises the possibility that synthesis of serotonin and aberrant expression of 5HT receptors may play an important role in tumorigenesis. Normal cells may acquire a transformed phenotype by acquiring the ability
20 to synthesize serotonin and/or serotonin receptors which can transduce second messenger pathways. Results presented here show (1) proliferating cells contain serotonin, (2) inhibition of serotonin synthesis inhibits cell proliferation by tumor cells but not by normal
25 cells, and (3) proliferation of tumor cells but not normal cells is inhibited by certain serotonin receptor antagonists.

SUMMARY OF THE INVENTION

30 A method of down-regulating proliferation of cells exhibiting a 5HT2-like receptor comprising, functionally decreasing the amount of serotonin available for binding to said 5HT2-like receptor or functionally reducing the availability of 5HT2-like receptor binding sites. This
35 method applies to neoplastic or tumor cells and activated T-cells. The activated T-cells can be IL-2 dependent or IL-2 independent. The proliferation of the cells is

decreased by administering a sufficient amount of a compound to inhibit the activity of the enzyme tryptophan hydroxylase thereby inhibiting serotonin synthesis. An example of such a compound is p-chlorophenylalanine.

5 A method of down-regulating proliferation of cells exhibiting a 5HT2-like receptor comprising functionally reducing the availability of 5HT2-like receptor binding sites, the proliferation is decreased by introducing an amount of at least one antagonist sufficient to bind to
10 said 5HT2-like receptor to interrupt cell proliferation. Said at least one antagonist is selected from the class of 5HT receptor ligands. Said class of 5HT receptor ligands includes ritanserin, mesulergine, mianserin, spiperone, mimotopes and antibodies to said 5HT2-like
15 receptor.

A method of up-regulating proliferation of cells exhibiting a 5HT2-like receptor comprising functionally increasing the availability of 5HT2-like receptor binding sites. The proliferation of said cells is increased by
20 introducing an amount of at least one agonist sufficient to enhance cell proliferation. Said at least one agonist is selected from the class of 5HT receptor ligands. Said class of 5HT receptor ligands include pelanserin, ketanserin, methyl serotonin, 8-OH-DPAT, and propranolol.

25 A method of treating a T-cell dependent disease state in a mammal comprising down-regulating proliferation of cells exhibiting a 5HT2-like receptor by functionally decreasing the amount of serotonin available for binding to said 5HT2-like receptor or functionally reducing the
30 availability of 5HT2-like receptor binding sites. The proliferation of the cells is decreased by administering an effective amount of a compound to inhibit the activity of the enzyme tryptophan hydroxylase thereby inhibiting serotonin synthesis. Such a compound includes
35 p-chlorophenylalanine.

A method of treating T-cell dependent disease state in a mammal comprising down-regulating the proliferation of cells exhibiting a 5HT2-like receptor by functionally

reducing the availability of 5HT2-like receptor binding sites wherein the proliferation of said cells is decreased by introducing an effective amount of at least one antagonist sufficient to bind to said 5HT2-like receptor to interrupt cell proliferation. Said at least one antagonist is selected from the class of 5HT receptor ligands. Said class of 5HT receptor ligands include ritanserin, mesulergine, pirenperone, spiperone, mimotopes and antibodies to said 5HT2-like receptor.

10 A method of treating a neoplastic disease state in a mammal comprising down-regulating proliferation of cells exhibiting a 5HT2-like receptor by functionally decreasing the amount of serotonin available for binding to said 5HT2-like receptor or functionally reducing the availability of 5HT2-like receptor binding sites. The proliferation of the cells is decreased by administering an effective amount of a compound to inhibit the activity of the enzyme tryptophan hydroxylase thereby inhibiting serotonin synthesis. Such compounds include p-chlorophenylalanine.

20 A method of treating a neoplastic disease state in a mammal comprising down-regulating proliferation of cells exhibiting a 5HT2-like receptor by functionally reducing the availability of 5HT2-like receptor binding sites wherein the proliferation of said cells is decreased by introducing an effective amount of at least one antagonist sufficient to bind to said 5HT2-like receptor to interrupt cell proliferation. Said at least one antagonist is selected from the class of 5HT receptor ligands. Said class of 5HT receptor ligands include ritanserin, mesulergine, pirenperone, spiperone, mimotopes and antibodies to said 5HT2-like receptors.

30 A method of treating an immune deficient disease state in a mammal by up-regulating proliferation of T-cells exhibiting a 5HT2-like receptor comprising functionally increasing the availability of 5HT2-like receptor binding sites. The proliferation of said cells

is increased by introducing an effective amount of at least one agonist sufficient to enhance cell proliferation. Said at least one agonist is selected from the class of 5HT receptor ligands. Said class of
5 5HT receptor ligands include pelanserin, ketanserin, methyl serotonin, 8-OH-DPAT, propranolol, and mianserin.

BRIEF DESCRIPTION OF THE DRAWINGS

- 10 Figure 1 is a graph demonstrating 5HT binding to Jurkat cells. Jurkat cells (1×10^6) were incubated with the indicated concentrations of (^3H)5HT for 6 min at 4°C in the absence (total binding) or presence of $50 \mu\text{M}$ 5HT (nonspecific binding). Specific binding ($\square$) is
15 expressed as the difference between these two values. Figure 1A shows the results graphed on a Scatchard plot. Figure 2 is a graph demonstrating kinetics of association and dissociation of (^3H)5HT to Jurkat cells. (A) Time course of association of (^3H)5HT to Jurkat cells.
20 Conditions were as described in Materials and Methods except that time of incubation was varied ($\square$). (B) Time course of dissociation of bound (^3H)5HT ($\square$) from Jurkat cells.
- 25 Figure 3 is a graph demonstrating comparison of the effect of OKT3 and 5HT on intracellular Ca^{2+} concentrations in Jurkat cells. Approximately 5 s after data collection was initiated. OKT3 ($1 \mu\text{g/ml}$) or 5HT ($5 \mu\text{M}$) was added directly to cells and data acquisition
30 continued for a total of 4 min. The relative intracellular calcium concentration was calculated from the ratio of violet fluorescence (calcium bound indo-1) to blue fluorescence (calcium-free indo-1) and plotted as a function of time.
- 35 Figure 4 is a graph demonstrating concentration dependence of 5HT mediated increases in intracellular

Ca²⁺. Jurkat cells, loaded with indo-1 were incubated with 5HT at 37°C for 2 min. Percent of cells with increased intracellular CA²⁺ (■) and total intracellular CA²⁺ concentration (□) was determined by measuring the
5 increase in fluorescence with the use of FACSTAR Plus as described in Materials and Methods. Percent positive cells contained 375 to 425 nM Ca²⁺ and negative cells contained 150 to 175 nM Ca²⁺.

10 Figure 5 is a graph demonstrating 5HT-mediated increase in phosphatidylinositol turnover. Jurkat cells were labeled with (³H)inositol for 48 h. Labeled cells were incubated with 3 μM 5HT (□) or 1 μg/ml OKT3 (■) for the indicated periods of time before harvest and analysis of
15 IP levels by anion exchange chromatography.

Figure 6A is a graph showing specific binding of 3H-5HT to T-cell blasts.

Figure 6B is a graph showing binding of ketanserin to
20 T-cell blasts.

Figures 6A-1 and 6B-1 depict Scatchard analysis of the binding data from Figures 6A and 6B, respectively.

Figure 7 is a graph showing competition experiments with
25 various 5HT receptor ligands.

Figure 8 is a graph showing the identification of 5HT in PBL.

30 Figure 9 is a graph showing effects of 5HT on proliferation of PBL.

Figure 10 is a graph showing analogues which bind to 5HT receptors and increase or decrease T-cell proliferation.

35 Figure 11 is a graph showing the activation of CD8+ suppressor T-cells by PWM requires 5HT.

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Figure 12 is a graph showing the effects PBL stimulated with OKT3 in presence or absence of analogues which bind to 5HT.

- 5 Figures 13A and 13B are graphs of properties of the serotonin receptor on tumor cells. (A) Jurkat cells were harvested from log phase growth and incubated at 3×10^6 /ml in a total volume of 600 μ l of RPMI 1640 media at $0-4^{\circ}\text{C}$ in the presence of 3H-5HT ($\blacklozenge$), 125I-LSD ($\blacklozenge$), 3H-ketanserin ($\blacksquare$), 3H-8OH-DPAT ($\blacksquare$) or 3H-DOB ($\square$) (NEN) in the
10 presence or absence of 50 M 5HT for 6 min. Binding equilibrium was reached during this period (not shown). Cells were collected on to nitrocellulose membranes (Millipore) and tubes and filters were washed three times
15 each with 4ml of cold PBS. Ascorbate (100 μ M) was included in the wash solutions to reduce nonspecific binding to filters (15). Specific binding is expressed as the difference between total binding and binding observed in the presence of 50 μ M 5HT. Figure 13A shows
20 a Scatchard analysis of binding data with 5HT ($\square$) and ketanserin ($\blacklozenge$). The K_d was 130 nM. (B) Cells were incubated at 0°C for 6 min in the presence of 100 μ M 3H-5HT and the indicated concentrations of the following 5HT receptor ligands: 5HT ($\square$), ketanserin ($\blacksquare$)
25 α -methyl serotonin ($\blacklozenge$), mesulergine ($\blacksquare$), 8OH-DPAT($\blacklozenge$), and ritanserin ($\square$), and bound 3H-5HT was determined as described above. Specific binding was determined in the presence of 50 μ M 5HT.
- 30 Figure 14 is a graph showing reversal of ritanserin mediated inhibition of tumor cell proliferation by 5HT receptor ligands. ME180 cells were cultured for 48 hr in the presence or absence of ritanserin (20 μ M) and in the presence or absence of the indicated concentrations of
35 the following 5HT receptor ligands: 5HT ($\square$), pelanserin ($\blacklozenge$), Ketanserin ($\blacksquare$), mianserin ($\blacklozenge$), 8OH-DPAT($\blacksquare$), propranolol ($\square$). Cells were labeled with 1 μ Ci ^3H -TdR

for the final 6 hr of culture and collected to measure incorporation into DNA. Results are expressed as the percent of control incorporation of ^3H -TdR into ME180 cell cultures in the absence of ritanserin or other
5 ligands. Incorporation of ^3H -TdR by ME180 cells was 40,679 $\pm$ 2548 in the absence of ritanserin and 2215 $\pm$ 433 in the presence of ritanserin. These ligands affect their specific 5HT receptor sites between 1-10 nM.

10 Figure 15 is a graph showing 5HT dependent changes in intracellular cAMP content of cell blasts.

Figure 16 is a graph showing the rate of change of cAMP content (○) and proliferation (●) in T cell cultures
15 after addition of 5HT.

Figure 17 is a graph showing inhibition of antiproliferative effects on gamma interferon (IFN). (A) ME-180 cells were cultured for three days with IFN in
20 the absence (□) or presence of 300 μM tryptophan (■) or 5 μM 5Htp (◆). (B) ME-180 cells were cultured for three days with 100 u/ml IFN in the presence of varying concentrations of 5Htp (□) or tryptophan (◆). Control cell proliferation was 88,765 $\pm$ 1256 cpm of ^3H -TdR after
25 a 4 hour pulse with 1 μCi ^3H -TdR.

Figure 18 is a graph showing a comparison of the ability of 5Htp and metabolites of 5Htp to reverse antiproliferative effects of gamma IFN. ME-180 cells
30 were cultured with 100 u/ml IFN in the presence or absence of the indicated concentrations of 5Htp (○), 5HT (●), melatonin (■), N-methyl serotonin (□) or 5-hydroxyindole acetic acid (x) for three days. Control proliferation was 73,456 $\pm$ 2289 cpm and proliferation in
35 the presence of IFN was 16,894 $\pm$ 1145.

Figure 19 is a graph showing cells loss tryptophan and 5HT after culture with gamma IFN. ME-180 cells were cultured for the indicated number of days in the presence (0) or absence (0) of 100 u/ml IFN , harvested and analyzed for 5HT content and tryptophan content by HPLC; UPPER GRAPH: 5HT; LOWER GRAPH: tryptophan.

Figure 20 is a graph showing 5Htp does not inhibit loss of extracellular tryptophan in cultures with gamma IFN. ME-180 cells were cultured in the absence (0) or presence of 100 u/ml IFN (●,□) and in the presence (●) or absence (□) of 10 μM 5Htp. Culture media was harvested on the indicated days and analyzed for tryptophan content by HPLC.

Figure 21 is a graph showing 5Htp reverses inhibition of cell proliferation by low tryptophan. ME-180 cells were cultured for 48 hr in RPMI-1640 media with the indicated amounts of tryptophan with (●) or without (○) 3 μM 5Htp. Results are expressed as incorporation of ³H-TdR after a 6 hr pulse on day 2.

DETAILED DESCRIPTION OF THE INVENTION

Initial studies were conducted to determine if 5HT receptor subtypes could be identified on human T lymphocytes and if 5HT receptors could mediate signal transduction events.

¹Abbreviations used in this paper: 5HT, serotonin, 5-hydroxytyptamine; DOB, (R)-(-)-4-bromo-2, 5-dimethoxy-amphetamine; 8OH-DPAT, 8-hydroxy-2-(di-n-propylamine) tetralin; IP, inositol phosphates; IC₅₀, concentration required to inhibit response by 50%; EC₅₀, concentration required to yield half-maximal response.

Reagents. ³H-5ht (20 Ci/mmol), ³H-ketanserin (65 Ci/mmol), ¹²⁵I lysergic acid diamide (LSD, 2200 Ci/mmol), ³H-8OH-DPAT (128 Ci/mmol) and

³H-myo-inositol (48 Ci/mmol) were obtained from New England Nuclear. FCS, RPMI 1640, and L-glutamine were obtained from GIBCO. Indo-1 was obtained from Sigma Chemical Co.

5

Cells. Jurkat cells were obtained from the American Type Culture Collection and were maintained in continuous culture in RPMI 1640 medium supplemented with 10% FCS without antibiotics but with 1 mM glutamine.

10

Preparation of membranes from Jurkat cells. Jurkat cells, 1-2 l cultures, were harvested by centrifugation, suspended in 10 mM tris-HCl, pH 7.5, containing 1 mM EDTA, incubated for 10 min on ice, and were homogenized with a Dounce homogenizer in 20-40 ml total volume. Homogenates were centrifuged at 10,000xg for 10 min to remove nuclei and cellular debris. The supernatant was centrifuged at 100,000xg for 1 hr. The pellet was suspended in tris-HCl buffer with EDTA at a final concentration of 7.7 mg/ml protein. This membrane preparation was stored at -70°C until use in binding assays.

Binding Assay Jurkat cells were harvested from log phase cultures and were incubated at 3.0×10^6 /ml in a total volume of 600 μ l or RPMI 1640 at 0-4°C in the presence of ³H-5HT with or without various 5HT agonists or antagonists (see text for details) for 6 min. Binding equilibrium was reached during this incubation period. Displacement experiments were performed by incubating cells with ³H-5HT, followed by incubation with unlabeled agonists or antagonists as described in the text. Cells were collected onto glass fiber filters and tubes and filters were washed 3 times each with 4 ml of cold PBS. Ascorbate (100 μ M) was included in the wash solutions when ¹²⁵I-LSD or ³H-8OH-DPAT binding was evaluated to reduce nonspecific binding to the filters (14-15).

Washing of each filter took less than 20 sec. Total binding of ^3H -5HT to Jurkat cells was typically 10,000-15,000 cpm in the presence of 100 nM ^3H -5HT (10^6 total cpm added). About 1500-3000 cpm remained bound in the presence of 100 μM 5HT (nonspecific binding). For association rate studies, cells were incubated for varying periods of time with 100 μM ^3H -5HT with and without 100 μM 5HT. Binding was stopped by addition of PBS and filtration. For dissociation rate studies, cells were incubated with 100 nM ^3H -5HT for 10 min. at 0-4°C. Next, 5HT was added to yield a final concentration of 100 μM and the reaction was stopped by addition of cold PBS and filtration of the samples. Binding assays were performed in duplicate a minimum of three times. Duplicate samples were within 5% of each other.

Similar procedures were employed when Jurkat membrane preparations were used in the binding assays except that binding assays were performed in tris-HCl buffer, pH 7.5, with 500-700 $\mu\text{g}/\text{ml}$ total protein.

Intracellular Ca^{++} measurements. Mobilization of intracellular Ca^{++} was evaluated by the indo-1 method using a modified Becton Dickinson FACSTAR Plus (16-17). Briefly, Jurkat cells were incubated with 2 μM indo-1 in DMEM with 10 mM HEPES for 45 min. at 37°C, washed three times and resuspended at 10^6 cells/ml in PBS. Aliquots (1 ml) were equilibrated to 37°C and placed in the modified sample station and data acquisition was initiated. Approximately 5 sec. after data collection began, 5HT or OKT3 (see text for concentrations) was added directly to the sample and data acquisition continued for a total of 4 minutes. Fluorescence emission was divided by a longpass filter (455 nm cutoff) placed 45° to the collecting lens and measured simultaneously through two bandpass filters, BP485/22 and BP395/20. Data collected in list mode was subsequently processed to yield a ratio of violet fluorescence (395/20 nm) to blue fluorescence (485/22 nm) as a function of time. Calcium concentration

was calculated using the formula:

$$[Ca^{++}] = Kd \cdot \frac{(R - R_{min})}{(R_{max} - R)} \cdot \frac{Sf2}{Sb2}$$

5

where Kd is the dissociation constant (250 nm); R, Rmin, and Rmax are the fluorescence intensity ratios at resting, zero, and saturation calcium concentrations, respectively; and Sf2/Sb2 is the ratio of fluorescence intensity of the calcium-free and calcium-bound dye (18).

10 The effect of concentration of 5HT on the response of individual Jurkat cells versus the total population was also evaluated. Cells were equilibrated to 37°C, 5HT was added and the cells were incubated for 2 min. prior to collecting 10,000 events in list mode. The data were subsequently analyzed for ratio of violet to blue fluorescence and percent of cells responding about a 2 standard deviation threshold. These experiments were performed a minimum of three times with similar results.

20 Inositol phosphate levels. Jurkat cells, 2×10^6 /ml, were labeled with 10 μ Ci/ml 3 H-myo-inositol in RPMI 1640 media with 1% dialyzed FCS for 48 hrs. Cells were harvested and washed twice in RPMI 1640 media without FCS. Cells were suspended in media without FCS at 1×10^6 /ml at 37°C, treated with 5-HT as described in the text, harvested at various times by centrifugation. Extracts were neutralized with 7 N KOH and analyzed for levels of IP by anion exchange chromatography as previously described (19). Duplicate samples were within 5% and experiments measuring inositol phosphate levels were performed three times with similar results.

35 Purification of PBMC. PBMC were obtained from the buffy coats of whole blood from normal human volunteers. After isolation on isolympH gradients (Pharmacia, Piscataway, NJ), PMBS were washed twice in PBS, suspended in a solution of 10% DMSO plus 90% FCS (GIBCO, Grand Island, NY) and stored in liquid nitrogen until use.

Cell surface Ag and proliferative responses of PBMC after storage in liquid nitrogen were similar to properties of fresh lymphocytes.

PWM and PHA were obtained from GIBCO (OKT3 was from ascites of BALB/c mice and used at a dilution of 1:1000), diluted in PBS and used at a dilution of 1/80 in culture. Purified recombinant or natural IL-2 were purchased from Boehringer Mannheim (Indianapolis, IN). One unit of IL-2 activity is defined as the amount required to cause 50% maximal proliferation of the IL-2 dependent cell line CTLL in a 24-h assay. Purified rIL-1, rIL-4, and rIL-6 were obtained from Genzyme (Boston, MA).

Cell cultures. PBMC, CD4+PBMC or CD4+ cells were cultured at 5×10^5 cells/ml in RPMI-1640 media supplemented with 10% FCS without antibiotics for 3 to 8 days in flat bottom 96-well microculture plates (Becton Dickinson) in 100 to 200 μ l total volume in a humidified atmosphere of 5% CO₂ in air. To measure (³H)TdR incorporation, 1 Ci of (³H)thymidine (2 Ci/mmol, Amersham International, Indianapolis, IN) was added to cultures in duplicate 18 to 20 h before cells were harvested on filter paper (PHD cell harvester, Cambridge, MA). Data are expressed as the average (³H)TdR incorporation of duplicate samples. Duplicate samples were within 5% and experiments were performed at least three times with similar results and were repeated in subsequent experiments as positive controls. Appl. Microbiol. 16:1706. Binding assays were performed as described on p15-16 for Jurkat cells.

30

Table 11

Inhibition of 5HT synthesis results in inhibition of proliferation by a tumor cell line. ME-180 cervical carcinoma cells were obtained from ATCC and were maintained as described (). Cells were cultured for 48 hrs and cultures were either analyzed for 5HT content or for cell proliferation by addition of ³H-TdR. Media

35

and cells were separated, 5HT was extracted and protein was precipitated with 70% ethanol, and samples were concentrated with a Speed-vac. Samples were analyzed for 5HT content by HPLC (Hewlet-Packard) equipped with a fluorescence detector (Beckman) with a 280 nm excitation filter and a 360nm emission filter. The two mobile phases consisted of A: 50mM triethylamine adjusted to pH 3.0 with phosphoric acid and B: 50mM triethylamine, pH 3.0 with 40% methanol. The gradient was linear from 100% A to 100 % B over 25 min (51). The retention times of known standards were as follows: 5HT, 3.50 min; 5OH-tryptophan, 4.77 min; tryptophan, 9.11 min, 5OH-indoleacetic acid, 12.5 min; and melatonin 21.69 min. Cells were also pulsed with 1 μ Ci 3H-TdR for the final 4 hrs of culture and collected to measure incorporation into DNA. Cultures were performed in triplicate. Actual synthesis of 5HT from tryptophan was also demonstrated by culturing cells with 3-H-tryptophan, extracting soluble components and analyzing 3H-5HT by thin layer chromatography with a solvent system of methyl propanol-ethyl acetate-ammonium hydroxide-water (45-30-17-8) (52). Under these conditions the Rf for tryptophan was 0.03 and the Rf for 5HT was 0.50.

