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8-METHOXYSORALEN-MEDIATED IMPAIRMENT OF HUMAN LYMPHOCYTE PROLIFERATION IN VITRO IN THE ABSENCE OF ULTRAVIOLET IRRADIATION: IMMUNOLOGICAL AND BIOCHEMICAL MECHANISMS

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8-METHOXYPсорALEN-MEDIATED IMPAIRMENT OF HUMAN LYMPHOCYTE
PROLIFERATION IN VITRO IN THE ABSENCE OF ULTRAVIOLET
IRRADIATION: IMMUNOLOGICAL AND BIOCHEMICAL MECHANISMS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

George Warren Cox, B.A.

* * * * *

The Ohio State University

1987

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Department of Pharmacology
To My Parents
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Journal of Immunopharmacology.

Cox, G.W., Orosz, C.G., Lewis, M.G., Olsen, R.G. and Fertel, R.H.  8-Methoxypsoralen suppresses interleukin-2 production and interleukin-2 receptor expression in normal human
lymphocytes. (manuscript in preparation)

Abstracts


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- Physiology
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- Enzymology
  - Dr. K. Richardson
- Radioisotopes
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I. Chemical structures of psoralens
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<th>Description</th>
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<tr>
<td>5'-AMP</td>
<td>5'-adenosine monophosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>3-CEP</td>
<td>3-carbethoxypsoralen</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HLA</td>
<td>human lymphocyte antigen</td>
</tr>
<tr>
<td>3H-TdR</td>
<td>tritiated-thymidine</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>Ki</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>K_M</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LCL</td>
<td>lymphoblastoid cell line</td>
</tr>
<tr>
<td>5-MIP</td>
<td>5-methylisopsoralen</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte culture reaction</td>
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<tr>
<td>5-MOP</td>
<td>5-methoxypsoralen</td>
</tr>
<tr>
<td>8-MOP</td>
<td>8-methoxypsoralen</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PUVA</td>
<td>psoralen + ultraviolet A irradiation</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Tac</td>
<td>T-cell activation antigen</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximal velocity</td>
</tr>
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INTRODUCTION

A. THE HISTORY AND USE OF PSORALENS IN MEDICINE

Psoralens were originally extracted from the plants Ammi majus (Egypt) and Psoralea corylifolia (India), and have been used to treat vitiligo since the year 2000 B.C. Psoralens have been found in more than two dozen plant species, including figs, celery, parsley and parsnip [1-3]. The naturally occurring and synthetic psoralens are furocoumarins, which are heterocyclic aromatic compounds that are derived chemically from the condensation of a furan ring with a coumarin ring (Plate I). Although this fusion can occur in several ways, only two forms are generally observed: (1) a linear, tricyclic structure which resembles the native psoralen, and (2) a nonlinear, angular structure called isopsoralen or angelicin.

The compound 8-methoxypsoralen (8-MOP) is used in combination with long-wave ultraviolet light (UVA; 320-400 nm) in what has come to be known as PUVA therapy (PUVA = psoralen + UVA irradiation). Photochemotherapy with PUVA is used to treat psoriasis [4], vitiligo [5], lichen planus [6] and mycosis fungoides [7]. The major short-term side effects of PUVA therapy include nausea,
PLATE I. CHEMICAL STRUCTURES OF PSORALENS.

8-Methoxypsoralen

\[
\text{OCH}_3
\]

\[
\text{C}_{12}\text{H}_8\text{O}_4 \quad \text{M.W. 216.18}
\]

5-Methoxypsoralen

\[
\text{OCH}_3
\]

\[
\text{C}_{12}\text{H}_8\text{O}_4 \quad \text{M.W. 216.18}
\]

3-Carbethoxypsoralen

\[
\text{OCH}_2\text{CH}_3
\]

\[
\text{C}_{14}\text{H}_{10}\text{O}_5 \quad \text{M.W. 258.23}
\]

5-Methylisopsoralen

\[
\text{CH}_3
\]

\[
\text{C}_{12}\text{H}_8\text{O}_3 \quad \text{M.W. 200.20}
\]
erythema and pruritus [8]. The major long-term side
effect may be an increased risk of squamous-cell carcinoma
in patients treated with PUVA [9].

B. THE EFFECT OF PSORALENS PLUS UVA IRRADIATION ON
DNA METABOLISM

It has been clearly established that upon UVA
irradiation the psoralens form both monoadducts and
diadducts with pyrimidine bases in DNA and RNA [10-13].
The reaction sequence for diadduct formation involves
three discrete steps: (1) intercalation of the psoralen
between the base pairs in double-stranded regions of the
nucleic acid structure; (2) absorption of 320-400 nm light
by the psoralen resulting in covalent photocycloaddition
of the 4',5'- or the 3,4-double bond to the 5,6-double
bond of the pyrimidine base, forming a monoadduct; and (3)
a second photocycloaddition of the 4',5'-monoadduct to a
second pyrimidine base situated on the opposite strand,
resulting in a diadduct. The 3,4-monoadduct cannot absorb
in the 320-400 nm region and, therefore, does not form a
diadduct. These reactions result in DNA cross-linking and
impaired DNA replication. An inhibition of DNA synthesis
by PUVA therapy has been postulated as the primary
mechanism by which 8-MOP works.
C. EVIDENCE FOR EFFECTS OF 8-MOP WHICH ARE INDEPENDENT OF UV-ASSOCIATED DNA DAMAGE

It is clear that 8-MOP's ability to inhibit DNA synthesis (and ultimately cell proliferation) following UVA irradiation is one mechanism by which 8-MOP is effective in the treatment of psoriasis. However, there is reason to suggest that 8-MOP may have cellular effects which are independent of DNA damage. For example, there are effects of PUVA therapy which extend beyond the time needed to repair DNA damage [14]. Additionally, while PUVA decreases keratinocyte proliferation, it also stimulates melanocyte proliferation and increases melanogenesis [15,16].

Although mechanisms of action of 8-MOP which are independent of UV-induced DNA damage have not been firmly established, evidence for effects of 8-MOP which are independent of UV-induced DNA damage have been published. For example, Albrightson et al. [17] reported that 8-MOP in the absence of UV light was capable of increasing the concentration of adenosine 3':5'-cyclic monophosphate in several types of cells in vitro. Furthermore, Laskin et al. [18] identified specific, saturable, high-affinity binding sites for 8-MOP on several types of cells and proposed that there are receptors for 8-MOP that mediate the diverse clinical responses observed with PUVA therapy.
Finally, it has been reported that psoralens can act as both inducers and inactivators of cytochrome P-450 [19,20].

D. THE EFFECT OF 8-MOP ON IMMUNE CELL FUNCTION

PUVA therapy has been reported to alter immune function. 8-MOP plus UV light can suppress contact hypersensitivity in the mouse [21], alter the proportion of circulating lymphocytes [22,23], deplete or alter Langerhans cells and blood monocytes [24-27], and inhibit spontaneous [28] and mitogen and alloantigen-induced lymphocyte proliferation [29-34]. The specific effect of 8-MOP on immune cells has been used to therapeutic advantage in at least one instance. Recently, 8-MOP was reported to be effective in a new and novel treatment for systemically disseminated cutaneous T-cell lymphoma [35].

Although the immunosuppressive effects of 8-MOP plus UV irradiation have been established, few studies have examined the potential immunosuppressive effects of 8-MOP or other psoralen derivatives in the absence of UV irradiation. Lischka and Decker [36] and Lischka [37], however, demonstrated that lymphocytes obtained from patients treated with 8-MOP alone had a decreased response to mitogenic stimulation. Additionally, treatment of cells with UVA did not decrease the response further, indicating that 8-MOP had an effect without UV
irradiation. 8-MOP alone has also been shown to cause a slight inhibition of the epidermal cell-lymphocyte reaction [31], and 0.1 ug/ml (approximately 0.5 uM) of 8-MOP has been demonstrated to cause a 22% inhibition of $^3$H-TdR incorporation into a human lymphoblastoid cell line without effecting DNA cross-linking [38]. Finally, Kraemer et al. [39] reported that treatment of lymphocytes with either 8-MOP or UVA alone decreased DNA synthesis, and in combination, the effect was additive at lower doses of 8-MOP and synergistic at high concentrations of 8-MOP. Nevertheless, there remains sufficient evidence to argue against an effect of 8-MOP independent of UV irradiation [22,40]. In most of these studies, however, investigators have administered 8-MOP to psoriatic patients and then performed tests of immune cell function prior to UV irradiation. Typically only one or two concentrations of 8-MOP were examined and usually only as appropriate controls for experiments performed with UV exposure.