As a model system, binding of 3 H-5HT to transformed human Jurkat T-cells was examined to determine whether T-cells may express specific binding sites for this neurotransmitter. These cells have been used by numerous investigators to explore signal transduction mechanisms following antigen or mitogen stimulation (20-21). Results in figure 1 show that binding of 3 H-5HT to Jurkat cells was detectable and that binding was saturable. Specific binding was determined by subtracting the amount of 3 H-5HT bound in the presence of 100 μ M unlabeled 5-HT from the amount of 3 H-5HT bound in the presence of the indicated concentrations (fig. 1) of 5-HT. At these concentrations of 5-HT, specific binding represented approximately 70-80% of total binding and maximum binding

of ^3H -5HT was obtained within 5 min. of incubation (fig. 2). Binding of 5HT reached a maximum at 1-2 μM 5HT. Figure 1A shows the Scatchard plot of the binding data. Binding data from three separate experiments yielded an average dissociation constant (K_d) of 90 nM. The site density for ^3H -5HT binding to Jurkat cells was 130 fmol per 10^6 cells or 80,000 receptors per cell. The Hill slope value was 0.8 for these data (not shown). A variety of binding conditions were investigated to determine their effect on affinity constants and receptor density. Inclusion of Mg^{++} ions up to 2 mM, pargyline up to 10 μM , EDTA up to 3 mM or imipramine (blocks uptake of 5HT) up to 10 μM did not alter observed binding constants or receptor densities (data not shown). All results shown here were derived from log phase cultures. Jurkat cells from cultures exceeding 10^6 cells/ml had significantly fewer 5HT receptors. These cultures also failed to respond to 5HT by mobilizing Ca^{++} or increasing inositol phosphate concentrations.

Isolated membrane preparations also bound 5HT with similar characteristics. Binding of 5HT reached saturation at 1-2 μM 5HT and the average K_d determined by Scatchard analysis was 160 nM. Kinetics of binding and dissociation of 5HT (see below) were also similar for intact cells and membrane preparations. Similar concentrations of 5HT or 5HT agonists or antagonists prevented binding of ^3H -5HT to both membrane preparations and to intact cells. The major difference between binding of 5HT to membrane preparations or to intact cells was in the calculated number of receptors/cell. Intact cells expressed 80,000 receptors/cell whereas calculations derived from binding data from isolated membrane preparations yielded 30,000 receptors per cell. At this point it is not certain where this difference arises from. However, since binding data are compared to the biological response of intact cells to 5HT as a function of 5HT concentration, only results of binding of

5HT to intact cells will be reported here.

Kinetics of binding were investigated. Results in figure 2a show that binding of ^3H -5HT was complete within 4 min. and half-maximal binding occurred with 30 sec. Binding was maintained for up to 10 min. From these data points an association rate constant (k_1) of $2.1 \times 10^{10} \text{ mol}^{-1} \text{ min}^{-1}$ was calculated from the equation: $k_1 = [1/t(a-b) \ln[b(a-x)/a(b-x)]]$ where a is the initial concentration of ^3H -5HT, b is the receptor concentration and x is the amount of receptor-ligand concentration at time t . The receptor-ligand complex readily dissociated in the presence of excess unlabeled 5HT. These results are shown in figure 2b. The pseudo-first order dissociation rate constant was 1.1 min^{-1} calculated from the following equation: $k_2 = (1/t) \ln(a/x)$ where a ^3H -5HT bound at time t (15). The ratio of k_2 divided by k_1 provides a calculated K_d of 180 nM. This value agrees reasonably well with the K_d calculated by Scatchard analysis.

Cell surface receptors for 5-HT have been classified into several subtypes on the basis of pharmacological as well as functional properties (12-13). Binding of 5-HT to 5-HT $1a$ or 5-HT $1b$ sites results in inhibition of stimulated adenylate cyclase activity while binding to 5-HT $1c$ or 5-HT 2 sites results in increase in inositol phosphate levels and intracellular Ca^{++} concentration. Jurkat cells were incubated with indo-1, a fluorescent dye sensitive to changes in intracellular Ca^{++} , and analyzed by flow cytometry as described in Methods after incubation in the presence or absence of $5 \mu\text{M}$ 5-HT or $1 \mu\text{g/ml}$ OKT3. These results are shown in figure 3. Cells incubated in PBS had a baseline calcium concentration of 150-175 nM and did not change intracellular Ca^{++} during the 10 min. observation period. Jurkat cells incubated with OKT3 increased intracellular Ca^{++} to a maximum of $2 \mu\text{M}$ within 2 min. before decreasing to a level of 700 nM. Jurkat cells incubated with $5 \mu\text{M}$

5HT increased Ca^{++} concentration to 400 nM within 2 min. Jurkat cells treated with 5HT did not show the large initial peak in $[\text{Ca}^{++}]$ which is characteristic of the response of Jurkat cells to OKT3 (20-21). Figure 4
5 compares the increase in intracellular Ca^{++} concentration in cultures of Jurkat cells to Ca^{++} concentration in individual Jurkat cells as a function of 5HT concentration. At the indicated 5HT concentrations cells could be divided into those with low Ca^{++} (150-175 nM)
10 and those with high Ca^{++} (375-425 nM). Thus the response of the entire culture shown in figure 3 is actually the average of individual cells which either have high or low intracellular Ca^{++} concentrations. Increases in intracellular Ca^{++} concentration or the number of cells
15 with increased intracellular Ca^{++} . The half-maximal response was between 100-300 nM 5-HT which was approximately equivalent to the dissociation constant obtained from the binding data.

Increased intracellular Ca^{++} concentrations can be
20 mediated by IP₃ which is produced by hydrolysis of phosphatidylinositol biophosphate (22). Binding of 5-HT to the 5HT₂ receptor family has been shown to increase levels of inositol phosphates in neural tissue. Jurkat T-cells were labeled for 48 hrs. with ³H-inositol and
25 stimulated with 3 μM 5-HT or with 1 $\mu\text{g/ml}$ OKT3. Cells were harvested at various times after addition of 5HT or OKT3 and analyzed for levels of IP by anion exchange chromatography. Results in fig. 5 show that IP levels increased after addition of 5HT or OKT3 and reached a
30 maximum within 1 min. Levels of IP decreased over the next ten minutes approaching baseline levels. Both 5HT and OKT3 yielded similar increases in IP levels under these conditions.

A number of 5-HT agonists or antagonists have been
35 employed to help discriminate among the different 5-HT receptor subtypes. Ketanserin, a 5-HT₂ receptor antagonist, alpha-methyl serotonin, a 5HT₂ receptor

agonist, and 5HT inhibited binding of ^3H -5HT to Jurkat cells. Concentrations which inhibited 50% of specific binding of ^3H -5HT (IC_{50}) were 20 μM , 3 μM and 0.8 μM for ketanserin, alpha-methyl serotonin and 5HT, respectively.

5 80H-DPAT, a specific agonist for the 5HT_{1a} receptor and ICS-205930, a specific antagonist of the 5HT₃ receptor failed to prevent 3H-5HT binding to Jurkat cells. Two other 5HT_{1c/2} receptor antagonists, mianserin and mesulergine, failed to inhibit 5HT binding to Jurkat

10 cells. These results are shown in table 1. Other 5HT₂ receptor antagonists such as ritanserin and pelanserin also failed to inhibit 5HT binding (not shown). ^{125}I -LSD, ^3H -ketanserin and ^3H -DOB have been used to label 5HT_{1c} or 5HT₂ sites in the central nervous system.

15 ^3H -80HDPAT has been used to label 5HT_{1a} sites in the central nervous system. Table 2 compares binding of these ligands to Jurkat cells as reflected by the K_d determined by Scatchard analysis. Specific binding was determined in the presence of 100 μM 5HT. Both

20 ^3H -ketanserin and ^3H -5HT labeled Jurkat cells whereas ^{125}I -LSD and ^3H -DOB failed to label Jurkat cells under the conditions employed. ^3H -80HDPAT also failed to label Jurkat cells. The binding constant for ketanserin was 100 nM. These 5HT receptor agonists and antagonists have

25 affinity constants in the range of 1-5 nM for their specific receptor classes.

The 5HT₂ agonist, alpha methyl serotonin, was also tested for its ability to stimulate Ca^{++} mobilization (table 3). Maximum Ca^{++} mobilization was obtained with

30 10 μM alpha methyl serotonin compared to 3 μM 5HT and the concentration required to yield half-maximal stimulation (EC_{50}) was 1 μM for alpha methyl serotonin compared to 200 nM 5HT. Values for displacement of bound 5HT were similar. The IC_{50} for displacement of bound 5HT was 800

35 nM for 5HT and 3 μM for alpha methyl serotonin. Table 4 compares the ability of several 5HT₂ antagonists to inhibit 5HT-mediated Ca^{++} mobilization in Jurkat cells.

Of the antagonists tested, only ketanserin inhibited 5HT-mediated Ca⁺⁺ mobilization. The IC₅₀ was 10 μ M.

Table 1

5

Inhibition of 5HT binding by 5HT receptor
agonists/antagonists

10	Agonist/Antagonist	Receptor Subtype	IC ₅₀ ^a
	5HT	endogenous	0.8 μ M
	ketanserin	5HT ₂	20 μ M
	alpha-methyl serotonin	5HT ₂	3 μ M
15	8-OH-DPAT	5HT _{1a}	>50 μ M
	ICS-205-930	5HT ₃	>20 μ M
	mesulergine	5HT _{1c/2}	>50 μ M
	mianserin	5HT _{1c/2}	>50 μ M

20 ^aBinding experiments were performed as described in the methods section with 2x10⁶ Jurkat cells in the presence of 100 nM ³H-5HT and various concentrations of agonists and antagonists.

25

30

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Table 2

5HT receptor ligand binding to Jurkat cells

5	5HT receptor ligand	Kd ^a	Kd ^b	5HT site
		(Jurkat)	(published values)	
10	³ H-ketanserin	100 nM	~1 nM	5HT2
	¹²⁵ I-LSD	>10 nM	~1 nM	5HT1c
	³ H-DOB	>100 nM	~1 nM	5HT2a
15	^a Jurkat cells were incubated with ³ H-ketanserin (1-500 nM), ¹²⁵ I-LSD (0.3-10 nM), or ³ H-DOB (0.5-200 nM) for 10 min. at 0-4°C. Specific binding was determined in the presence of 100 μM 5HT.			
20	Published values were obtained from references 12 and 13.			

Table 3

Ca++ mobilization by 5HT2 agonists

25	Agonist	Displacement of	Ca++ Mobilization	
		3H-5HT	IC50 ^a	EC50 ^b
30	5HT		800 nM	200 nM
	alpha-methyl serotonin		3 μM	1 μM

^aData for determination of displacement of 3H-5HT
 35 were obtained from figure 5.

^bEC₅₀'s were determined by incubating Jurkat cells with various concentrations of 5HT₂ agonists and following change in Ca⁺⁺ concentration as a function of time. The rate of change of Ca⁺⁺ is linear for the first 60 sec. and is complete 2-3 min. after addition of 5HT agonists.

Table 4

Inhibition of Ca⁺⁺ mobilization by 5HT₂ antagonists

10	Ca ⁺⁺ Mobilization	
	[Ca ⁺⁺], nM ^a	IC ₅₀ ^b
	5HT (1 μ M)	425
	+ Ketanserin	285
15	+ Pelanserin	435
	+ Mianserin	440
	+ Mesulergine	460

^aJurkat cells (1×10^6 /ml) were incubated for 5 min. in the presence of 10 μ M of the indicated antagonists before addition of 5HT. Ca⁺⁺ mobilization in Jurkat cells was determined by flow cytometry as described in the methods section 2 min. after addition of 5HT. At this time the response to 5HT is still linear with respect to time (Fig. 4).

^bIC₅₀ is the concentration of agonist which inhibits 50% of Ca⁺⁺ mobilization by Jurkat cells in the presence of 1 μ M 5HT.

The neurotransmitter, 5HT, is released during inflammation and has been suggested to play an important role in delayed type hypersensitivity responses (3,4,10). Antagonists of 5HT, such as ketanserin or ritanserin, have been shown to prevent DTH responses in animal models (10-11). Further, treatment of certain antigen specific T-cell clones with 5HT antagonists blocks their ability to transfer DTH responses to naive recipients. However,

functional 5HT receptors which transduce intracellular signals through second messengers have not been defined on T-cells. The purpose of experiments described here was to determine by both biochemical and pharmacological analysis whether functional receptors for 5HT could be identified on human T-cells and whether they were similar to subtypes already defined on other cell types present in the central or peripheral nervous system.

Receptor subtypes for 5HT have been characterized functionally, pharmacologically and molecularly (12-13). The following designations have been employed; the 5HT1 family consists of 5HT1a, 5HT1b, and 5HT1d; the 5HT2 family consists of 5HT1c, 5HT2a and 5HT2b, and the 5HT3 family presently consists of only one member. cDNA's encoding 5HT1a, 5HT1c and 5HT2 receptors have been identified and all encode proteins which are G protein-linked receptors with seven membrane spanning units (23-25). Functionally, binding of 5HT to 5HT1a sites results in modulation of adenylate cyclase activity (26), alterations in potassium ion channels, and inhibition of nerve cell transmissions (12-13). Specific agonists have also been identified which exclusively bind to 5HT1a sites. Two of these are 8OH-DPAT and ipsapirone. Binding of 5HT to 5HT1c or 5HT2 sites results in increased phosphatidylinositol turnover and intracellular Ca^{++} mobilization. A number of 5HT2 antagonists have been identified which interact with 5HT1c and 5HT2 sites with equivalent affinities and examples include mesulergine and mianserin. Other 5HT2 antagonists include ketanserin, ritanserin, and pelanserin. These ligands have been used to label 5HT2 sites, possess nanomolar affinities for 5HT2 sites and inhibit 5HT-mediated phosphatidyl-inositol turnover in target tissues (12-13). The 5HT2 family may contain additional heterogeneity. 5HT sites which mediate phosphatidyl inositol turnover have also been identified in the hippocampus region of the brain as well as in the

limbic forebrain. In these tissues, phosphatidylinositol turnover is only inhibited by very high concentrations of ketanserin (compare 1-10 μ M to the normal affinity of ketanserin for 5HT₂ sites of 1-10 nM (27-28). 5HT₃ receptors appear to be localized primarily in the peripheral nervous system. These receptors are not affected by 5HT₁ and/or 5HT₂ selective ligands. However, specific ligands, such as ICS 205903, have been identified which can block the effects of 5HT in the periphery (12-13).

The K_d for 5HT binding to Jurkat cells was 90 nM when determined by Scatchard analysis and was 180 nM when determined by kinetic analysis. The site density for 5HT receptors was 130 fmol per million Jurkat cells or 80,000 sites per cell. 5HT stimulated phosphatidylinositol turnover and increases in intracellular Ca⁺⁺ in these cells. Saturation of 5HT binding as well as maximum Ca⁺⁺ response was observed at 1-3 μ M 5HT. Half-maximal Ca⁺⁺ responses were seen at 200 nM 5HT which is close to the K_d for 5HT binding to Jurkat cells. Binding of ³H-5HT and 5HT mediated Ca⁺⁺ mobilization were not affected by the specific 5HT_{1a} agonist 80H-DPAT or by the specific 5HT₃ ligand ICS-205930. Further, 5HT or 80H-DPAT did not modulate cAMP concentrations in Jurkat cells (data not shown). These data are consistent with binding of 5HT to the 5HT₂ receptor family. Additionally, alpha methyl serotonin maleate, a 5HT₂ agonist, displaced bound 5HT and stimulated Ca⁺⁺ mobilization in Jurkat cells. However, ketanserin bound to Jurkat cells with only weak affinity (K_d of 100 nM) and no specific binding of ¹²⁵I-LSD (displaced by 5HT) to Jurkat cells could be detected. Also, mianserin, mesulergine, pelanserin, and ritanserin (all 5HT₂ antagonists) did not inhibit 5HT binding to Jurkat cells at concentrations $\leq$ 20 μ M and did not inhibit 5HT mediated Ca⁺⁺ mobilization. Ketanserin only displaced 5HT and inhibited Ca⁺⁺ mobilization at high concentrations (50% at 10 μ M. Further, ³H-DOB, a

specific 5HT_{2a} ligand, did not label Jurkat cells. Effects of 5HT antagonists were equivalent when 5HT binding was compared to Ca⁺⁺ mobilization studies. Taken together these data suggest that the 5HT receptor on
5 Jurkat cells is not a 5HT_{1c}, 5HT₂ or 5HT_{2b} receptor. It appears to be more similar to the one found on the hippocampus which binds 5HT and mediates phosphatidyl-inositol turnover but is only inhibited by ketanserin at micromolar concentrations (27-28). This
10 suggests that additional heterogeneity exists in the 5HT₂ family.

Data presented here describe 5HT receptors on human Jurkat T-cells and show that the receptor stimulates phosphatidyl-inositol turnover and increases in
15 intracellular Ca⁺⁺ concentration in these cells. The increase in levels of inositol phosphate caused by 5HT was similar to the increase caused by OKT3. The increase in Ca⁺⁺ concentration by 5HT was from 175 nM to 400 nM within 2 min. The increase in Ca⁺⁺ concentration caused
20 by OKT3 was to a maximum of 2 μ M within 2 min. This increase rapidly decreased to 700 nM within an additional min. These data do not address the biological or immunological consequences of interaction of 5HT with its
25 receptors on Jurkat cells. However, phosphatidylinositol turnover represents a critical element of signal transduction in T-cell activation (for review see ref. 29) and stimulation of this pathway by 5HT should have important immunological consequences. Sufficient 5HT may
30 exist at sites of inflammation to modulate T-cell function. 5HT is a major storage product of platelets and is released upon platelet aggregation which occurs at sites of inflammation (2-4). Interestingly, 5HT levels in platelets are depressed in humans with autoimmune
35 disease such as rheumatoid arthritis and systemic lupus erythematosus (30). 5HT₂ antagonists have been shown to exacerbate inflammatory and arthritic responses to streptococcal cell wall extracts in normally resistant

rats (31). However, in this instance it is not clear that these effects are mediated through 5HT receptors on T cells. With the availability of specific 5HT antagonists or agonists it should be possible to define the immunological consequences of interaction between 5HT and 5HT receptors on activated T cells and better understand the role of 5HT receptors on T cells in immune and inflammatory responses in vivo. Finally, similar types of 5HT receptors are present on human peripheral T cell blasts but are absent on resting human peripheral T cells (in preparation).

Following the demonstration that a novel 5HT-2-like receptor was present on transformed cells (Jurkat cell) further experiments were conducted to demonstrate that the receptor was present on human T lymphocytes (activated T cells).

5HT receptors on activated T cells. The 5HT receptor on Jurkat cells has a weak affinity for ^3H -5HT (100 nM) and for ketanserin (100 nM) when compared to classic 5HT₂ receptors. Specific binding of ^3H -5HT to T cell blasts was detectable and saturable. About 80% of ^3H -5HT bound was displaced by 100 μM 5HT. Binding saturated at 1-3 μM 5HT. At saturation, 200-250 fmol 5HT bound per 10⁶ T cells. Binding of 5HT saturated between 1-3 μM 5HT. The K_d , determined by Scatchard analysis was 180 nM (average of three separate experiments). T cell blasts bound 224 fmol 5HT per 10⁶ cells which represents a site density of 120,000 receptors per cell. These results are shown in Figure 6A. Figure 6A-1 shows the Scatchard analysis of the binding data. Binding data from three separate experiments yielded an average dissociation constant of 200 nM. Figure 6B shows binding of ketanserin to T cell blasts. Binding was saturable and T cell blasts expressed similar numbers of ketanserin and 5HT receptors. Specific binding of ketanserin was determined in the presence or absence of 100 μM 5HT.

Figure 6B-1 shows the Scatchard analysis of

ketanserin binding data which yields an average dissociation constant of 400 nM. Taken together, these data support the notion that there is a single receptor on T cell blasts which binds both 5HT and ketanserin within these concentration ranges. Competition experiments with various 5HT receptor ligands showed that 5HT, α -methyl serotonin and ketanserin could compete with H-5HT for binding to T cells, Figure 7. IC₅₀'s were 0.1 μ M, 0.06 μ M and 20 μ M, respectively. Other 5HT receptor ligands such as 8OH-DPAT (specific for 5HT_{1a} receptors), ICS-205930 (specific for 5HT₃ receptors), mianserin (specific for 5HT_{1c} and 5HT₂ receptors) and spiperone (specific for 5HT₂ receptors) did not compete with 5HT for binding to T cell blasts. The properties of the 5HT receptor on T cell blasts are comparable to the properties of the 5HT receptor found on Jurkat cells and appear distinct from the well characterized receptors found in nervous tissue.

A number of different T cell populations were evaluated for the presence of 5HT receptors. Resting lymphocytes expressed low levels of receptors (24,000/cell) when compared to T cell blasts (150,000/cell). Both CD4+ and CD8+ T cell lines expressed elevated levels of 5HT receptors (143,000 and 171,000 receptors/cell, respectively while CD3+,4-,8- T cell lines only expressed 53,000 receptors/cell which is only slightly more than that observed on resting lymphocytes. In other experiments, expression of 5HT receptors was examined as a function of time after stimulation with different T cell mitogens. Increase in the number of receptors was linear for 72 hr after stimulation. Three T cell mitogens, PHA, PWM, or OKT3, did not yield substantially different increases in the 5HT receptor number on T cells (Table 8).

35

Table 8

	Cells	5 HT Binding	
		Fmol/10 ⁶ cells	Receptors/cell
5	PBL	40 $\pm$ 3	24,000
	T Cell Blasts	250 $\pm$ 11	150,000
10	CD4+ T Cell Line	238 $\pm$ 9	143,000
	CD8+ T Cell Line	285 $\pm$ 5	171,000
15	CD3+4-8-T Cell Line	88 $\pm$ 6	52,800

In lymphocytes, elevation of intracellular cAMP is generally associated with the inhibition of lymphocyte proliferation and lymphocyte effector function (57). Conversely, elevation of intracellular Ca⁺⁺ is generally found to be required for activation of lymphocytes by antigenic or mitogenic signals and subsequent lymphocyte proliferation and effector function (53). Results presented here show that the 5HT_{1a} receptor can mediate an increase in intracellular Ca⁺⁺ in Jurkat cells. This should clearly enhance lymphocyte proliferation and effector function. The 5HT_{1a} receptor also regulates adenylate cyclase and levels of cAMP in lymphocytes. Depending upon the activation state of adenylate cyclase 5HT_{1a} agonists may either elevate or suppress levels cAMP and may either enhance or suppress lymphocyte proliferation and function.

Biological response of Jurkat cells and T cell blasts to 5HT, the specific 5HT_{1a} agonist, 8OHDPAT, and the 5HT_{1a} antagonist, spiperone.

5	JURKAT		T CELL BLASTS	
	Ca++	cAMP	Ca++	cAMP
	Conditions (nM)	(pmol)	(nM)	(pmol)
--	200	4.7	260	1.8
5HT+	610	4.9	260	0.6
10 5HT+ spiperone	225	4.7	250	1.4
8OHDPAT	470	4.8	255	0.5
8OHDPAT+ spiperone	280	4.6	265	1.6

For Ca++ measurements, cells were labeled with Indo-1 and intracellular Ca++ concentrations were monitored for 5 min at in the presence or absence of 3 μ M 5HT, 10 nM 8OHDPAT with or without 100 nM spiperone. Results are expressed as the concentration of intracellular Ca++. cAMP was extracted from cells after various treatments described above and quantitative as described. All cells were pretreated with 10 μ M forskolin to activate adenylate cyclase. Results are expressed as pmol cAMP/million cells. Comparison of the biological and pharmacological responses of 5HT and the 5HT_{1a} specific agonist, 8OHDPAT, in T cells.

30	BIOLOGICAL RESPONSE			PHARMACOLOGICAL RESPONSE	
	EC50		IC50	IC50	
	Ligands	Ca++	cAMP	Ca++	cAMP (binding data)
	8OHDPAT	5 nM	3 nM		1.5 nM
	5HT	200 nM	400 nM		3.0 nM
	spiperone			60 nM	70 nM 50.0 nM

Measurements of intracellular Ca++ were performed with Jurkat cells and measurements of cAMP were performed with activated T cells. Jurkat membranes were labeled

with 1 nM 8OHDPAT and displacement experiments were performed in the presence of varying concentrations of the indicated ligands.

Identification of 5HT in PBL. Resting PBL were
5 cultured in the presence or absence of PHA, PWM or OKT3 for varying periods of time, harvested and analyzed for 5HT content. These results are shown in figure 8. PBL contained 4 pmol 5HT/10 cells at culture initiation. This level was maintained for 4 d but was diminished by
10 day 7. By contrast, stimulation of PBL with either OKT3 or PHA resulted in loss of over 75% of 5HT content within 72 hr, about the time 5HT receptors have achieved maximum levels. Stimulation of PBL with PWM resulted in an initial increase in 5HT levels by 50% over the first 48
15 hr of culture and then a gradual decrease. These cells still maintained about 2 pmol 5HT/10 cells on day 7.

Effects of 5HT on proliferation of PBL.

Proliferation of PBL stimulated by PHA is partially inhibited by relatively high concentrations of 5HT.
20 Results in Figure 9 reproduce this finding and also show the effect of 5HT on proliferation of PBL to two other T cell mitogens, OKT3 and PWM. Proliferation of PBL in the presence of OKT3 was unaffected by addition of 5HT. By contrast, addition of 5HT to cultures stimulated with PWM
25 resulted in a three-fold increase in incorporation of ³H-TdR. Half-maximal increase in incorporation was obtained was 15 μ M 5HT. This was observed when 5HT was added on days 3-5 of the culture period, the time at which 5HT receptors are maximally expressed (see table
30 V). Addition of 5HT earlier in the culture period also resulted in a three-fold enhancement of proliferation but more 5HT (100 μ M) was required to achieve this effect. One possibility to explain this difference is that the activity of 5HT is mediated through 5HT receptors which
35 don't appear until day 3 and metabolism or oxidation of 5HT early in the culture period reduces the effective concentration of 5HT in the culture medium by day 3.

Several analogues which bind to 5HT receptors and which interact with the 5HT receptor on activated T cells were tested for their ability to stimulate or inhibit proliferation of PBL stimulated with PWM. Of those tested, 5HT, ketanserin (5HT₂ receptor antagonist) and α -methyl serotonin (5HT₂ receptor agonist) stimulated T cell proliferation by about three-fold at concentrations between 10-30 μ M. Two other 5HT₂ receptor antagonists, ritanserin (5HT₂ selective) and mianserin (5HT₂ and 5HT_{1c} selective) failed to stimulate T cell proliferation. In fact ritanserin was somewhat inhibitory at the higher concentrations. These results are shown in figure 10. Both CD4+ and CD8+ T cells proliferate in cultures of PBL stimulated with PWM. In mixed cultures of CD4+ and CD8+ lymphocytes, the CD8+ lymphocyte makes up the major percentage of proliferating cells on day 7. Results in figure 5 also show that the ability to stimulate proliferation of PBL cultures with 5HT or 5HT receptor ligands is lost if CD8+ lymphocytes are eliminated by antibody and complement treatment.

Results presented above indicated that T cell blasts express 5HT receptors, that 5HT enhances T cell proliferation stimulated by PWM, and that T cells contain 5HT. This raises the possibility that 5HT may be important to the normal proliferation of T cells activated by PWM. Inhibitors of tryptophan hydroxylase, the rate limiting step in 5HT synthesis, have been used to deplete stores of 5HT inside cells. Results in table 9 show that inhibition of 5HT synthesis by p-chlorophenylalanine (pCPA) also partially inhibited proliferation of cells stimulated by PWM. Addition of 5HT or ketanserin to these cultures reversed the inhibitory effects of pCPA.

Table 9

Inhibition of 5HT synthesis suppresses
T Cell proliferation

	PBL	PWM	pCPA (2 mm)	5HT (10 mm)	Ketanserin (10 mm)	³ H-TdR (cpm)
5						
10	+	-	-	-	-	1,728 $\pm$ 210
	+	+	-	-	-	11,954 $\pm$ 1022
	+	+	+	-	-	4,306 $\pm$ 688
15	+	+	+	+	-	9,726 $\pm$ 1614
	+	+	+	-	+	10,068 $\pm$ 469

20 Activation of CD8+ suppressor T cells by PWM requires
5HT. CD8+ suppressor T cells make up the largest
percentage of proliferating cells in cultures of PBL
stimulated with PWM. Since inhibition of 5HT synthesis
by pCPA inhibited proliferation in PWM stimulated
25 cultures, it suggested that pCPA may also inhibit
expression of suppressor cell function. These results
are shown in figure 11. Cultures of PBL were stimulated
with PWM in the presence or absence of pCPA to reduce
5HT content. Some cultures were also treated with
30 ketanserin to provide an agonist for the 5HT receptor to
possibly reverse the effects of pCPA. After 7 d,
cultures were washed, treated with mitomycin C and added
to fresh cultures of CD4+ T cells stimulated with PWM to
test for suppression cell activity. The results show
35 that cells for PWM stimulated cultures suppressed
proliferation of CD4+ T cells in the presence of PWM by a
maximum of 70% at a 1:2 ratio of suppressor cells to

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responding cells. Suppression of proliferation by 50% was obtained with 7×10 cells or at a ratio of 7 responding cells to 1 suppressor cell. The presence of pCPA in the primary culture used to produce suppressor cells completely blocked generation of suppressor cell activity. When ketanserin was included in cultures with pCPA, suppressor cell activity was normal when compared to controls.

Other T cell mitogens such as PHA or OKT3 stimulate proliferation of both CD4+ and CD8+ T cells but do not result in the expression of suppressor cell activity by CD8+ T cells within the first week of culture. PBL stimulated with either OKT3 or PHA lose their 5HT content within 72 hrs while cultures stimulated with PWM retain 5HT content for at least 7 d. Addition of pCPA to cultures stimulated with either PHA or OKT3 did not inhibit proliferation (not shown). To determine whether 5HT content may be related to suppressor cell activation, PBL were stimulated with OKT3 in the presence or absence of the T cell 5HT receptor agonist, ketanserin (5HT receptor antagonist), and tested for suppressor cell activity after 5 days of culture. T cell blasts, harvested from cultures stimulated with OKT3 lacked detectable suppressor cell activity. By contrast, T cell blasts from cultures stimulated with both OKT3 and ketanserin expressed suppressor cell activity when tested on second cultures. These results are shown in figure 12.

5HT as a co-mitogen for CD8+ T lymphocytes.

Proliferation of CD8+ T lymphocytes in response to PWM requires CD4+ T cells. The three-fold enhancement of proliferation of PBL cultures caused by 5HT or 5HT receptor ligands was lost after eliminating CD8+ T cells. Therefore, it seemed possible that 5HT may serve as a co-mitogen for CD8+ T cells and stimulate proliferation in the presence of PWM. These results are shown in Figure 9. Cultures of CD8+ T cells failed to proliferate

in the presence of PWM or in the presence of PWM and IL2. By contrast, cultures containing CD8+ T cells and PWM proliferated in the presence of either 5HT or ketanserin. The level of proliferation was comparable to that when
5 CD4+ T cells were added to cultures of CD8+ T cells. Addition of 5HT or ketanserin alone did not stimulate proliferation of CD8+ T cells. Taken together, these data suggest that 5HT is a co-mitogen for CD8+ suppressor T cells.

10 Modulation of cAMP levels in activated T cells by 5HT. Two signal transduction pathways which have been linked to specific 5HT receptor subtypes are modulation of adenylate cyclase and activation of phospholipase C. Activation of phospholipase C results in elevation of
15 inositol phosphates and increase in intracellular Ca++ concentration. In Jurkat cells, 5HT stimulates elevation of inositol phosphates and intracellular Ca++ concentration. Therefore, changes in inositol phosphate levels and intracellular Ca++ were measured in T cell
20 blasts (PHA) in response to 5HT and, as a positive control, in response to OKT3. Results in Table 7 summarize these experiments. In contrast to Jurkat cells, 5HT did not cause a change in inositol phosphate levels or in intracellular Ca++ concentration. As a
25 positive control, OKT3 stimulated an increase in both inositol phosphate concentrations and in intracellular Ca++ concentration in T cell blasts. Since T cell blasts did not respond to 5HT by altering inositol phosphate levels and intracellular Ca++ concentrations,
30 intracellular cAMP levels were compared in the presence and absence of 5HT. Three cell types were compared: resting T cells, T cell blasts and Jurkat cells, and cAMP levels were compared in the presence and absence of forskolin. These results are shown in Table 8. Resting
35 T cells did not change intracellular cAMP levels in response to 5HT where as activated T cells increased cAMP by over 2 fold in response to 5HT (5 μ M). Jurkat cells

did not alter cAMP levels in response to 5HT. Forskolin activates adenylate cyclase and causes an increase in intracellular cAMP. Forskolin increased cAMP concentration in resting T cells, activated T cells and Jurkat cells. Forskolin-dependent increase in cAMP levels was inhibited by addition of 5HT to activated T cells but not to resting T cells or to Jurkat cells. Figure 2 shows the concentration of 5HT required to cause an increase in cAMP levels in activated T cells, or in the presence of forskolin, a decrease in cAMP levels in activated T cells. Changes in cAMP levels were proportional to 5HT concentrations between 30 nM and 3 μ M. The half-maximal response was between 200-800 nM 5HT which was similar to the Kd obtained from the binding data. Addition of PMA also results in an increase in cAMP levels in T cell blasts or in Jurkat cells. In contrast to the case with forskolin, 5HT failed to inhibit the PMA-dependent increase in cAMP in either cell type. These results are shown in Table 9.

Stimulation of proliferation of CD8+ T cells by 5HT.
Changes in intracellular cAMP concentrations are known to affect the function and proliferations of activated T cells (53). Based on the above results, 5HT may regulate intracellular cAMP levels differently in different subpopulations on activated T cells, or in activated T cells exposed to different stimuli. Results in Table VII compare changes in cAMP concentrations in T cell blasts initially stimulated with PHA, OKT3 or PWM. T cells were harvested during peak proliferative responses and assayed for cAMP content in the presence or absence of 5HT. Cultures were also incubated with 5HT for the final 48 hr of culture to determine any affect on proliferation. 5HT caused a 2-fold increase in cAMP levels in T cells stimulated with PHA, a slight increase in cAMP in cells cultured with OKT3, but caused a 60% decrease in cAMP levels in T cells stimulated with PWM. Proliferation responses of the T cell cultures with the different

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stimuli in the presence or absence of 5HT appeared to reflect their levels of intracellular cAMP. Thus, 5HT slightly inhibited proliferation of T cells in response to PHA but stimulated proliferation of T cells in response to PWM by over 3-fold. Additionally, elimination of CD8+ T cells before stimulation with PWM eliminated the co-mitogenic effect of 5HT in these cultures. Thus, 5HT can either enhance or suppress T cell proliferation, apparently by regulating cAMP levels. The direction of the response seems to depend upon the mitogenic stimulus and the T cell subpopulation responding to that stimulus. Results in Figure 3 show that the proliferative response of PWM activated T cells to 5HT is fairly rapid. Increases in proliferation in the presence of 5HT were detectable within hours and reached maximum levels with 40 hours. Levels of cAMP were decreased to 60% of control with 30 min and to 40% of control over the next 40 hr of culture. Results in Figure 4 show the concentration dependence of the co-mitogenic effect of 5HT on PWM-activated T cells. 5HT was added to cultures 48 hr before assay of proliferation. Maximal stimulation of proliferation was observed at 30 μ M 5HT and the IC50 was 10 μ M 5HT.

The consequences of addition of 5HT to T cells depends upon the phenotype of the responding cells. Stimulation of T cell proliferation with OKT3 or PHA yields T cell blasts of both CD4 and CD8 phenotypes while stimulation of cultures of T cells with PWM yields T cell blasts primarily of the CD8 phenotype. Responses of these two types of cultures to 5HT is also different. Addition of 5HT to cultures stimulated with PHA or OKT3 results in elevation of cAMP and slight inhibition of T cell proliferation. By contrast, addition of 5HT to cultures stimulated with PWM causes a 60% decrease in cAMP levels and a 3-4 fold increase in T cell proliferation. Cells proliferating in these cultures are primarily CD8+ T cells as measured by FMS (not shown) and

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exhibit suppressor cell but not cytotoxic activity. Elimination of CD8+ T cells eliminates the co-mitogenic effect of 5HT in these cultures. 5HT is also a growth factor for certain other typed of cells such as smooth
5 muscle cells or certain types of fibroblasts (46,47). This suggests that the growth factor properties of 5HT are not limited to cells of the immune system.

Results presented clearly show that T cells express receptors for 5HT upon activation and that 5HT can
10 influence the proliferation and function of activated T cells. It is well established that 5HT is a major component of the secretory granules of platelets and should be released at sites of inflammation (2-4). Thus 5HT should be present at sites of inflammation to influence T
15 cell function. More recently it has been shown that purified resting T cells also contain 5HT (50). T cells release 5HT into the media in response to IFN gamma. Under these conditions, the concentration achieved in media is approximately 300 nM/ml/10 T cells. This is
20 very similar to the Kd of the T cell 5HT receptor. If 5HT is released by T cells, it could play an important role in regulation of T cell function by T cells. It is well established that CD8+ T cells do not proliferate in response to PWM in the absence of CD4+ T cells (54-56).
25 Thus it is interesting to hypothesize that 5HT could be released by CD4+T cells in response to IFN gamma and act as a growth factor for CD8+ T cells. Rheumatoid arthritis is a major disease of a large group of rheumatic diseases. Rheumatic diseases with an autoimmune
30 component include rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome and Behcet's disease. The arthritis of rheumatoid arthritis can result in destruction of the
35 joint with consequent deformity. The disease is not confined to joints; vasculitis, caused by immune complexes, can involve the skin, the eye, and the lung.

The arthritis results from a complex interaction of synovial cells with various cellular elements (and their soluble products) that infiltrate from the circulation into the synovial lining of joints. This leads to massive proliferation and activation of synovial cells. The properties of synovial cells in tissue culture have been likened to those of transformed cells. For a more detailed discussion on the sequence of events leading to joint lesions, see Fundamental Immunology 2ed., Paul, (1989) pp 846-849.

A form of experimental arthritis is adjuvant arthritis, which is a purely T cell-mediated autoimmune disease. This form of arthritis can be induced in susceptible strains of rats (e.g. Lewis rats) by injection of Mycobacterium tuberculosis in oil (complete Freund's adjuvant). There is a structural resemblance between mycobacterial peptidoglycans and proteoglycans in joint cartilage. A nonpeptide of a Mycobacterium tuberculosis antigen contains the epitope recognized by T cells mediating adjuvant arthritis. In addition, T cells from patients with rheumatoid arthritis respond to this shared epitope. Paul, supra.

Protocol: Adjuvant Arthritis

Complete Freund's Adjuvant (CFA) is made by supplementing extra heavy mineral oil with 10 mg/ml heat killed Mycobacterium butyrium or Mycobacterium tuberculosis H37Ra. On day 0, female Lewis rats (200-225 g) are given 0.1 ml injection of this adjuvant (100 µg/animal) subcutaneously into the right hind footpad (injected paw). A primary inflammatory reaction occurs in the injected foot. This response subsides by day 5 and is followed on days 9-12 by a secondary, chronic inflammatory/arthritis response in both the injected foot and the contralateral non-injected left foot. Measurements of both footpad and ankle diameter of the day 2 primary response and the days 12 and 16 secondary response (both feet) are made using a hand-held

engineer's caliper. Results are expressed as the mean change swelling + or - S.E. from the day 0 uninjected paw diameters.

Drugs are dissolved or suspended in water, physiologic saline or a mixture of either of the latter in 2% polyethylene glycol 400/0.1% Tween 80 and administered by normal routes (orally, intravenously or intramuscularly) either prior to or after establishment of disease.

Adjuvant arthritis is clearly mediated by T cells and activated T cells clones have been isolated which can transfer the disease to a naive animal. Ritanserin inhibits development of the disease at 10 or 30 mg/kg. Ketanserin does not prevent development of the disease.

15

Table 10

Ritanserin Data - Adjuvant Arthritis

Exp #1.

2 Response - Day 16

20	Mean	Ankle Diam ± S.E. (mm)	% Inhib	Status
	Control	2.55 ± .44		
	Control			Extrm.
25	+ Ritanserin	0.93 ± .31	63.5	Active
	30 mg/kg i.p.			
	(Days - 1,0,1,5,7,9,12)			
	+ Ketanserin	2.09 ± .46	23.7	Inactive
30	Exp. #2			
	Control	2.35 ± .56		
	Control			
	+ Ritanserin	1.39 ± .30	40.3*	Active
	30 mg/kg p.o.			
35	(Days - 1,0,3,6,8,10,13)			
	+ Pelanserin	2.44 ± .34	4.3	Inactive

Exp. #3					
	Control	3.28	$\pm$.44		
	Control				
	+ Ritanserin	2.33	$\pm$.43	29.0	Inactive
5	3.0 mg/kg p.o.				
	+ Ritanserin	0.89	$\pm$.21	72.9	Extrm.
	10 mg/kg p.o.				Active

*Difference in activity may be explained by slightly
10 different dosing schedule.

Materials. Purified recombinant human IFN was purchased
from Amgen (Thousand Oaks, CA) and diluted in media
before use. 5-OH-tryptophan, 5HT, tryptophan,
5-OH-indoleacetic acid, and melatonin were obtained from
15 Sigma and p-chlorophenylalanine (pCPA) was obtained from
Research Biochemicals, Inc. (Natick, MA). RPMI 1640
media, fetal calf serum (FCS), sodium pyruvate and sodium
glutamate were from GIBCO. Flat-bottom multi-well tissue
culture dishes were from Becton Dickinson or from Costar.
20 Solvents for HPLC were from Aldrich and were HPLC grade.