Preliminary reports suggest that 8-MOP may have an effect on lymphocyte function which is independent of its UV-associated interaction with DNA. An investigation of the possible mechanisms by which 8-MOP can alter lymphocyte function requires a basic understanding of the process of lymphocyte activation.
E. MITOGENIC LYMPHOCYTE ACTIVATION AND PROLIFERATION

The activation of normal resting peripheral blood lymphocytes involves a complex cascade of molecular and biochemical events which ultimately results in cellular proliferation and differentiation. These include changes in: (1) the expression of protooncogenes [41-44]; (2) the intracellular concentration of calcium [45-48]; (3) the concentration of cyclic nucleotides [49-51]; (4) the metabolism of arachidonic acid [52-56]; and (5) the activation of protein kinase C [57-59].

The use of mitogens to study lymphocyte transformation (or activation and proliferation) began in 1960 with the discovery by Peter Nowell that the lectin phytohemagglutinin (PHA) could transform small resting lymphocytes into proliferating lymphoblasts [60]. Lectins, a group of carbohydrate-binding proteins, were originally characterized by their ability to bind to the surface of erythrocytes and cause cell agglutination. It was, in fact, the ability of lectins to cause hemagglutination that lead to the discovery of their mitogenicity for lymphocytes. It is generally accepted that lectins initiate lymphocyte proliferation by binding to receptors on the cell surface [61]. The nature of these receptors, however, and the precise mechanism by which lectin binding induces activation has not been
completely elucidated. Although lectins bind preferentially to a single sugar moiety, for example, N-acetyl-D-galactoseamine (PHA) or D-glucose, D-mannose (Con A) [62], binding specificity alone cannot account for the differences between mitogenic and nonmitogenic lectins. For example, the T cell mitogens (Con A and PHA) bind equally well to both T and B cells [61,63], but activate primarily T cells. In addition, there does not appear to be a correlation between the number of available lectin binding sites (or binding affinity) and mitogenicity. It has been demonstrated that only 3–6% of the Con A binding sites need to be occupied to obtain maximum lymphocyte transformation [63,64]. Mitogenicity may, however, result from cross-linking of lectin receptors, which results in conformational changes in the membrane [65]. Regardless of the nature of the initial interaction of lectin with the surface of the lymphocyte, the sequence of activation and proliferation events which results is similar to those which occur following antigen stimulation. Lectins induce blastogenesis, cell division, and ultimately the expression of differentiated lymphocyte function.

In general, mitogens stimulate resting (G₀) lymphocytes to enter the G₁ phase of the cell cycle. Early RNA and protein synthesis results in the production
and secretion of the lymphokine growth factor interleukin-2 (IL-2) [66,67] and the expression of IL-2 receptors [68]. Interaction of IL-2 with its receptors on the surface of activated T cells results in the expression of transferrin receptors [69,70]. The binding of transferrin to its receptors in the late G₁ phase of the cell cycle results in the G₁ to S phase transition and ultimately cell division. A diagramatic summary of lymphocyte activation is shown in Figure 1.