Cell Cultures. The human cervical carcinoma cell line,
ME-180, was obtained from American Type Culture
Collection (Rockville, MD) and was maintained in tissue
culture flasks in RPMI 1640 medium with 10% FCS without
25 antibiotics in a humidified atmosphere of 5% CO₂ in air
at 37°C.

Growth Inhibition Assays. ME-180 cells were plated in
complete media at 1×10^5 cells/ml, 100 μ l/well in 96 well
plates and were cultured in the presence or absence of
30 varying amounts of IFN for 3 d. On the third day,
cultures were pulsed with 1 μ Ci ³H-TdR for 5 hr and were
harvested on filter paper. Cultures were performed in
duplicate a minimum of three times with similar results;
many were repeated as positive controls for subsequent
35 experiments. Duplicates were within 10% of each other.
Incorporation of ³H-TdR was determined with a liquid
scintillation spectrometer.

Extraction and analysis of tryptophan, 5-OH-tryptophan, and 5HT. After various treatments for various periods of time as described in the text, cells and media were separated and mixed with ethanol to achieve a final concentration of 70%. Samples were stored at 0-4°C overnight before removing precipitates and cell debris by centrifugation. Samples were concentrated to dryness with a Speed-vac and dissolved in 20 µl of buffer A before analysis by HPLC (Hewlet-Packard) (13). The HPLC instrument was equipped with a fluorescence detector (Gilson) with a 280 nm excitation filter and a 360 nm emission filter. The two mobile phases consisted of A; 50 nM triethylamine adjusted to pH 3.0 with phosphoric acid and B: buffer A with 40% methanol. The gradient was linear from 100% A to 100% B over 25 min. The retention times of known standards were as follows: 5HT, 3.50 min; 5-OH-tryptophan, 4.77 min; tryptophan, 9.11 min; 5-OH-indoleacetic acid, 12.51 min; and melatonin, 21.69 min. Identification of peaks in cell extracts with known standards was achieved by mixing known amounts of standards with cell extracts and showing identity of peaks on the chromatograms. Recovery of tryptophan and 5HT from cell extracts was greater than 90% as determined by mixing known amounts of standards with cells before initiating the extraction procedure. Experiments quantitating levels of 5HT and tryptophan were performed three times each with similar results.

Reversal of IFN-mediated growth inhibition by tryptophan or 5Htp. Inhibition of cell proliferation by IFN is caused by loss of tryptophan catalyzed by indoleamine 2,3-dioxygenase in certain tumor cell lines. Addition of exogenous tryptophan reduces inhibition of cell proliferation by IFN (Ref 6-9 and Figure 1). Tryptophan, an essential amino acid, is required for protein synthesis and for synthesis of the neurotransmitters, 5HT and melatonin. Results in Figure 1 show that tryptophan, as well as 5Htp, reduces IFN

-mediated inhibition of cell proliferation. On a molar basis, 5Htp is greater than 500-fold more effective than tryptophan at inhibiting IFN activity against ME-180 cells (Figure 17B). 5Htp is a precursor for 5HT synthesis and is not used for protein synthesis. This raises the possibility that inhibition of 5HT synthesis may be an important result of tryptophan depletion in cultures of cells exposed to IFN. Results in Figure 2 compare IFN -mediated inhibition of cell proliferation. Optimal results were obtained when compounds were added on day one and two of the three day culture. Under these conditions only 5Htp blocked IFN activity. Other 5Htp metabolites, 5HT, melatonin, N-Ac-5HT or 5-OH-indoleacetic acid, also products of 5Htp metabolism, failed to block IFN-mediated inhibition of cell proliferation.

IFN depresses intracellular levels of both tryptophan and tHT. Taken together, the above results raise the possibility that loss of tryptophan in culture media results in loss of 5Htp or its metabolites, such as 5HT, and this leads to inhibition of cell proliferation. Figure 3 shows the level of intracellular tryptophan and 5HT in cells after varying periods of time in culture with IFN. Cells contained both tryptophan and 5HT; both were lost upon culture with IFN. 5Htp was undetectable in cell extracts. Synthesis of 5Htp is the rate limiting step in 5HT synthesis and 5Htp does not generally accumulate in cells.

5Htp restores levels of 5HT and cell proliferation in IFN treated cells. Tumor cells were cultured with inhibitory amounts of IFN in the presence or absence of 5Htp to determine if 5Htp restored 5HT levels (Table 5). Treatment with IFN resulted in a decrease in 5HT to undetectable levels. In the presence of 5Htp, levels of 5HT were maintained. In fact, cultures of cells with IFN and 5Htp contained four-fold higher levels of 5HT than untreated cultures. Addition of 5Htp also restored cell proliferation in these cultures.

5Htp does not prevent the loss of tryptophan from media in the presence of IFN. Two possible explanations for the ability of 5Htp to prevent IFN-mediated inhibition of cell proliferation are that it prevents the loss of tryptophan from media or that it restores intracellular 5HT levels. Results in Figure 4 show that 5Htp does not inhibit loss of extracellular tryptophan in cultures of cells exposed to IFN. Control cultures of ME-180 cells did not consume significant amounts of tryptophan in media over the 72 hr culture period. By contrast, cultures of cells treated with IFN consumed 50% of tryptophan in 24 hr and 95% within 48 hr. The rate of loss of tryptophan in media for IFN treated cultures was not inhibited by addition of 5Htp. Under these conditions, levels of 5HT and cell proliferation were restored to control values.

Inhibition of cell proliferation by low concentrations of tryptophan is reduced by 5Htp. Proliferation of tumor cells in the presence of varying concentrations of methionine or leucine reaches maximum rates between 10-20 μ M amino acid. By contrast, proliferation of tumor cells in the presence of varying concentrations of tryptophan reaches maximum rates between 50-100 μ M amino acid (6). Figure 5 compares proliferation of ME-180 cells in the presence of varying concentrations of tryptophan with or without 3 μ M 5Htp. In the absence of 5Htp, maximum proliferation was obtained with 50 μ M tryptophan. In the presence of 5Htp, maximum proliferation was obtained with 10 μ M tryptophan. Under these conditions, addition of 5Htp did not affect the ability of cells to incorporate 3 H-tryptophan into proteins. This indicates that 5Htp is not converted to tryptophan by ME-180 cells and used for protein synthesis (not shown).

Inhibition of 5HT synthesis inhibits proliferation by ME-180 cells. The above results suggest that lowering intracellular 5HT should inhibit proliferation of ME-180

cells. Therefore, ME-1870 cells were cultured with pCPA, a specific inhibitor of tryptophan hydroxylase, to determine if this resulted in loss of intracellular 5HT and inhibition of cell proliferation. The results are shown in Table 6. Culture of ME-180 cells for 48 hr with pCPA resulted in loss of tHT and inhibition of cell proliferation but did not cause a loss in extracellular tryptophan. Loss of 5HT and inhibition of cell proliferation was prevented by addition of 5Htp. Thus, depletion of 5HT by two independent mechanisms, indoleamine 2,3 dioxygenase catalyzed oxidation of tryptophan or inhibition of tryptophan hydroxylase, resulted in inhibition of tumor cell proliferation. In both instances, tumor cell proliferation was recovered by restoring 5HT levels with 5Htp.

Additional tumor cell lines, as well as normal diploid cells, were examined to determine if they also contained 5HT, whether pCPA lowered intracellular 5HT concentrations and inhibited proliferation and whether 5Htp would restore 5HT levels and reverse inhibition of proliferation. These results are shown in Table 7. All tumor cell lines examined contained 5HT and proliferation of four of five cell lines was inhibited by pCPA. Inhibition of proliferation by tumor cell lines was largely reversed by addition of 5Htp. This suggests that many tumor cell lines may require 5HT or metabolites of 5HT for proliferation. By contrast, proliferation of several different types of normal diploid cells was not inhibited by pCPA.

30

35

Table 5

5Htp restores 5HT levels and proliferation in IFN-treated ME-180 cells.

5	Intracellular Levels*		
	Culture Conditions	5HT (pmol/mg protein)	Proliferation (³ H-TdR)
10	ME-180 Cells	18	98,069 ± 1025
	+IFN	1	19,789 ± 874
	+IFN +5Htp	80	99,672 ± 1984
	+5Htp	32	96,331 ± 1593
15	*ME-180 cell cultures were for three days and were initiated at 2x10 ⁵ cells/ml. IFN (300 µ/ml) was added at initiation, and 5Htp (3 µM final concentration) was added every day. Samples were harvested and analyzed for 5HT by HPLC.		

20

Table 6

Inhibition of tryptophan hydroxylase depresses 5HT levels and inhibits cell proliferation.

25	Culture Conditions	Intra-cellular*	Extra-cellular	Pro-lifer-ation
		5HT (pmol/mg protein)	Tryp (um)	(³ H-TdR)
30	ME-180 Cells	16.5	12	44443 ± 2244
	+pCPA	3.1	25	16642 ± 1723
	+pCPA			
	+5Htp	37.1	n.d.	42221 ± 4128
35	+5Htp	44.6	n.d.	42982 ± 2629

*ME-180 cells were cultured for 48 hr in the presence of 2mM pCPA and/or 10 uM 5Htp. Culture media or cells were harvested and analyzed for tryptophan and 5HT by HPLC.

5

Table 7

Inhibition of 5HT synthesis inhibits
proliferation by tumor cells.

Cell	5HT Content	Proliferation (cpmx1000)		
		Control	+pCPA	+pCPA +5-OH-trypt
10 Population*	(mol/mg protein)			
<u>Transformed Cell Lines</u>				
ME-180	13 $\pm$ 2	44.7	12.6	42.2
15 HT29	24 $\pm$ 3	56.7	14.1	47.4
Jurkat	31 $\pm$ 3	85.6	24.0	40.2
MOLT-4	22 $\pm$ 5	34.3	36.5	31.5
U937	39 $\pm$ 6	194.0	71.8	155.6
BT20	N.D.	29.0	6.5	29.7
20 <u>Normal Diploid Cells</u>				
T cell Blasts	25 $\pm$ 2	45.9	37.1	41.4
Fibroblasts	29 $\pm$ 4	10.3	9.3	10.2
(lung)				
Fibroblasts	N.D.	4.8	3.9	4.5
25 (synovial)				
<u>Endothelial</u>	<u>N.D.</u>	<u>7.5</u>	<u>7.9</u>	<u>7.5</u>

*Different human cell lines were obtained from ATCC. Their culture conditions have been described (6). Sera were dialyzed before use to remove 5HT. ME-180 (Cervical carcinoma), BT20 (breast carcinoma), HT29 (colon adenocarcinoma), U937 (histocytic lymphoma, monocyte lineage), Jurkat (T cell lymphoma), MOLT-4 (acute lymphoblastic leukemia), endothelial cells (primary cultures), synovial fibroblasts (primary cultures), lung fibroblasts (MRC-5; long term line) or primary cultures of T cell blasts were cultured for 48 hr.

Serotonin was found to be present in neoplastic or tumor cells and the 5HT₂-like receptor was found to be present on tumor cells. It has been demonstrated that like activated T cells, tumor cell proliferation can be regulated by serotonin receptor agonists and antagonists as well as by inhibition of serotonin synthesis.

In nervous tissue, serotonin (5-hydroxytryptamine, 5HT) is synthesized by hydroxylation and decarboxylation of tryptophan and is stored in granules (35-36). It is released in response to appropriate stimuli and binds to specific receptors on neighboring cells activating second messenger pathways (37-39). The rate limiting step in serotonin synthesis is the level of tryptophan hydroxylase activity. A specific enzyme inhibitor of tryptophan hydroxylase, p-chlorophenylalanine (pCPA), has been employed to deplete serotonin levels within cells (40-41). Human cervical carcinoma cells (ME-180) were treated with pCPA for 48 hr in the presence or absence of 5-OH-tryptophan and analyzed for 5HT content or for rates of cell proliferation. Cultured cells contained 13 ± 2 pmol/mg protein 5HT which was reduced to 4 ± 1 pmol/mg protein 5HT after treatment with pCPA. Similarly, media from cultured cells contained 44 nM 5HT which was reduced to 10 nM by culturing with pCPA. Addition of 5-OH-tryptophan restored 5HT levels to that of control cells. Treatment of ME-180 cells with pCPA also reduced H-TdR incorporation by 70%. Inhibition was also largely reversed by 5-OH-tryptophan (Table VIII). Several additional tumor cells lines and normal diploid cell lines were also analyzed for 5HT content and cultured for 48 hr in the presence of pCPA to reduce 5HT levels (table IX). The concentration of 5HT in cell extracts ranged between 10-40 pmol/mg protein. 5-OH-tryptophan, the precursor of 5HT, was not detectable in cell extracts. Culturing cells in the presence of pCPA inhibited cell proliferation in 5 of 6 tumor cell lines examined but did not significantly affect proliferation of normal diploid

cells. Addition of 5-OH-tryptophan to cell cultures treated with pCPA largely reversed inhibition of proliferation in tumor cell cultures but did not appreciably affect proliferation in cultures of normal
5 diploid cells. Thus, depletion of 5HT resulted in inhibition of tumor cell proliferation but not normal cell proliferation and restoration of 5HT levels by addition of 5-OH-tryptophan restored proliferation in these cells.

10 Serotonin accumulated in media at concentrations which can transduce second messenger pathways through 5HT receptors (37-38) and 5HT receptors which transduce second messenger pathways (inositol phosphate release and changes in intracellular Ca^{++}) have been identified on
15 Jurkat cells but not on Molt-4 cells (42). Therefore a number of 5HT receptor agonists and antagonists were tested to determine if they would also inhibit tumor cell proliferation in a 5HT reversible manner. Of those tested, ritanserin, a 5HT₂ receptor antagonist 40,
20 inhibited tumor cell proliferation by 70-95% (five of six lines tested) at concentration between 10-50 μ M but did not inhibit proliferation by normal diploid cells. Lower concentrations of ritanserin (<10 μ M) did not inhibit proliferation by tumor cells. Addition of 5HT partially
25 reversed the inhibitory effects of ritanserin (Table X). Proliferation by the one tumor cell line, Molt-4, which was not inhibited by pCPA, was also not inhibited by ritanserin. Additionally, while pCPA lowered concentrations of 5HT in cell extracts, ritanserin
30 actually increased 5HT content in cell extracts. For example, extracts from ME-180 cells contained 15.5 pmol/mg protein of 5HT while extracts from cultures treated with ritanserin contained 58.8 pmol/mg protein of 5HT. Thus, both inhibitors of 5HT synthesis as well as
35 5HT receptor antagonists inhibited proliferation by tumor cells but not by normal diploid cells. A number of ligands which bind 5HT receptors were tested for their

ability to prevent ritanserin-mediated inhibition of tumor cell proliferation. These results are shown in figure 14. Ketanserin and pelanserin, also 5HT₂ receptor antagonists, mianserin, a 5HT_{1c} receptor antagonist and 8-OH-DPAT and propranolol, a 5HT₁ receptor agonist and antagonist, respectively, partially prevented inhibition of proliferation by ritanserin in a concentration dependant manner. These data which largely parallel binding data on Jurket cells have failed to classify these 5HT receptors into one of the well characterized 5HT receptor subtype (42). Interestingly, ritanserin, ketanserin and pelanserin are all 5HT₂ receptor antagonists with similar pharmacological and biochemical properties but ritanserin inhibits tumor cell proliferation and ketanserin and pelanserin prevent inhibition by ritanserin. This is not to imply that these data are sufficient to define a new 5HT receptor subtype. Analysis of these receptors in much greater detail will be required to completely define these 5HT receptor types. Taken together these data raise the possibility that 5HT and 5HT receptors may be required for proliferation of certain tumor cell lines in tissue culture. Some of the characteristics of this proliferation is consistent with an autocrine pathway of growth factor action where a cell releases its own growth factors which bind to cell surface receptors and stimulate cell function (43-44). Data presented here show that 5HT is produced by tumor cells and that it is required for proliferation. Studies with ligands known to bind to 5HT receptors support the notion that 5HT may act through cell surface receptors to stimulate proliferation. In this regard it is important to note that 5HT has been shown to be a growth factor for both quiescent fibroblasts as well as smooth muscle cells (45-47). In these instances exogenous as opposed to endogenous 5HT was employed to stimulate DNA synthesis. These results are consistent with many current notions of

tumorigenesis where normal diploid cells may become transformed by acquiring the ability to produce their own growth factors or growth factor receptors (48-49). Production of 5HT and 5HT receptors may be one way which normal cells acquire a permanent transformed phenotype. Whether tumor cells synthesize serotonin and require serotonin for proliferation in vivo is an important question which is unanswered.

10	Cultures	5HT		Proliferation
		intracellular (pmol/mg protein)	extracellular (cpm) (nM)	
15	ME-180 Cells	13 $\pm$ 2	44 $\pm$ 3	44,700 $\pm$ 5620
	+pCPA	4 $\pm$ 1	10 $\pm$ 2	12,600 $\pm$ 1220
	+pCPA+5-OH-trypt	16 $\pm$ 4	56 $\pm$ 4	42,200 $\pm$ 4220

20

Table 12

Inhibition of 5HT synthesis inhibits proliferation by tumor cells but not by normal diploid cells. Different human cell lines were obtained from ATCC. Their culture conditions have been described (50). Sera were dialyzed before use to remove 5HT. ME180 (cervical carcinoma), BT20 (breast carcinom), HT29 (colon adenocarcinoma), U937 (histocytic lymphoma, monocyte lineage), Jurkat (T cell lymphoma), MOLT-4 (acute lymphoblastic leukemia), endothelial cells (primary cultures), synovial fibroblasts (primary cultures), lung fibroblasts (MRC-5; long term line) or primary cultures of T cell blasts were cultured for 48 hr. Other conditions were as in Table 11.

35

5		5HT Content	Proliferation		
	Cell	(pmol/mg	(cpm x 1000)		
	Population	Protein)			
		Control	+pCPA	+pCPA	
			+pCPA	+5-OH-trypt	
10					
	<u>Transformed Cell Lines</u>				
	ME180	13 $\pm$ 2	44.7	12.6	42.2
	HT29	24 $\pm$ 3	56.7	14.1	47.4
15	Jurkat	31 $\pm$ 3	85.6	24.0	40.2
	MOLT-4	22 $\pm$ 5	34.3	36.5	31.5
	U937	39 $\pm$ 6	194.0	71.8	155.6
	BT20	N.D.	29.0	6.5	29.7
	<u>Normal Diploid Cells</u>				
20	T cell				
	Blasts	25 $\pm$ 2	45.9	37.1	41.4
	Fibro-				
	blasts	29 $\pm$ 4	10.3	9.3	10.2
	(lung)				
25	Fibro-				
	blasts	N.D.	4.8	3.9	4.5
	(synovial)				
	Endothelial	N.D.	7.5	7.9	7.5
30					
35					

Table 13

Inhibition of proliferation of tumor cells by a 5HT receptor antagonist and reversal by 5HT. Tumor cell lines and normal diploid cells were cultured for 48 hrs in the presence or absence of 30 μ M retanserine (a 5HT₂ receptor antagonist which is active against a 5HT₂ site at concentrations of 1-10 nM) with or without 1 μ M 5HT. Addition of 5HT did not affect control proliferation. Cultures were set up in triplicate and the standard error was less than 10% (data not shown).

	Cell Population	3H-TdR Incorporation (cpm x 1000)		
		Control	+ritanserine	+ritanserine +5HT
	<u>Transformed Cell Lines</u>			
	ME180	40.7	2.2	21.4
	Jurkat	75.8	0.4	51.6
20	HT29	159.5	25.3	58.4
	U937	68.9	15.2	45.8
	MOLT-4	34.5	32.8	55.5
	BT20	27.3	3.1	19.1
	<u>Normal Diploid Cells</u>			
25	Fibroblasts	10.3	8.7	8.0
	T Cell Blasts	43.9	40.5	45.5
	Fibroblasts (synovial)	10.9	14.4	11.5
30	Endothelial cells	7.4	7.4	6.4

In addition to agonists and antagonists that can be chemically synthesized, it is within the scope of this invention to generous mimotapes and/or antibodies to the 5HT₂-like receptor.

The identification of the novel 5HT2-like receptor allows for the generation of antibodies to the receptor. The antibodies have application as a probe for research and as a therapeutic. Techniques for generating
5 antibodies are well known in the art. For reference to methods see Fundamental Immunology, Second Edition, supra Chapter 12. It is recognized that a plurality of "types" of antibodies can be generated including polyclonal, monoclonal, chimeric, humanized and human. What these
10 antibodies "types" have in common is the ability to recognize an epitope or epitopes specific to the novel 5HT2-like receptor.

With the identification of the novel 5HT2-like receptor, purified receptor can be used as a probe for
15 screening libraries of random peptide sequence to identify peptide that specifically bind to proteins. Alternatively, antibodies generated against the purified receptor can be used as a probe. For techniques on constructing a library of peptides, see Cwirla et al.,
20 Peptides on page: A vast library of peptides for identifying ligands, Proc.Natl. Acad. Sci. USA, Vol. 87, 6378-6382, August 1990. For methods of screening see Devlin et al., Random Peptide Libraries: A Source of Specific Protein Binding Molecules, Science, Vol. 249,
25 404-406, 27 July 1990 and Scott and Smith, Searching for Peptide Ligands with an Epitope Library Science, Vol. 249, 386-390, 27 July 1990.

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What is claimed is:

1. A method of regulating the functions of activated T cells exhibiting a 5HT1a receptor comprising reducing the availability of 5HT1a receptor binding sites.
2. The method of claim 1 wherein the availability of 5HT1a receptor binding sites is reduced by introducing an effective amount of at least one antagonist selected from the class of 5HT receptors ligands.
3. The method of claim 2 wherein said class of 5HT receptor ligands includes spiperone, mimotopes and antibodies to said 5HT1a receptor.
4. A method of regulating the function of activated T cells exhibiting a 5HT1a receptor comprising functionally decreasing the amount of serotonin available for binding to said 5HT1a receptor.
5. The method of claim 4 wherein the function of activated T cells is decreased by administering an effective amount of a compound to inhibit the activity of the enzyme tryptophan hydroxylase thereby inhibiting serotonin synthesis.
6. A method of up-regulating the function of activated T cells exhibiting 5HT1a receptors comprising functionally increasing the availability of 5HT1a receptor binding sites.
7. The method of claim 6 wherein the function of activated T cells is increased by introducing an amount of at least one agonist selected from the class of 5HT1a receptor ligands including 8-hydroxydipropylaminotetralin HBr.
8. A method of treating a T-cell dependent disease state in a mammal comprising down-regulating the function of cells exhibiting 5HT1a receptors by reducing the availability of 5HT1a receptor binding sites.
9. The method of claim 8 wherein the function of cells is reduced by introducing an effective amount of at least

one antagonist selected from the class of 5HT receptor ligands, sufficient to bind to said 5HT receptor to interrupt cell function.

10. The method of claim 9 wherein said class of 5HT
5 receptor ligands includes spirerone, mimotopes and antibodies to said 5HT receptors.

11. A method of treating a T-cell dependent disease state in a mammal comprising down-regulating proliferation of cells exhibiting 5HT1a receptors by functionally
10 decreasing the amount of serotonin available to said for binding to said 5HT receptor.

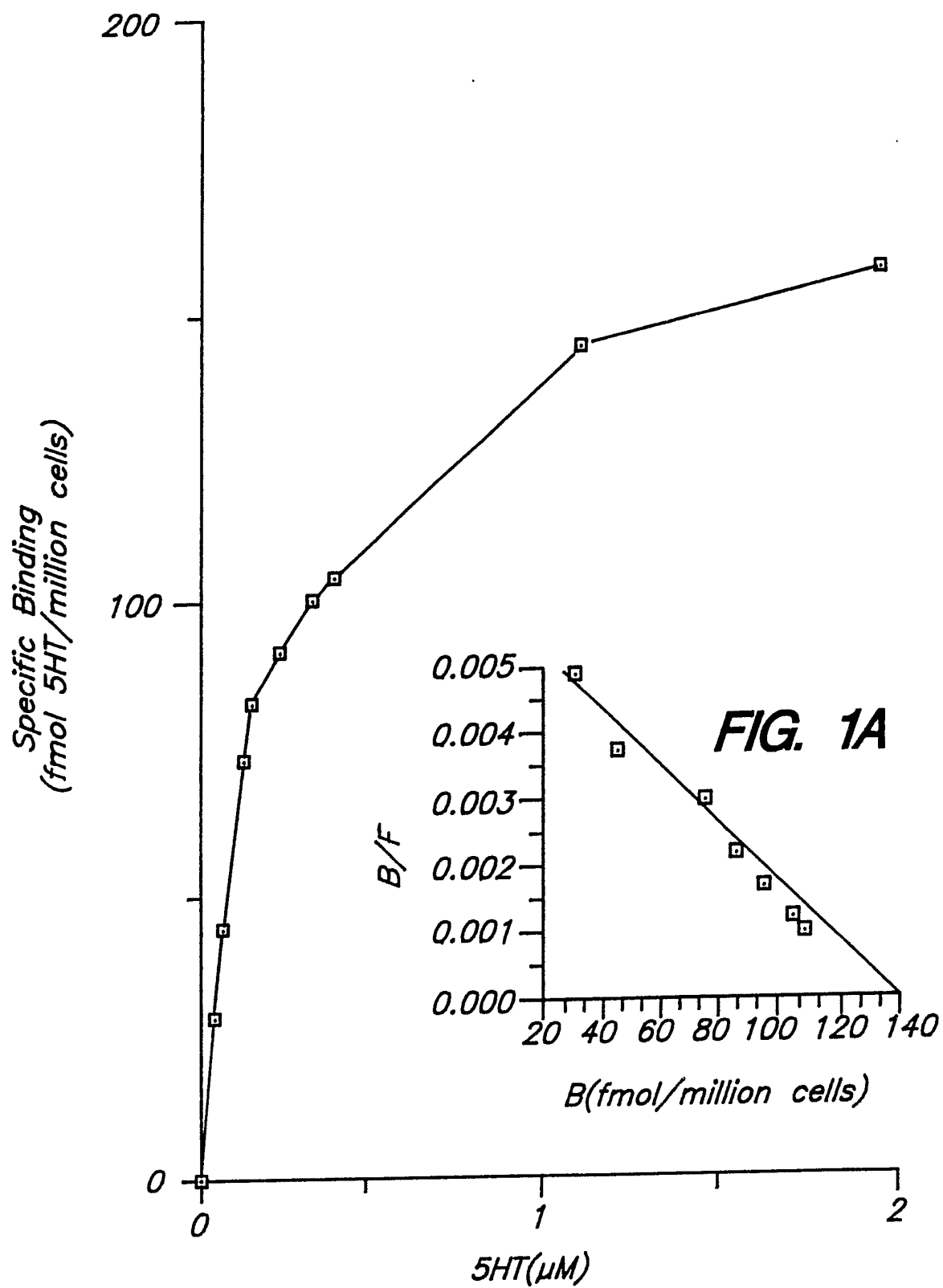
12. The method of claim 11 wherein the proliferation of activated T cells is decreased by administering an effective amount of a compound to inhibit the activity of
15 the enzyme tryptophan hydroxylase thereby inhibiting serotonin synthesis.

13. The method of claim 12 wherein the proliferation of activated T cells is decreased by introducing an effective amount of at least one antagonist selected from
20 the class of 5HT receptor ligands including ritanserin, mianserin, spiperin, mimotope and antibodies to said 5HT1a receptor.

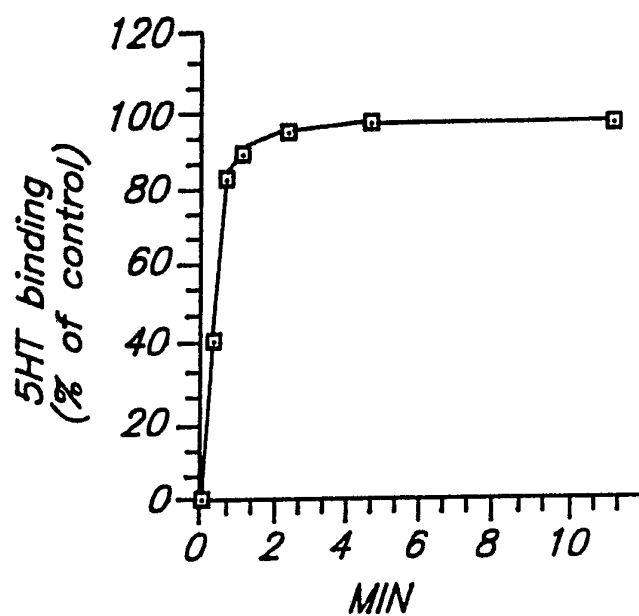
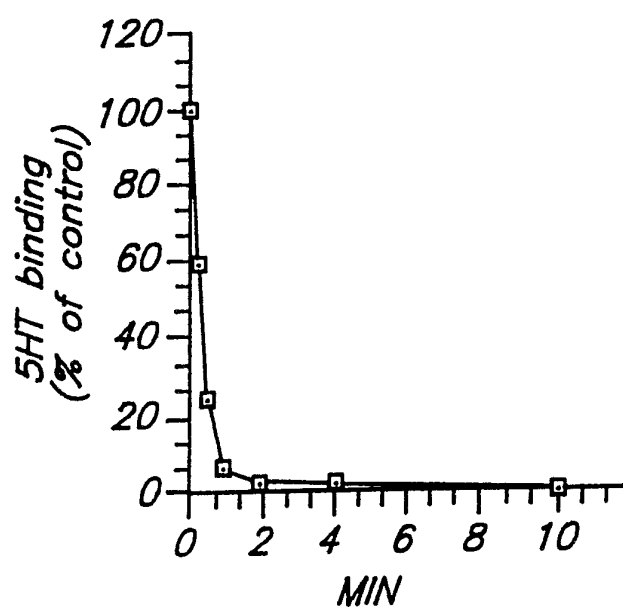
14. A method of treating an immune deficient disease state in a mammal comprising up-regulating the function
25 of activated T cells exhibiting 5HT1a receptors by functionally increasing the availability of 5HT1a receptor binding sites by introducing an effective amount of at least one agonist selected from the class of 5HT1a receptor ligands including 5HT, Ketanserin, and L-methyl
30 5HT said effective amount sufficient to enhance cell function.

15. A method of up-regulating the function of the subpopulation CD8+ of activated T cells exhibiting a 5HT1a receptor comprising an effective amount of serotonin
35 sufficient to increase cell function.

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**FIG. 1**

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**FIG. 2A****FIG. 2B**

3 / 23

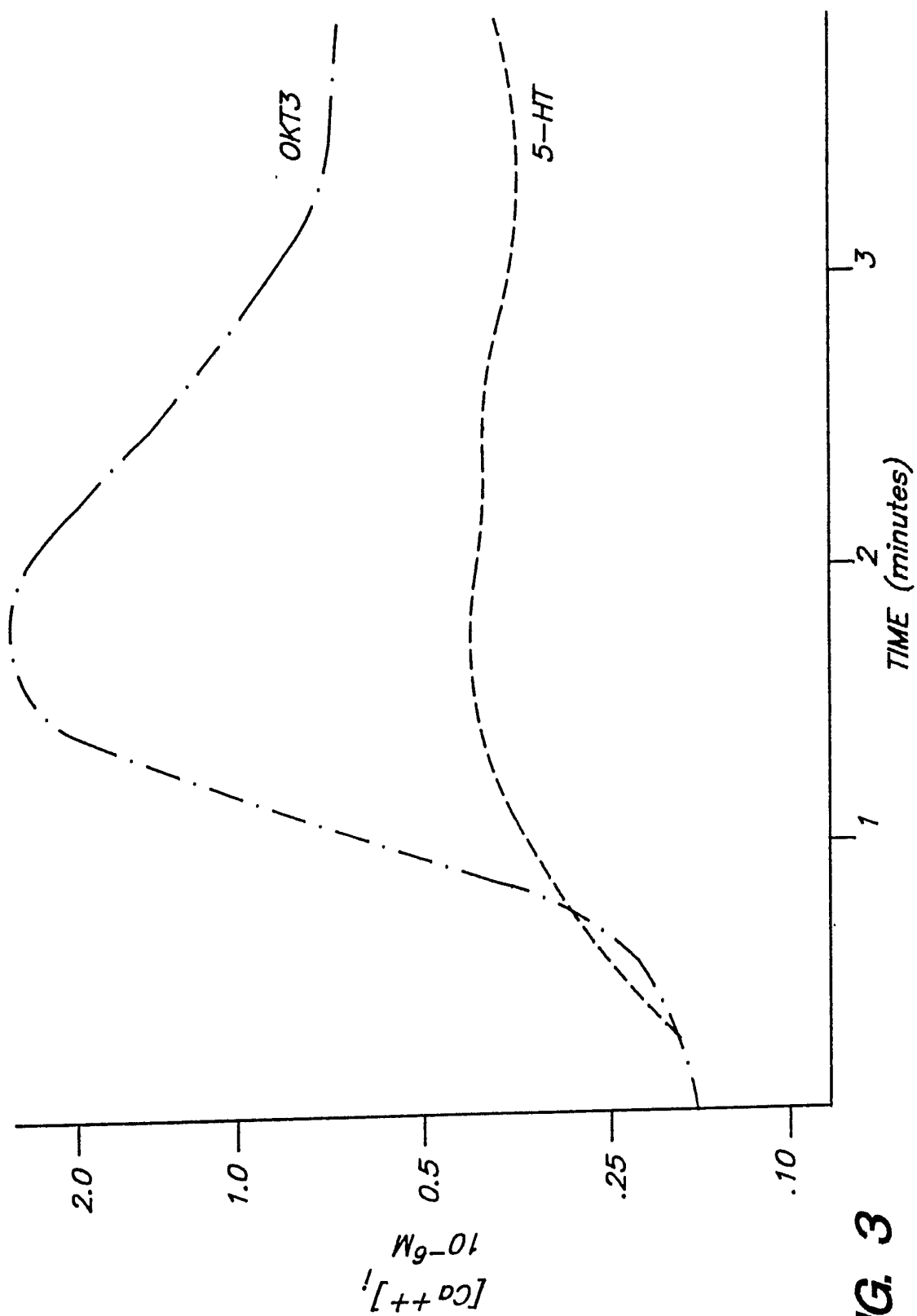
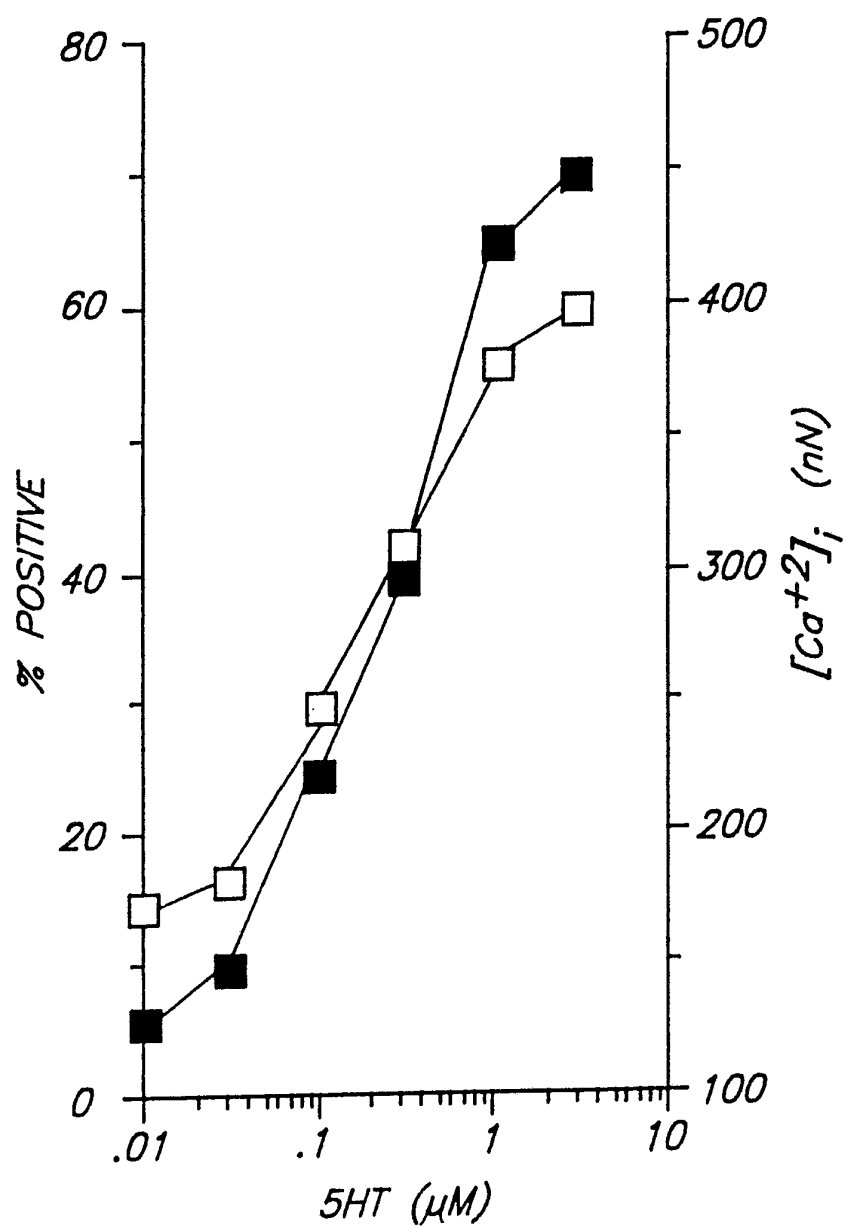


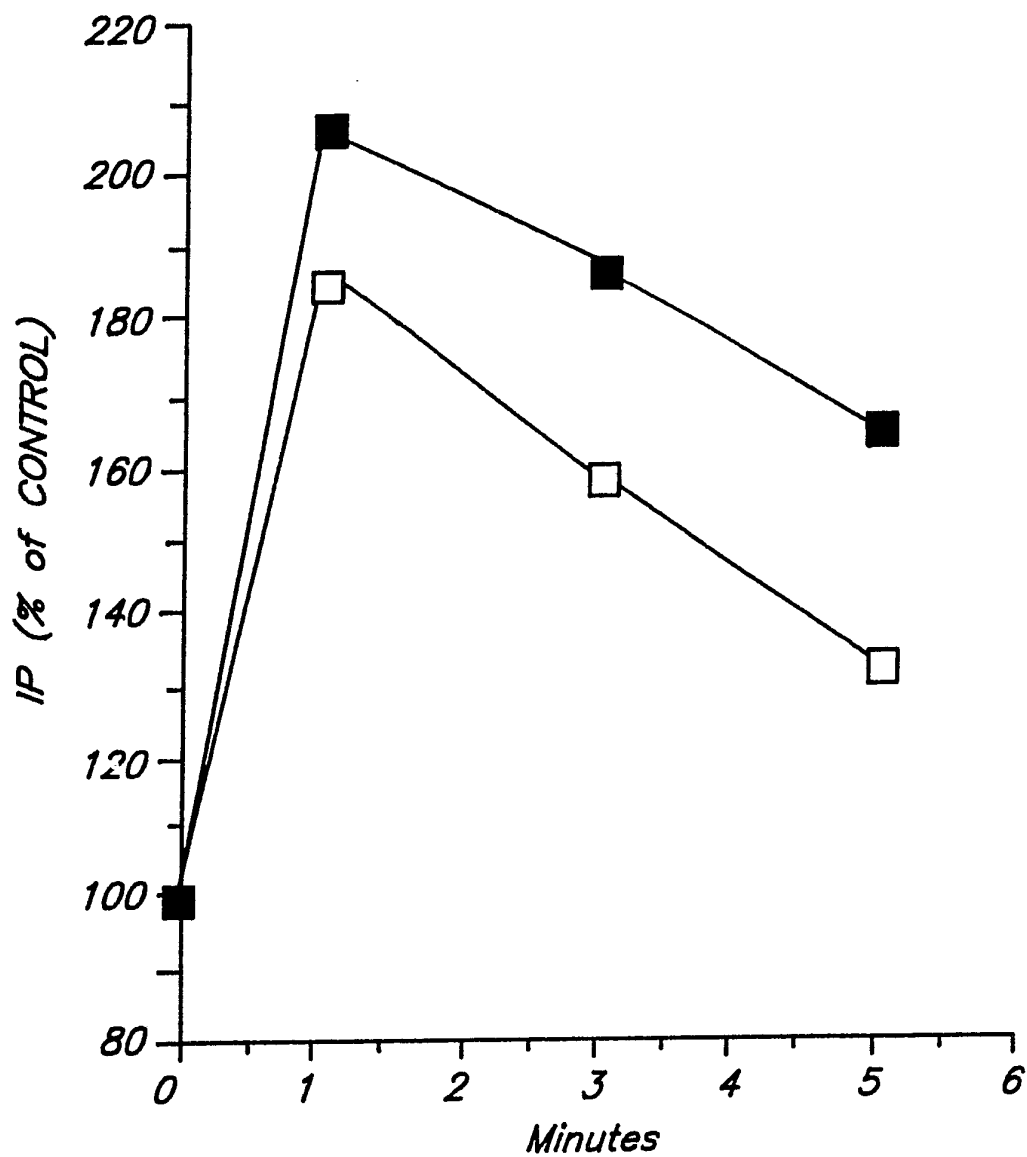
FIG. 3

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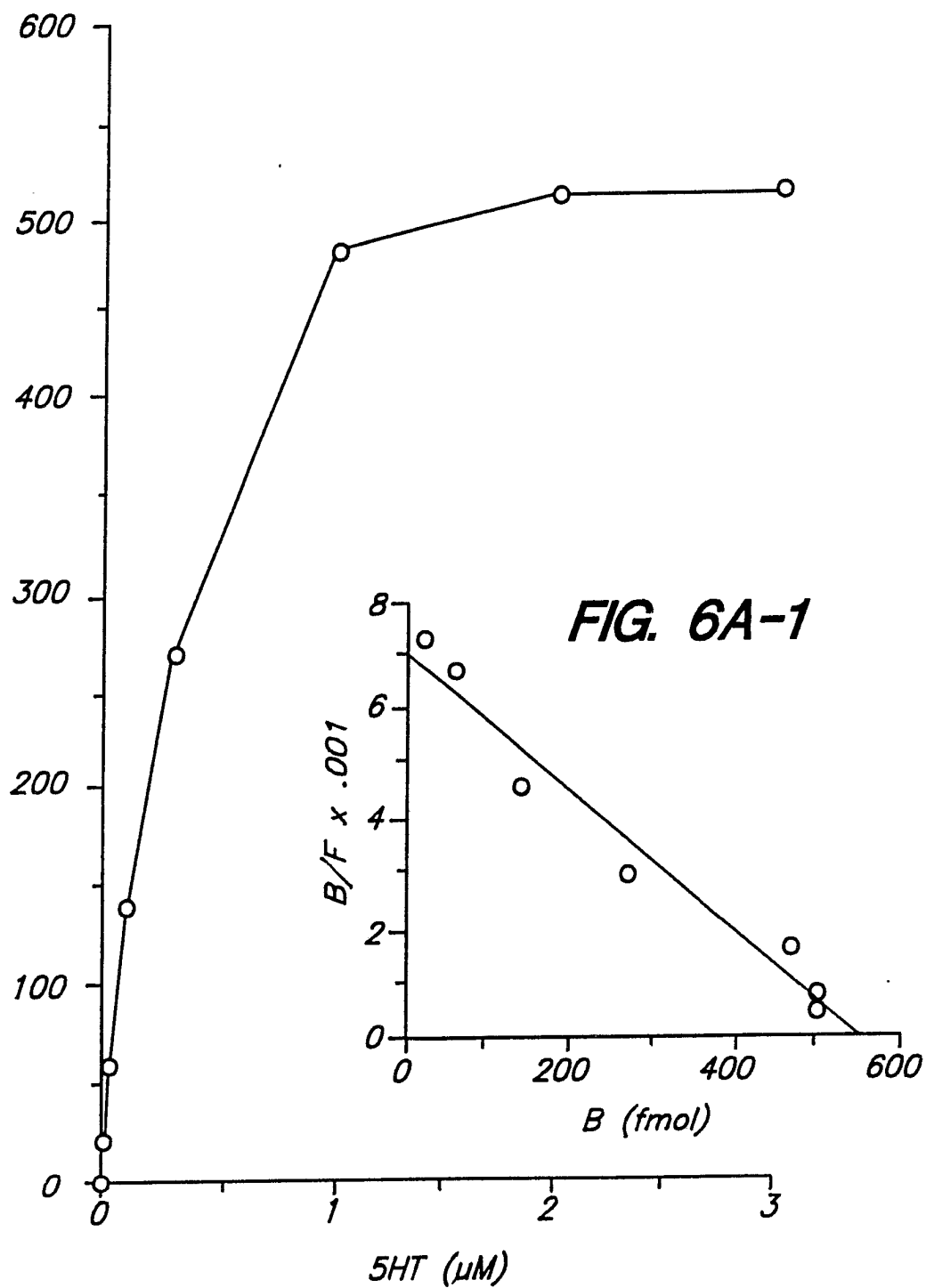
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**FIG. 4**