Interleukin-2 and its receptors

Several endogenous growth factors, or cytokines, are produced during the course of a normal immune response. Perhaps the most extensively characterized factor is the lymphokine interleukin-2 (IL-2), which plays a critical role in T lymphocyte immunoregulation. IL-2 (formally referred to as T-cell growth factor) was discovered in 1976, when Morgan et al. [71] observed that the conditioned medium from lectin-stimulated mononuclear cells contained a mitogenic substance which was capable of supporting the continuous proliferation of lectin-activated T cells. Several advances in IL-2 research have since aided in the elucidation of the mechanisms of T lymphocyte activation and proliferation. In particular, IL-2 has been purified [72,73], sequenced [74] and cloned [75]. In addition, a rapid bioassay, with a lower limit
FIGURE 1. A MODEL OF T LYMPHOCYTE ACTIVATION.

of sensitivity of 1-10 pM, has been developed for the quantitation of IL-2 activity in culture supernatants [76,77]. Monoclonal antibodies to IL-2 have also been prepared [78].

The nucleotide sequence of IL-2 cDNA obtained by Taniguchi et al. [75] predicted that the mature secreted human IL-2 is a polypeptide containing 133 amino acids with a calculated molecular weight of approximately 15,000 daltons. Although IL-2 is variably glycosylated, the carbohydrate components are not believed to play a part in T cell proliferation, but rather, may be involved in the clearance of IL-2 [79]. The observation that there is only one copy of the IL-2 gene [80] suggests that there is only one IL-2 polypeptide.

Early experiments demonstrated that two signals are required for IL-2 production. Smith et al. found that adherent cells (macrophages) were required for IL-2 production [81] and that this requirement was related to the production and secretion of the monokine IL-1 [82]. Therefore, both lectin (or antigen) and a soluble factor from monocytes/macrophages (IL-1) are required for IL-2 production. It is clear that once the initial signal is provided by lectin, the production of IL-2 (and subsequent responsiveness to IL-2) is the limiting mitogenic factor in T cell proliferation. If IL-2 is withheld from cells
stimulated with lectin, the cells cease to proliferate and
die as if cultured without lectin.

The presence of IL-2 receptors was initially
suggested by the ability of activated cells to absorb IL-2
activity from crude supernatant preparations [83]. The
development of the anti-Tac monoclonal antibody (Tac
refers to an "activation antigen " on T cells), however,
facilitated the characterization of the IL-2 receptor
[84]. Evidence that the Tac antigen was the IL-2 receptor
came from a number of studies which demonstrated that: (1)
anti-Tac blocked radiolabeled IL-2 binding in a
concentration-dependent manner and also suppressed
IL-2-dependent T cell proliferation at concentrations that
were similar to those which blocked IL-2 receptor binding
[85]; (2) IL-2 blocked the binding of radiolabeled
anti-Tac to activated T cells [86]; (3) IL-2 and anti-Tac
which were coupled to affinity supports both bound the
same 55,000 dalton glycoprotein from extracts of activated
T cells [87]; and (4) both anti-Tac and anti-IL-2
antibodies precipitated the same 65,000-70,000 dalton band
after IL-2 was covalently cross-linked to its receptor
[86].

The IL-2 receptor is a glycoprotein with an apparent
molecular weight of approximately 55,000 daltons and
50,000 daltons on the surface of normal activated T cells
and HTLV-infected HUT-102B2 cells, respectively [85-87]. The IL-2 receptor is encoded by a single gene which has been cloned and localized [88-90].

There are two classes of IL-2 receptors which are characterized according to their binding affinities for radiolabeled IL-2. Cells treated with PHA for 3 days have approximately 2,000-5,000 high affinity binding sites/cell with an apparent $K_d$ of 3-5 pM, and 20,000-30,000 low affinity binding sites/cell with an apparent $K_d$ of 15-40 nM [91,92]. Therefore, the high affinity receptors comprise only 5-15% of the total number of IL-2 receptors. Similar binding studies with radiolabeled anti-Tac did not distinguish between the high and low affinity classes of IL-2 receptors [93]. Furthermore, only the high affinity receptors are thought to mediate the physiological response to IL-2 [91,92]. The biological significance of the low affinity receptors remains unclear.