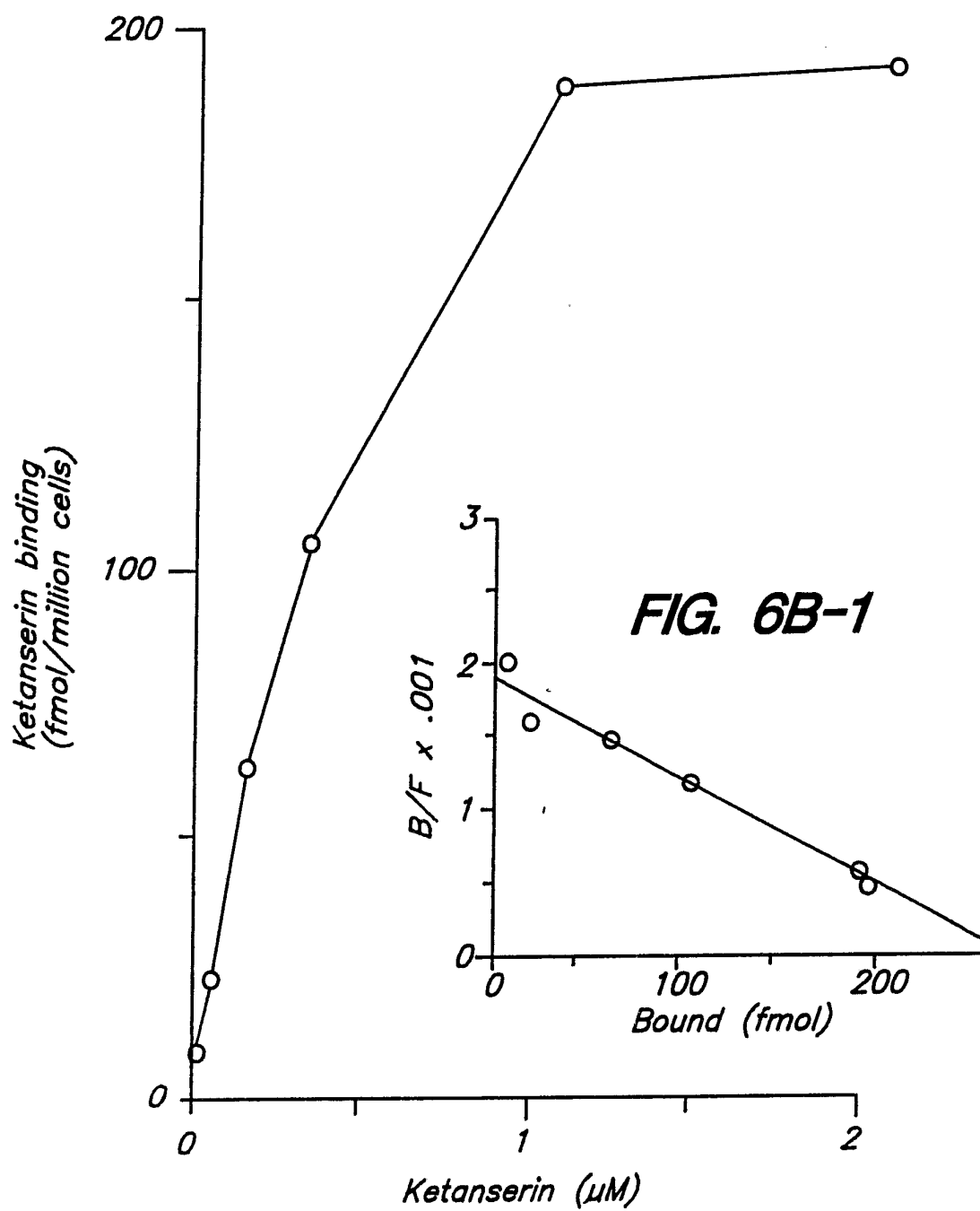
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**FIG. 5****SUBSTITUTE SHEET**

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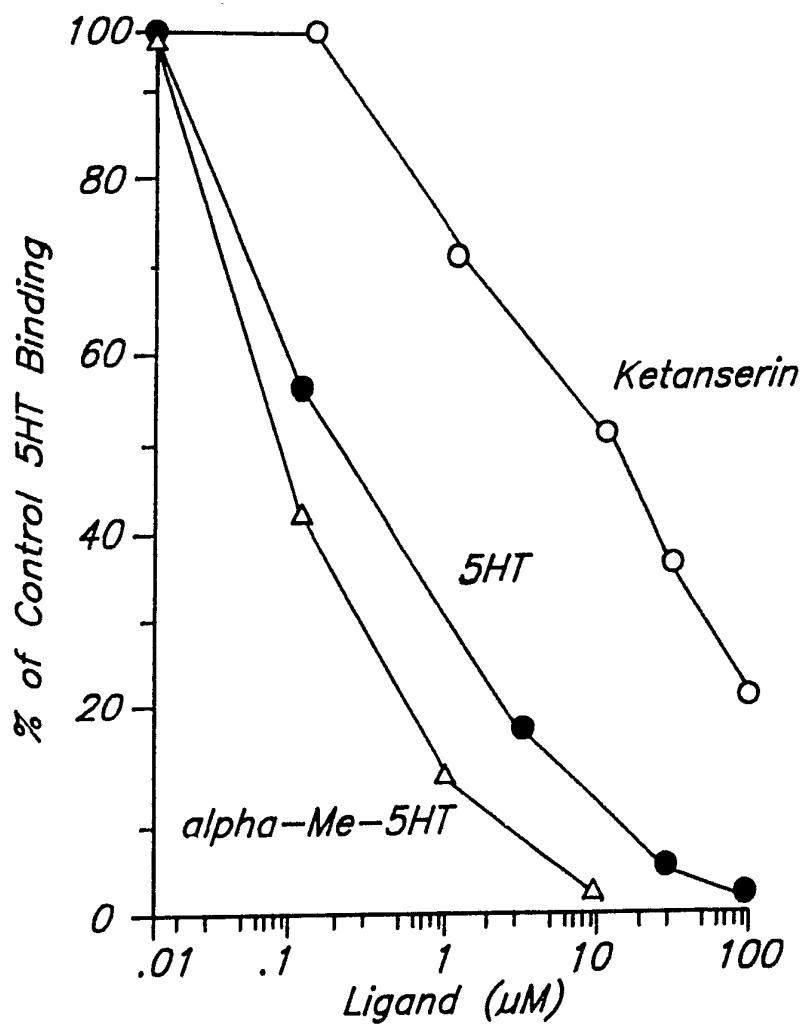
**FIG. 6A**

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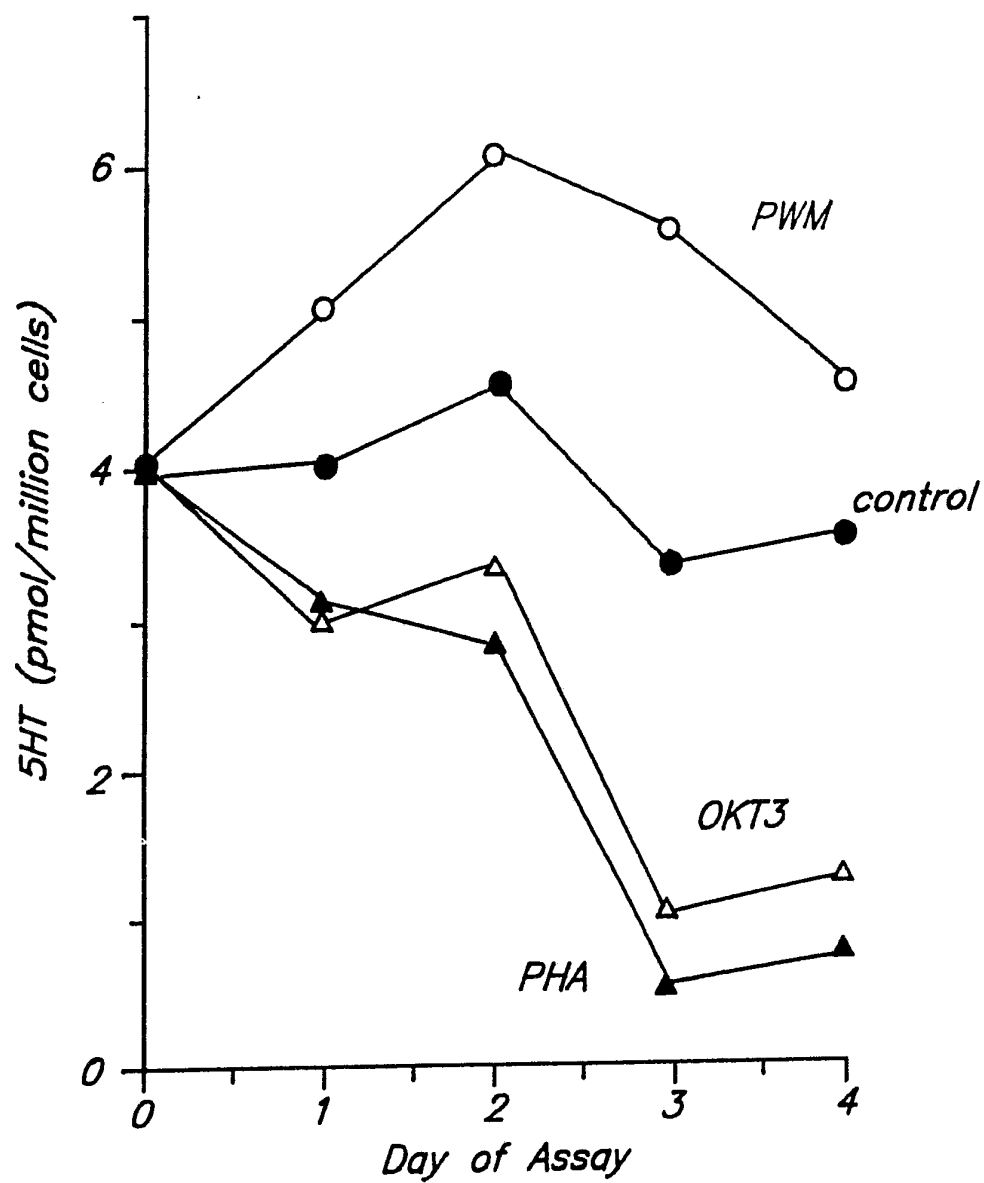
**FIG. 6B**

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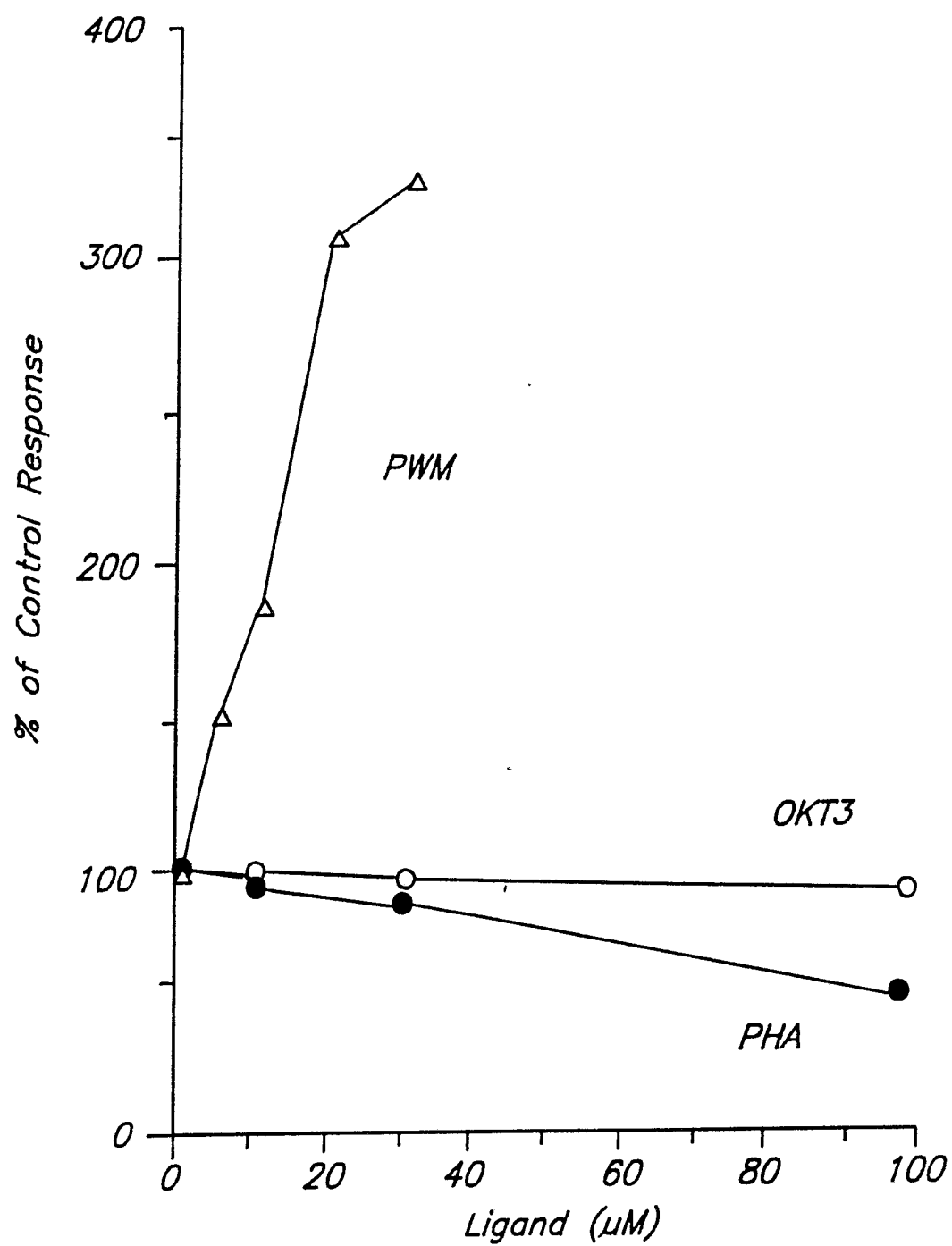
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**FIG. 7****SUBSTITUTE SHEET**

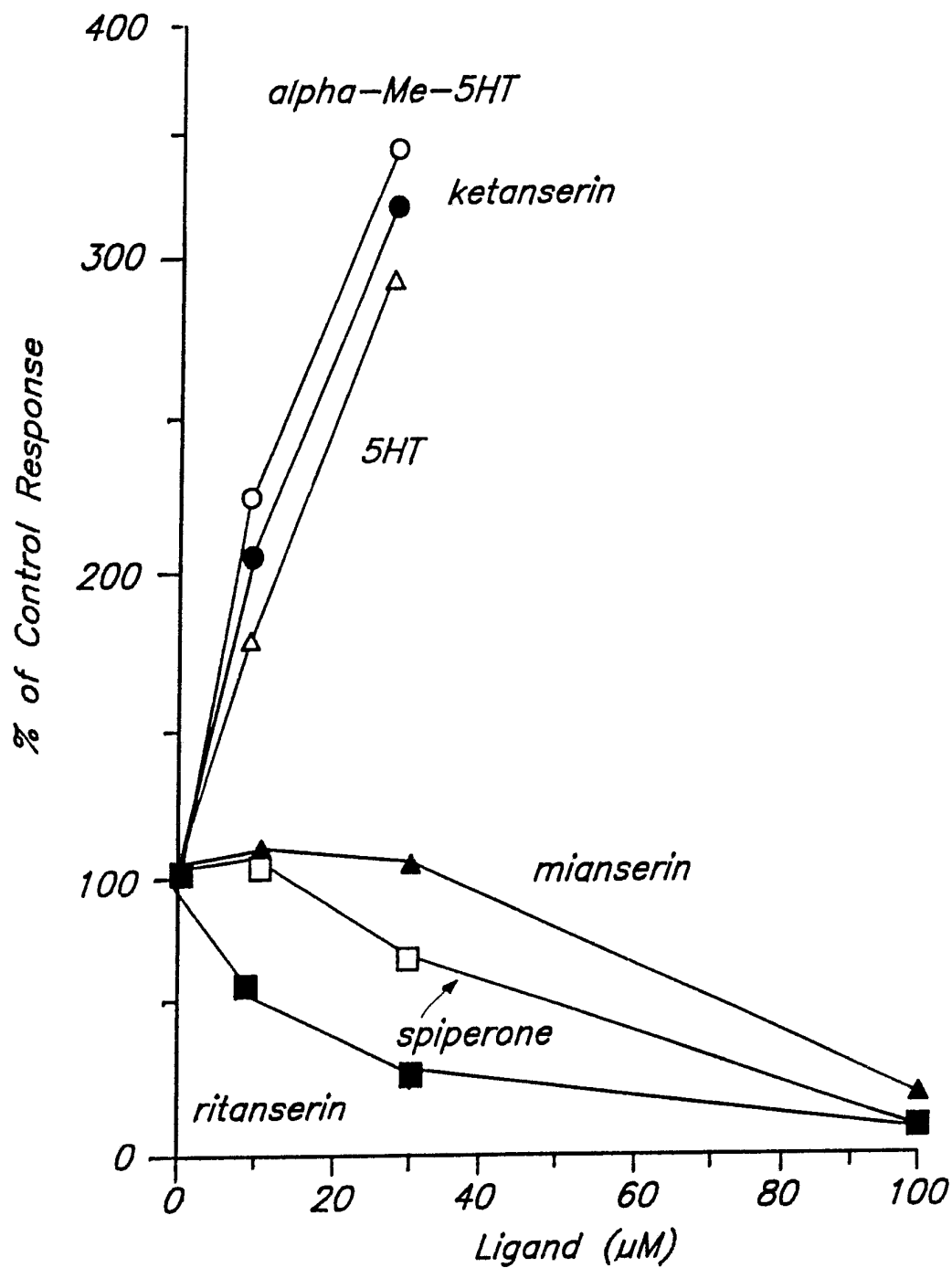
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**FIG. 8**

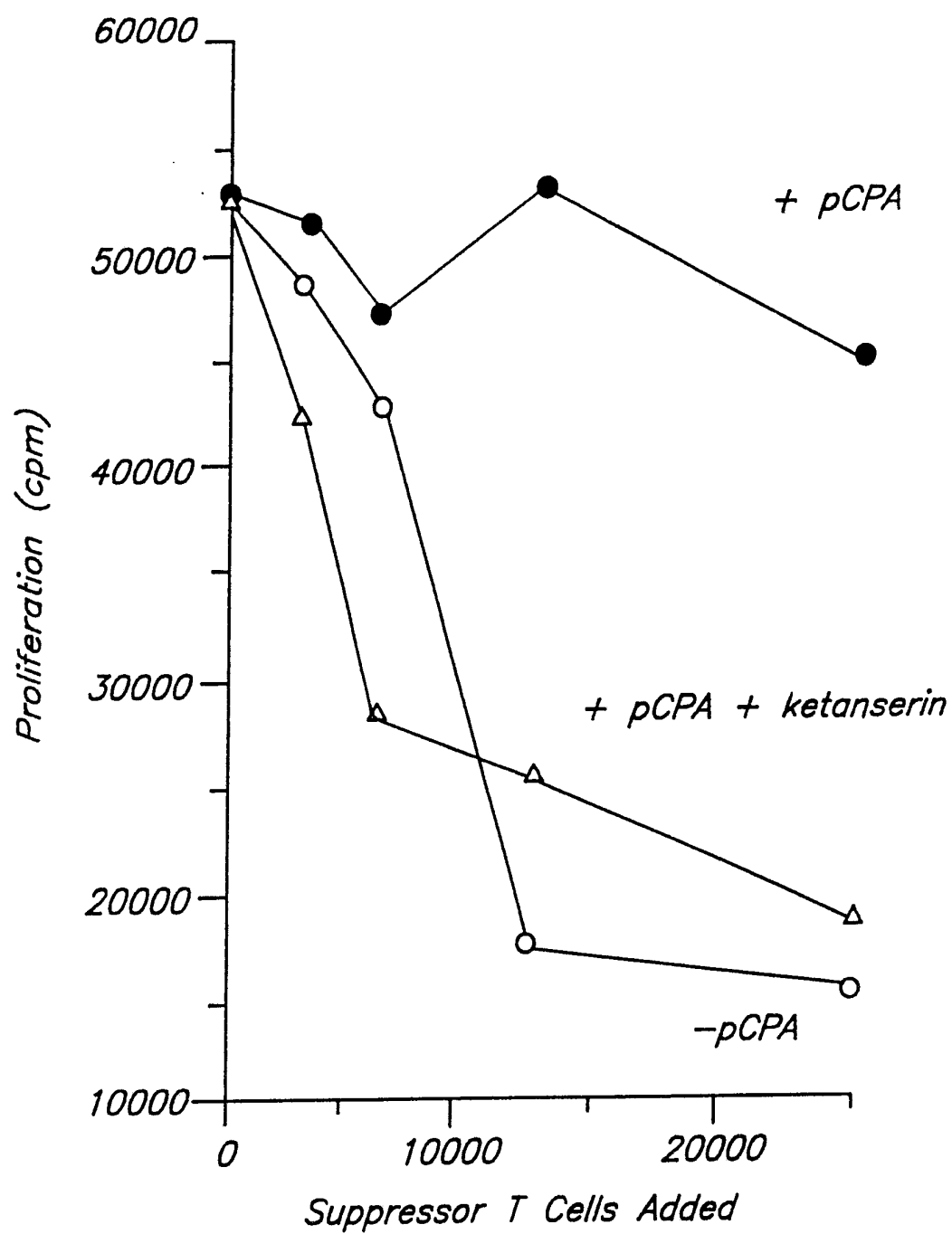
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**FIG. 9**

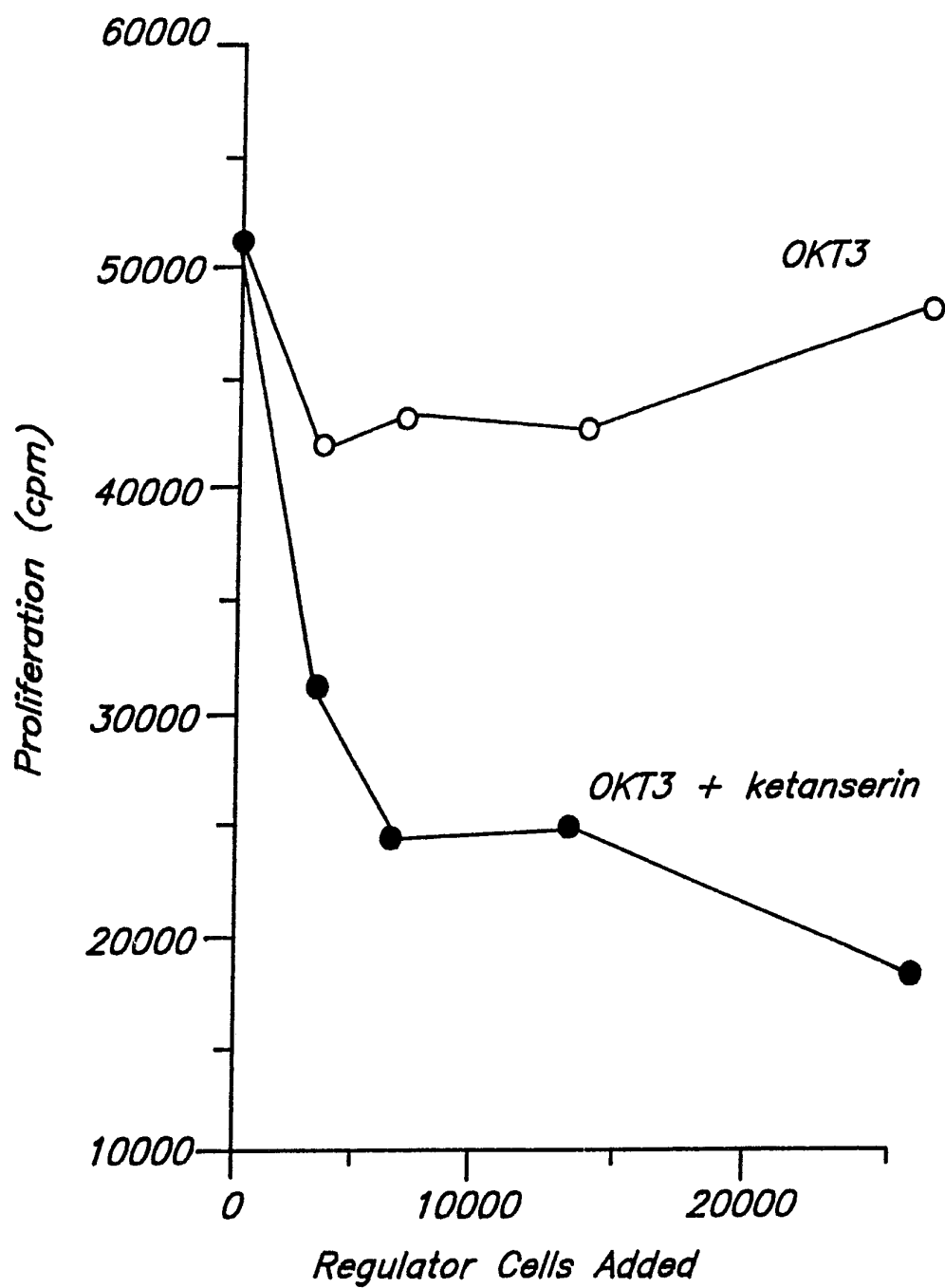
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**FIG. 10**

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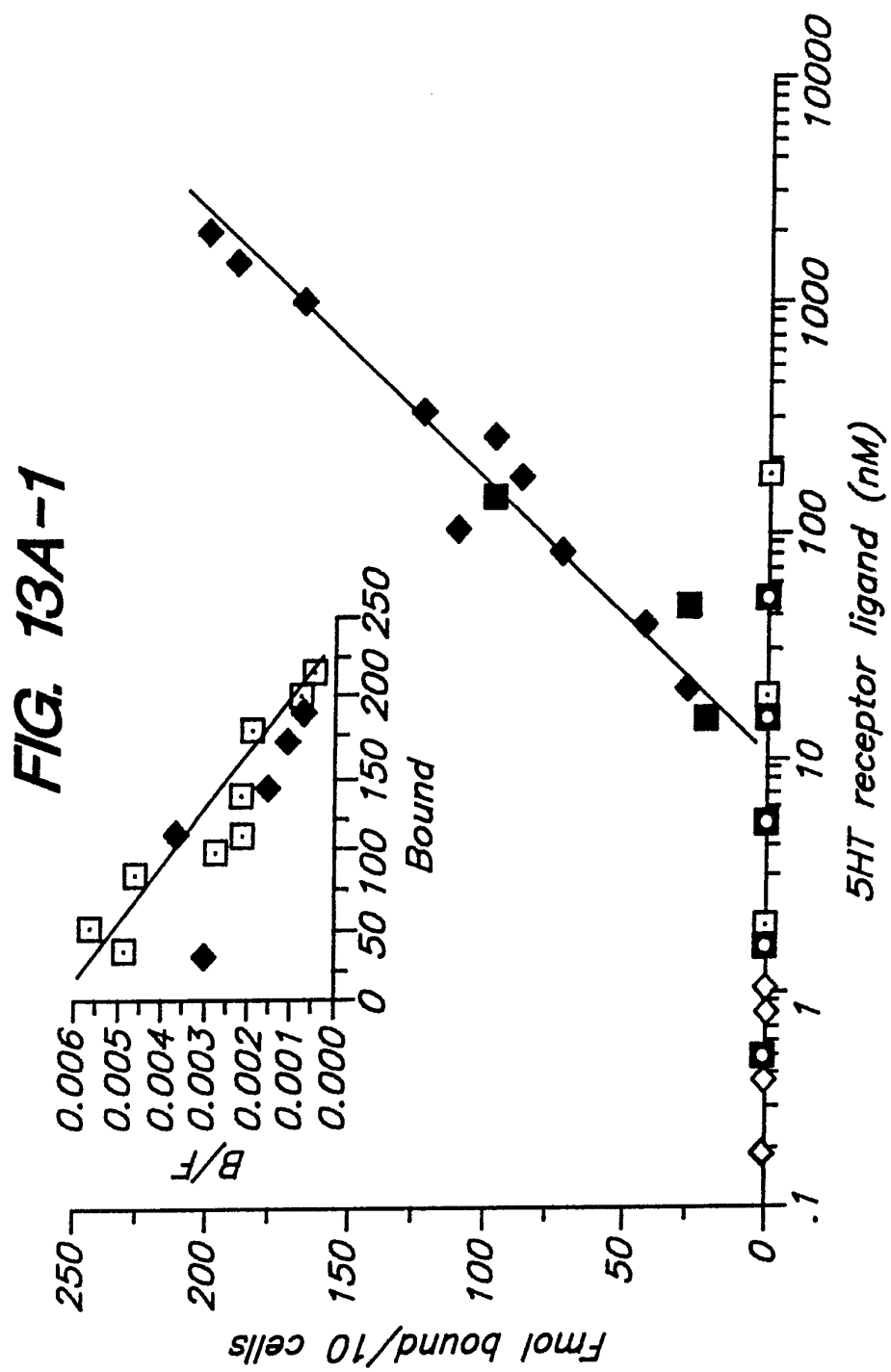
**FIG. 11**

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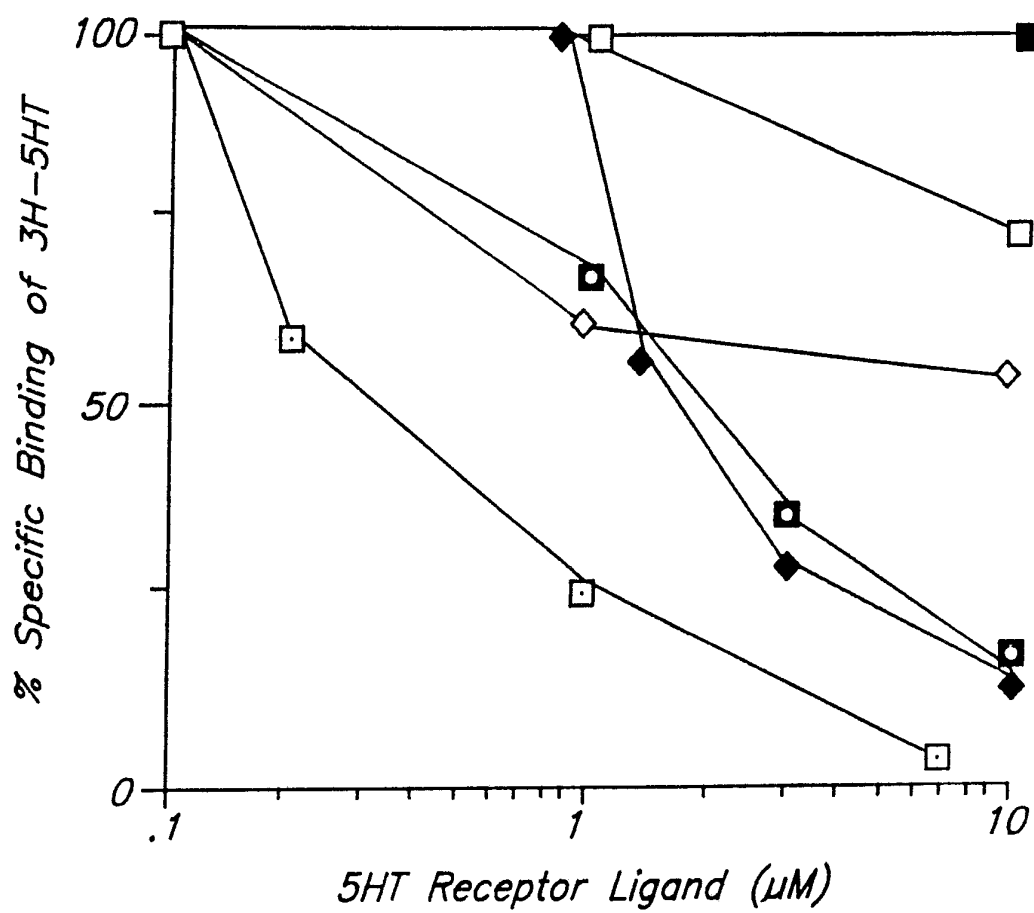
**FIG. 12**

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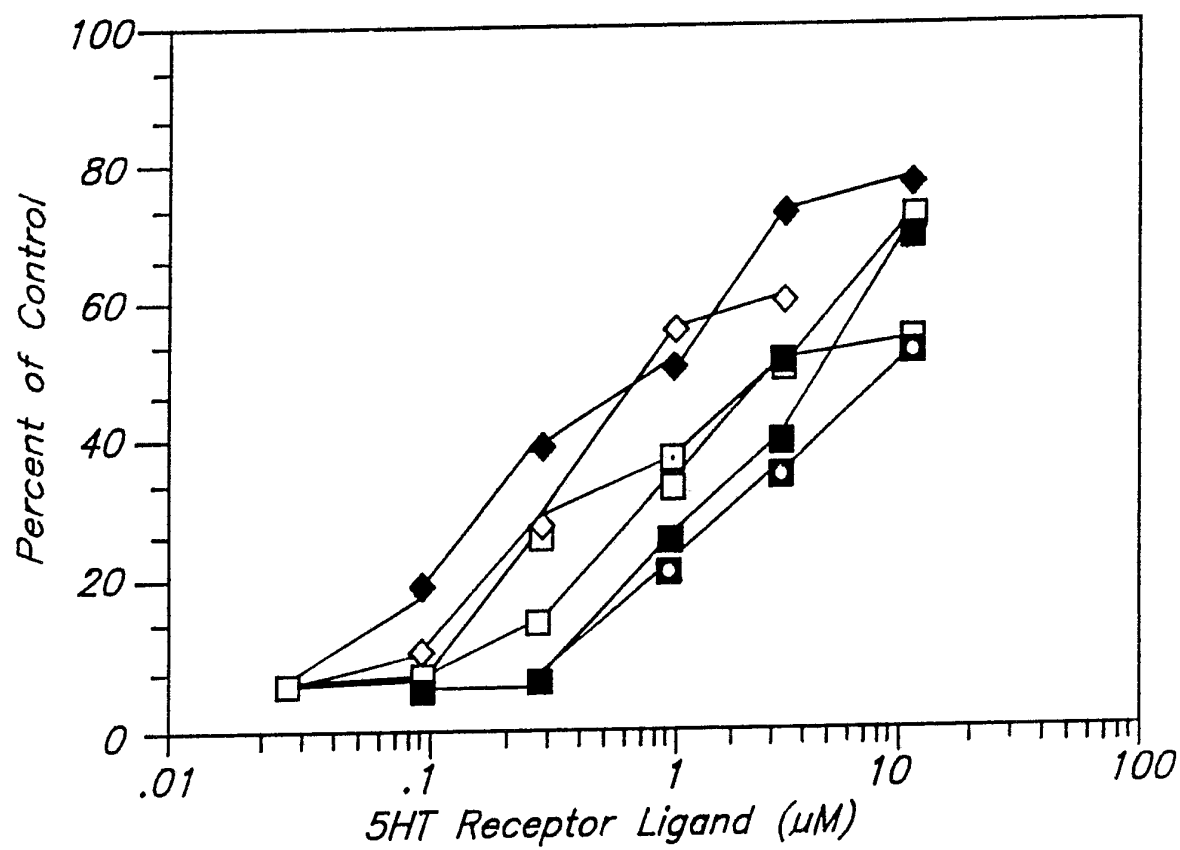
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**FIG. 13A**

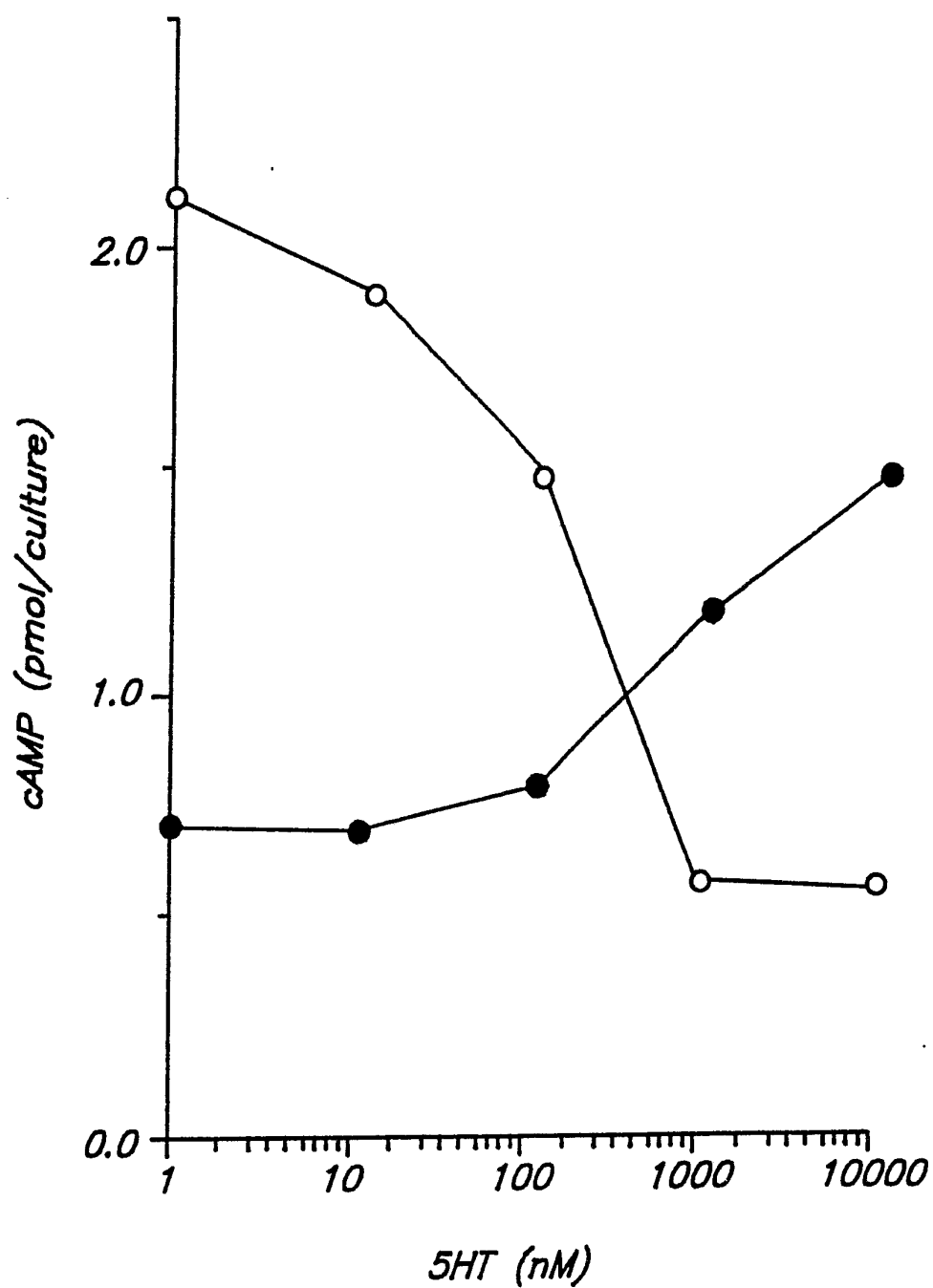
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**FIG. 13B**