The biochemical or cellular mediators which regulate the production and/or secretion of IL-2 and the expression of IL-2 receptors have not been identified. However, there is some evidence which indicates that adenosine 3':5'-cyclic monophosphate (cAMP) may be involved in IL-2 production [94].
F. THE ROLE OF CYCLIC AMP IN LYMPHOCYTE PROLIFERATION

An elevation of the concentration of cAMP within cells is generally associated with growth arrest and quiescence [95]. However, the precise role of cAMP in regulating the onset of DNA synthesis and proliferation in stimulated lymphocytes is not well characterized. Early reports demonstrated an increased level of cAMP in human peripheral blood lymphocytes incubated with Con A or PHA [96-98]. Other reports have not corroborated these findings [99,100], or have noted that both mitogenic as well as nonmitogenic lectins can increase cAMP, suggesting that the elevation of cAMP was unrelated to lymphocyte activation [101]. There is also some evidence to suggest that both a rise and fall in cAMP is required for the onset of lectin-induced DNA synthesis. Wang et al. [49] reported that prevention of con A-induced cAMP elevation in mouse lymphocytes inhibited the onset of DNA synthesis, while sustained elevation of cAMP caused a blockade of DNA synthesis. In contrast, other investigators have proposed a primary role for cGMP in the proliferative response to mitogens. Hadden et al. [100] and Schumm et al. [102] have observed large increases in lymphocyte cGMP levels in response to lectin stimulation. Additionally, PHA stimulation of human lymphocytes was reported to cause an increase of guanylate cyclase activity, and no effect on
cyclic nucleotide phosphodiesterases or adenylate cyclase [50]. As in the case of cAMP, there are conflicting reports for an effect of mitogens on cGMP concentration [103,104].

Although there are many conflicting reports over which cyclic nucleotide regulates mitogen-induced lymphocyte proliferation, there appears to be no dispute that experimental elevation of intracellular cAMP inhibits mitogen-induced stimulation. For example, aminophylline, isoproterenol, prostaglandins, and dibutyryl cAMP can inhibit PHA-induced lymphocyte stimulation [97]. Cyclic AMP has also been shown to modulate several other immune cell functions [94,105-108].

G. HYPOTHESIS

There is evidence from this laboratory that 8-MOP in the absence of UV irradiation increases the concentration of cAMP in several cells in vitro, including human peripheral blood mononuclear cells [17]. There is also evidence that there may be an immune component to the etiology of psoriasis [109,110]. It is now hypothesized that 8-MOP impairs human lymphocyte proliferation in vitro in the absence of UV irradiation. One mechanism by which 8-MOP impairs lymphocyte proliferation is by altering the cyclic nucleotide metabolism within these cells.
METHODS

I. CELL CULTURE TECHNIQUES

A. MATERIALS

Cell culture medium supplements

Fetal bovine serum (FBS) (lot # 300369 and 300435) was obtained from KC Biological, Lenexa, KS. FBS was heat-inactivated in a 57°C water bath for 30 minutes prior to use.

Penicillin and streptomycin sulfate were both obtained from Sigma Chemical Co., St. Louis, MO. A stock solution of $1 \times 10^5$ U/ml of penicillin and 0.1 g/ml of streptomycin was prepared in distilled water.

L-Glutamine was obtained as a 200 mM (100X) stock solution from GIBCO, Grand Island, N.Y.

Complete cell culture medium

The complete cell culture medium consisted of Roswell Park Memorial Institute 1640 medium (RPMI 1640) (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated FBS, 25 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin.
Balanced salt solutions

Hank's balanced salt solution (HBSS) (GIBCO, Grand Island, N.Y.) was prepared without phenol red indicator.