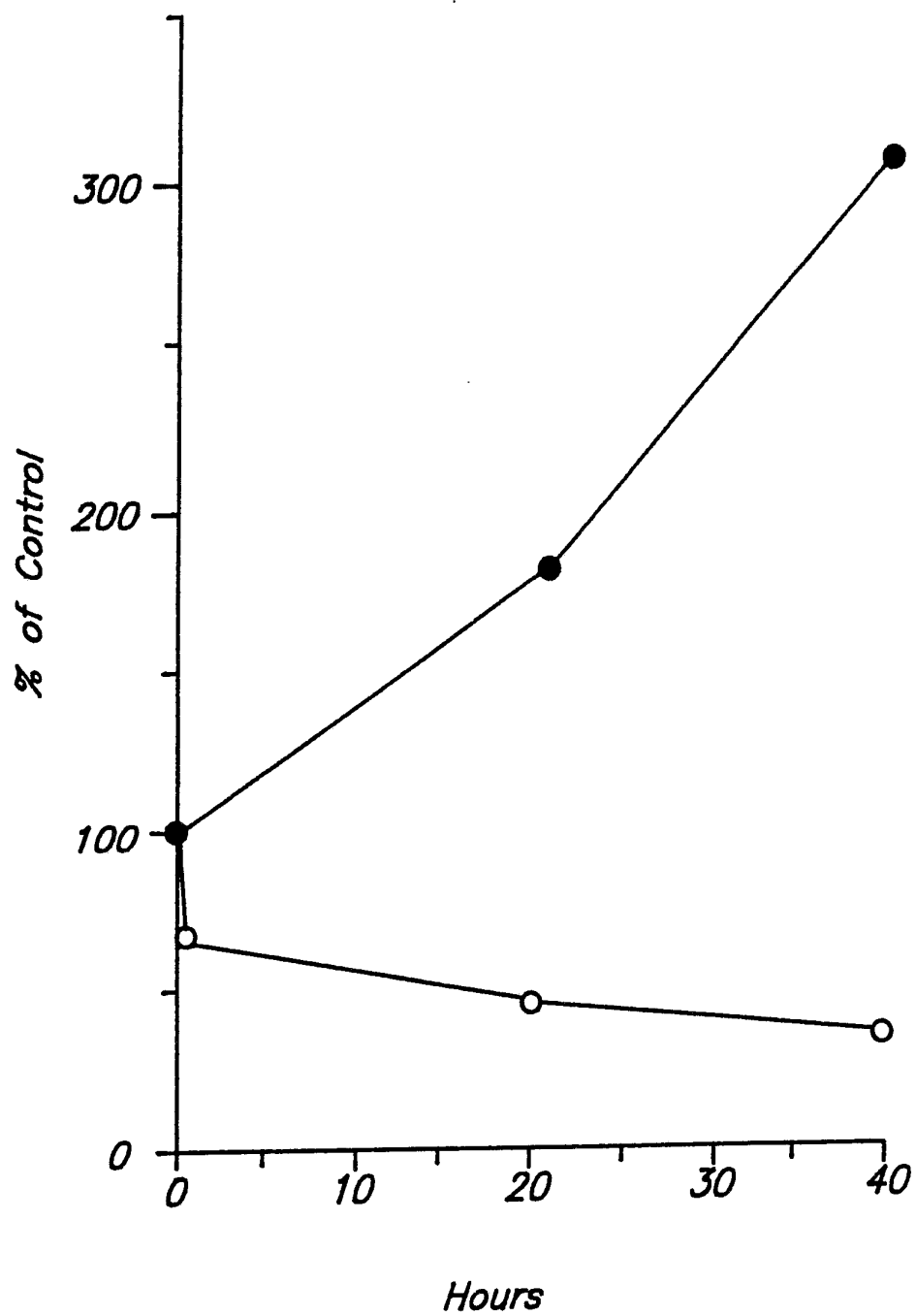
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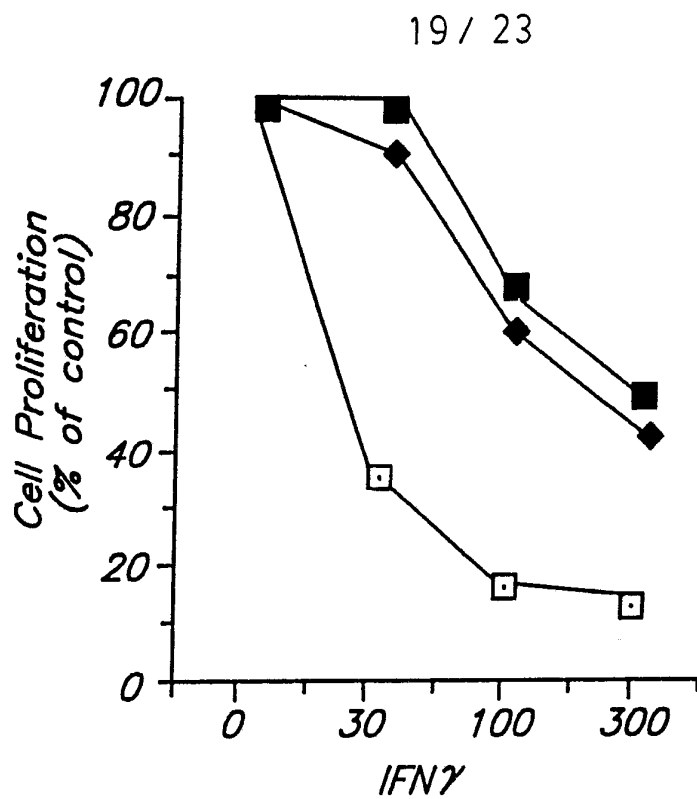
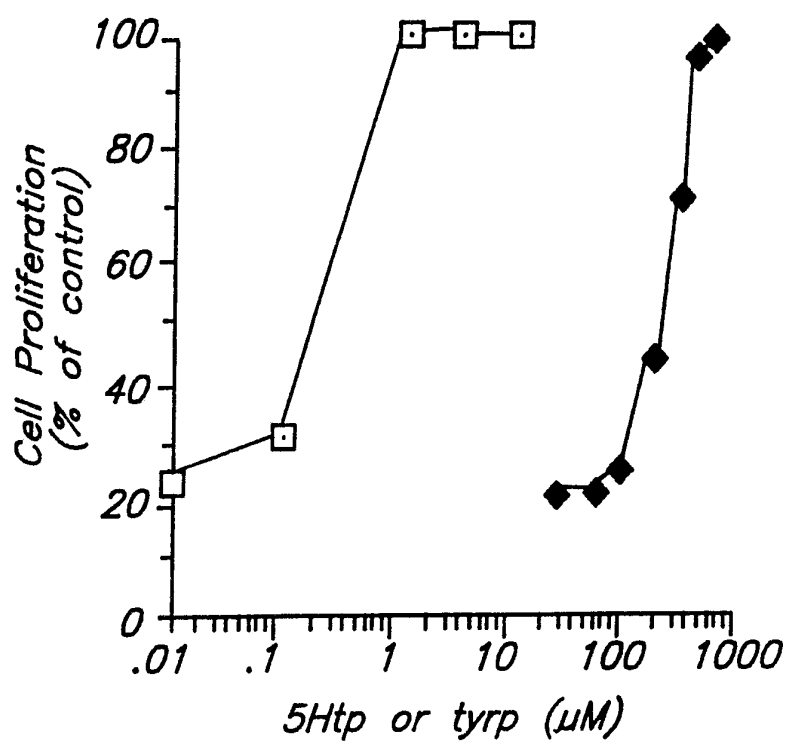
**FIG. 14**

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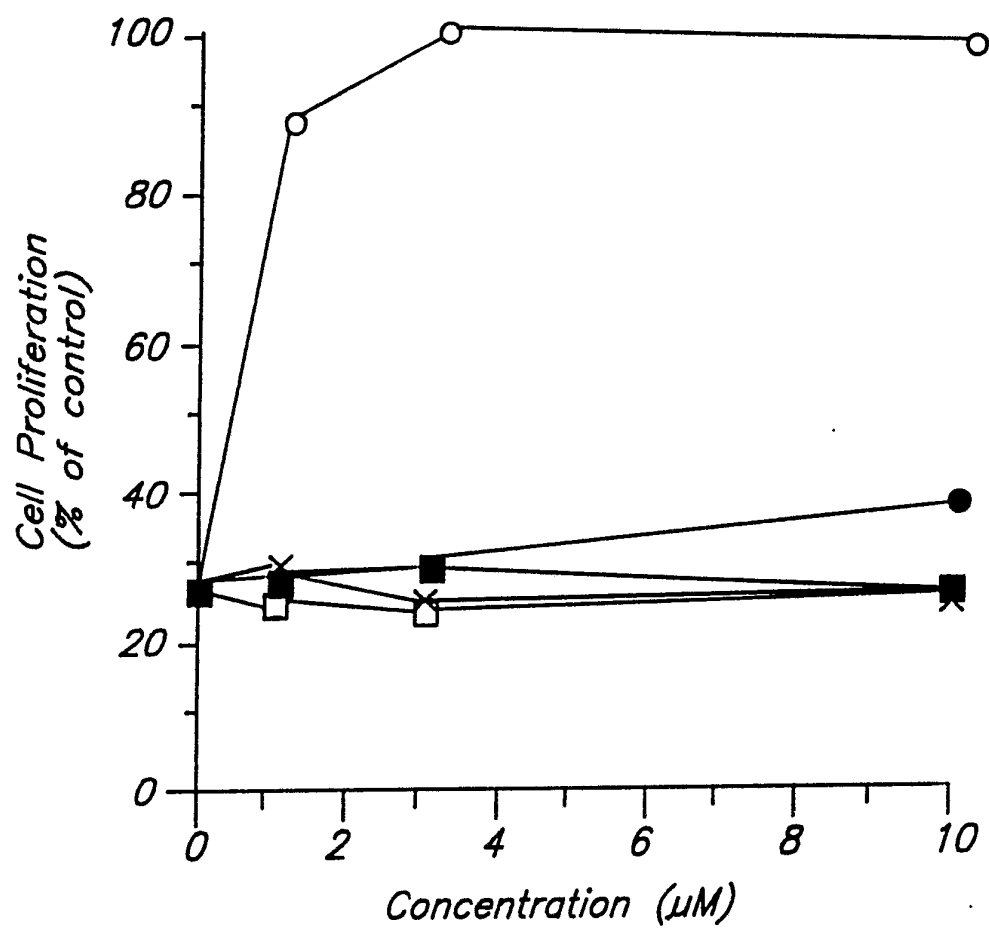
**FIG. 15**

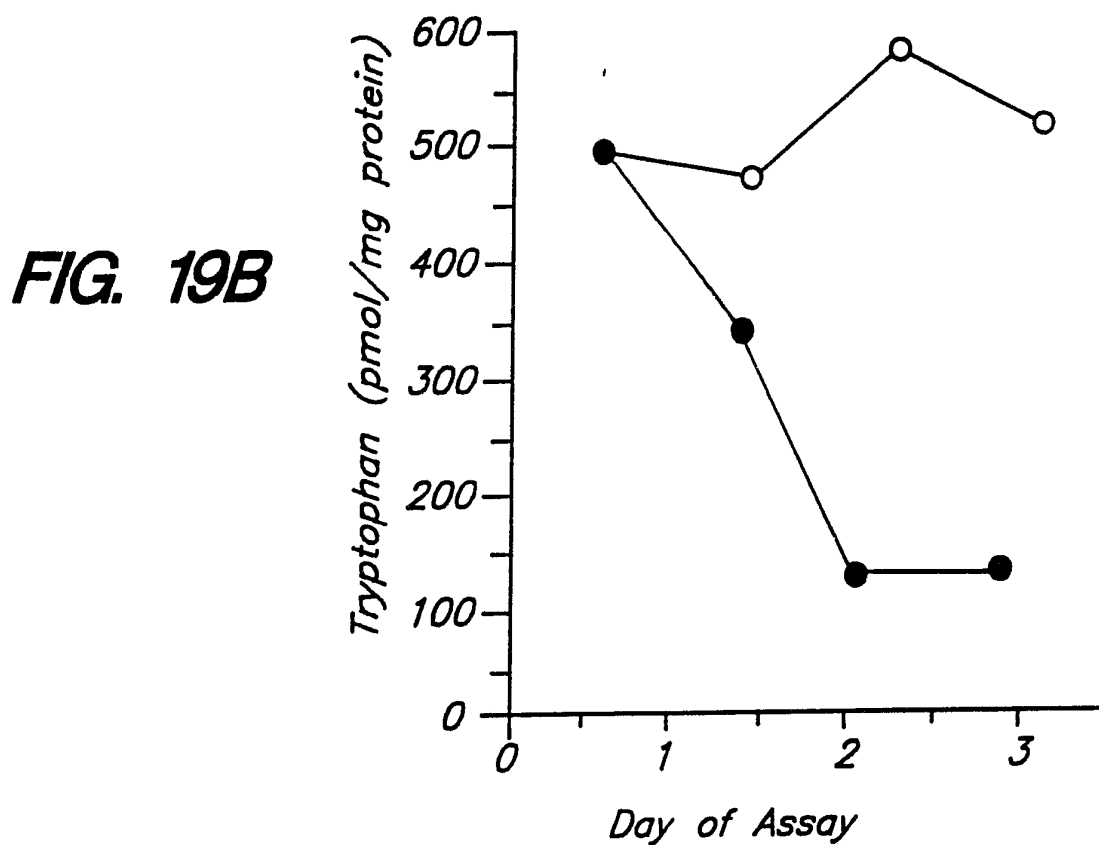
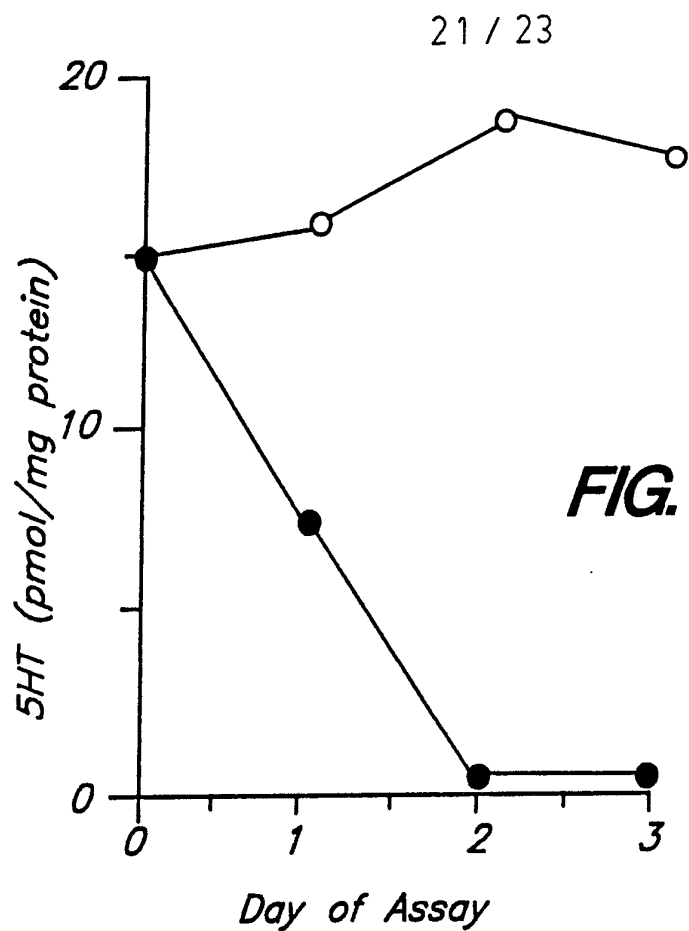
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**FIG. 16****SUBSTITUTE SHEET**

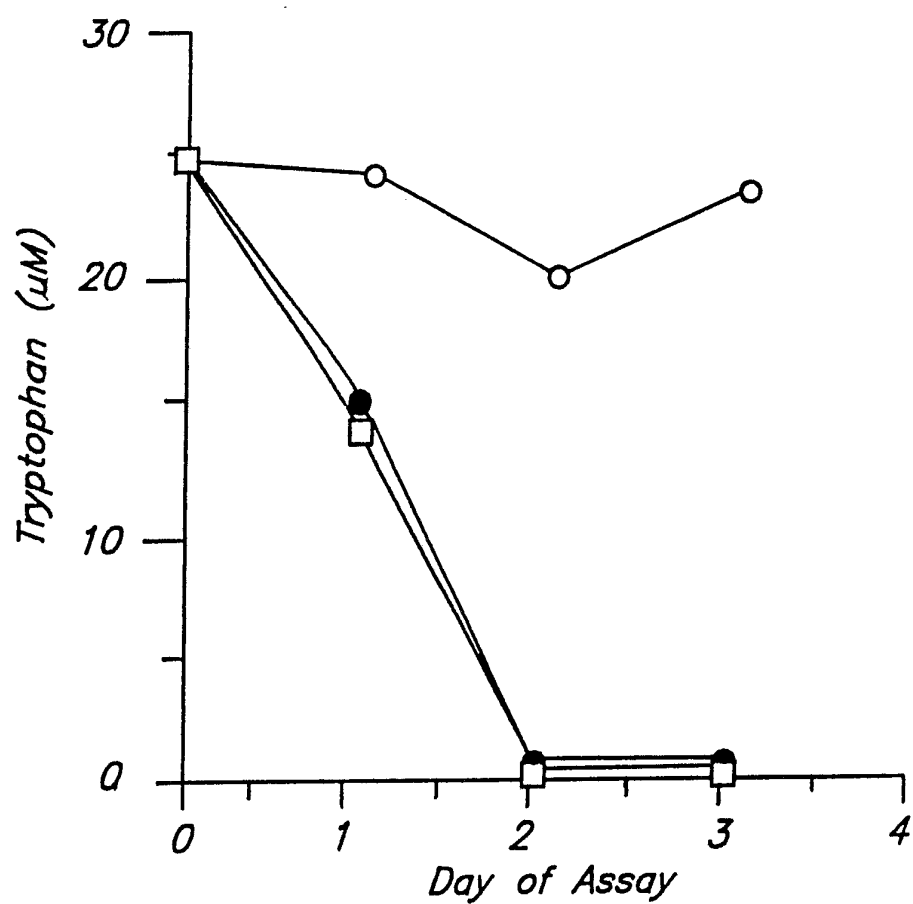
**FIG. 17A****FIG. 17B**

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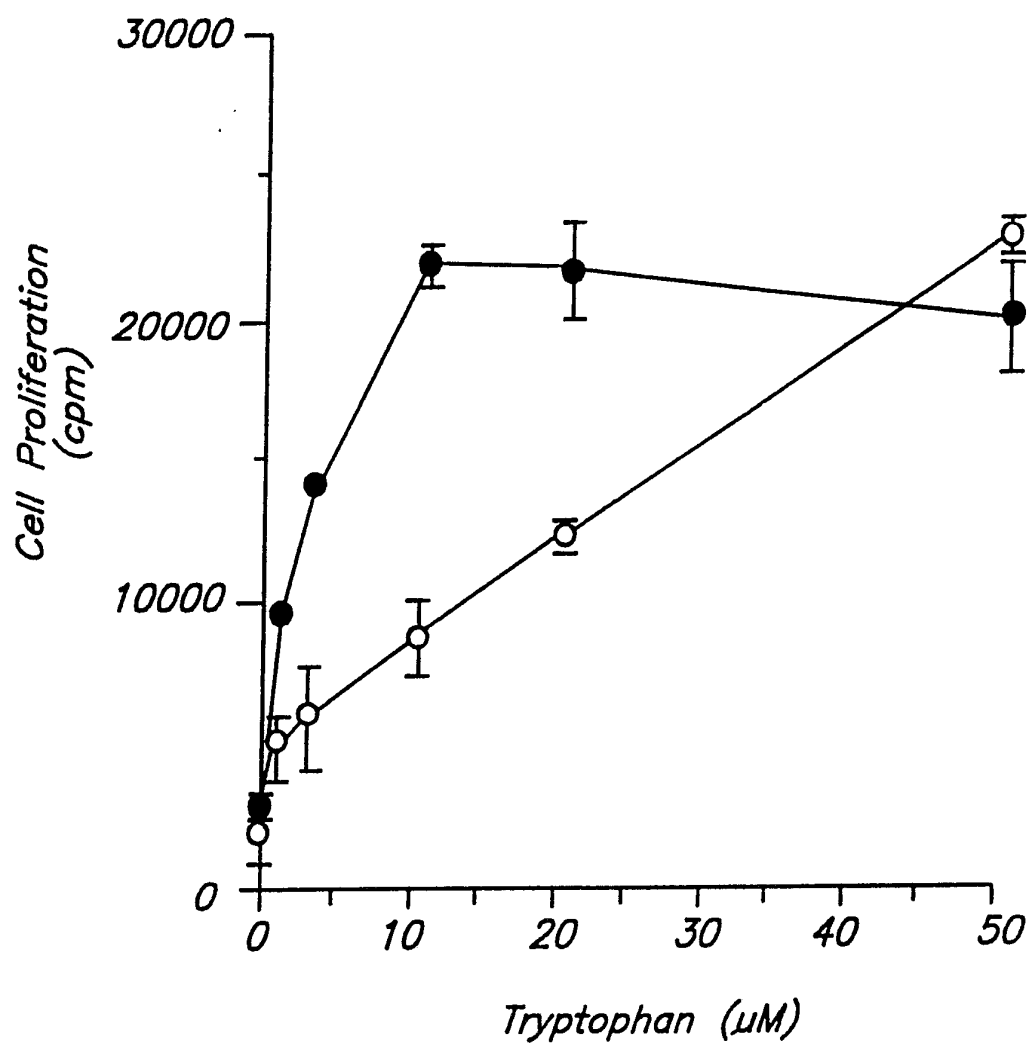
**FIG. 18**



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**FIG. 20**

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**FIG. 21**