Phosphate buffered saline (PBS) was prepared at pH 7.2 in distilled water without calcium or magnesium as follows:

- NaCl 8.0 g/L
- KCl 0.2 g/L
- Na₂HPO₄ 1.15 g/L
- KH₂PO₄ 0.2 g/L

B. METHODS

Isolation and purification of normal human peripheral blood mononuclear cells (PBMC)

Peripheral venous blood was obtained from healthy adult volunteers by venipuncture and collected in sterile vacutainer tubes containing sodium heparin. PBMC were isolated and purified by density-gradient centrifugation [111]. Fifteen ml of whole blood was transferred to a 50 ml conical-bottomed polystyrene centrifuge tube (Corning Glass Works, Corning, N.Y.) and diluted 1:2 with PBS. Approximately 17 ml of Ficoll-Paque (Pharmacia, Piscataway, N.J.) was carefully layered under the blood (without intermixing) using a sterile aspirating needle and a 20 cc disposable syringe. The tube was then centrifuged at 400 x g (approximately 1400 rpm) for 30 minutes at 18-20°C using a Beckman J-6B centrifuge.
After centrifuging there were four distinct layers within the tube. From top to bottom these contained: (1) plasma and platelets; (2) lymphocytes and monocytes (PBMC); (3) Ficoll-Paque; and (4) granulocytes and erythrocytes. After carefully discarding the upper layer, a sterile siliconized pasteur pipette was used to transfer the narrow lymphocyte layer to a clean 50 ml centrifuge tube. A sufficient volume of PBS was added to fill the tube and the tube was then inverted several times to insure adequate resuspension and washing of the cells. The tube was centrifuged at 90 x g for 10 minutes at 18-20°C. The resulting supernatant fraction was discarded and the lymphocyte pellet washed and centrifuged once more to ensure removal of contaminating platelets. The final cellular pellet contained primarily PBMC and was resuspended in complete medium at the appropriate concentration (usually 1 x 10⁶/ml).

**Culture and maintenance of the MOLT-4 and JURKAT cells**

The MOLT-4 cell line was obtained from the American Type Culture Collection, Rockville, MD. This suspension culture was originally derived from the peripheral blood of a 19 year-old male with acute lymphoblastic leukemia in relapse and is a stable T-cell leukemia [112]. MOLT-4 cells were suspended in complete medium and cultured in upright T-75 or T-150 cm² plastic flasks (Corning Glass
Works, Corning, N.Y.) at 37°C in a humidified, 5% CO₂ atmosphere incubator (Forma Scientific, Marietta, OH). Cells were maintained by subculture every 3 days; at this time they were resuspended at a concentration of $5 \times 10^5$ viable cells/ml by removal of an appropriate volume of cell suspension and addition of fresh medium or by collection of cells by centrifugation and complete replacement of medium. A maximum of $1-2 \times 10^6$ viable cells/ml was always obtained.

The *JURKAT* 77 (clone 6.8) cell line was a gift from Dr. Kendall Smith of Dartmouth Medical School. This cell line is a human T-cell leukemia which can be induced by phytohemagglutinin (PHA) and/or phorbol myristic acetate (PMA) to produce large quantities of interleukin-2 (IL-2) [113]. *JURKAT* cells were cultured and maintained in the same manner as the MOLT-4 cells, except that *JURKAT* cells were maintained at concentrations between $8 \times 10^4$ and $6 \times 10^5$ viable cells/ml.

**Counting and determination of cell viability**

Cells were counted using a hemacytometer. New methylene blue (0.05% in PBS) was used to routinely stain cells for counting and morphologic identification. Trypan blue (0.4% in normal saline) was used to determine cell viability; trypan blue is excluded by live cells and stains dead cells. Typically, 0.1 ml of cell suspension
was added to 0.1 ml of either stain (1:2 dilution) and allowed to stand for 2 minutes before loading onto the hemacytometer. Each of the four large corner squares of a Neubauer type hemacytometer was counted. When the cell count was low (less than 25/square) both sides of the hemacytometer were counted. When the cell count was too high for easy counting, a greater initial dilution of the cell suspension was made prior to the addition of the stain. The following equation was used to calculate the number of cells/ml in the original cell suspension:

\[
\frac{\text{# cells counted} \times \text{dilution factor} \times 10^4}{\text{# squares counted}}
\]

Cryostorage of cells

Cryostorage was used to retain a viable stock of MOLT-4 and JURKAT cells between periods of culture. Cells in log growth phase were washed and resuspended at approximately 2 x 10^6 viable cells/ml in complete medium containing 10% dimethylsulfoxide (DMSO), a cryoprotectant. One ml of this suspension was placed in a 2 ml sterile cryogenic vial (Wheaton, Millville, N.J.) and the vial was then placed approximately 2 inches above liquid nitrogen for 20 minutes. This allowed for a slow, vapor-phase freezing period after which the vials were placed in liquid nitrogen. Upon removal from the liquid nitrogen, the cells were rapidly thawed by gently shaking the vials
in a 37°C water bath. The 1 ml aliquot of cell suspension was added to 25 ml of fresh complete medium, washed and centrifuged. The supernatant fraction was discarded and fresh complete medium was added to adjust the viable cell concentration to within the appropriate range.

II. IMMUNOLOGICAL TECHNIQUES

A. MATERIALS

Lectin mitogens

Phytohemagglutinin (M form) (lot # 12N6352) (PHA) was obtained from GIBCO, Grand Island, N.Y. The lyophilized PHA was rehydrated with 10 ml of sterile distilled water to give a 100% stock solution. This stock solution was aliquoted and stored frozen at -70°C and remained stable for at least 2 months.

Concanavalin A (Con A) was obtained from Sigma Chemical Co., St. Louis, MO and was initially prepared as a 38 mg/ml stock solution in saturated sodium chloride. This was then diluted to 4 mg/ml with RPMI 1640, aliquoted and stored frozen at -70°C.

Interleukin-2 (IL-2)

IL-2 was obtained as delectinated, PHA-induced human IL-2 (lot # 25293 and 26208) (Cellular Products Inc., Buffalo, N.Y.). This IL-2 was free from both B cell
growth factor (BCGF) and immune interferon contamination. This preparation was assayed as previously described [76] and quantitated relative to a laboratory standard which had been quantitated relative to a JURKAT-derived IL-2 standard provided by the Biological Response Modifiers Program of the National Cancer Institute, Frederick, MD. When used at a final concentration of 10% (v:v) (approximately 80 U/ml) this IL-2 preparation produced at least a ten-fold increase in $^3$H-thymidine incorporation into unstimulated PBMC, using the culture conditions described below for the lymphocyte proliferation assay. Additionally, this preparation had been analyzed for its ability to stimulate proliferation of 7-10 day old, PHA transformed human T cells. When such T cells were cultured in a growth assay at an initial cell concentration of $2 \times 10^5$/ml, this preparation induced a minimum 5 to 8-fold increase in cell concentration at a final concentration of approximately 10% IL-2, within 5 days. The IL-2 was thawed upon receiving, aliquoted and stored frozen at -70°C. The preparation remained stable for at least 6 months.

$^3$H-Thymidine ($^3$H-TdR)

$^3$H-TdR (lot # 2161-248 and 2257-132) was obtained as (methyl-$^3$H)-thymidine (6.7 Ci/mmol) from New England Nuclear Research Products, Boston, MA. One ml of 1 mCi/ml
of $^3$H-TdR was diluted with 19 ml of sterile PBS to make a stock concentration of 0.5 uCi/10 ul which was then stored at 4°C.

CTLL-20 cells

The murine IL-2-dependent cell line CTLL-20 was used to detect and quantitate IL-2 activity and was provided and maintained in culture by Dr. Charles Orosz of The Ohio State University College of Medicine. CTLL-20 cells were maintained by biweekly transfer of $1 \times 10^4$ viable cells into upright T-25 cm$^2$ tissue culture flasks containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 2% FBS and 5% supernatant from Con A-stimulated rat splenocyte cultures as a source of T cell growth factors [114].

MLA-144 supernatant

Culture supernatant from the gibbon T lymphosarcoma cell line MLA-144 [115] was used as a source of exogenous IL-2 to support the proliferation of CTLL-20 cells in the IL-2 supernatant assay (see below). Maintenance of the cell line and preparation of the supernatant was performed by Dr. Charles Orosz (O.S.U.). The stock supernatant preparation used in these experiments contained 116 U of IL-2 per ml of medium.
Monoclonal reagents

Anti-interleukin-2 receptor (CD 25) (lot # J0412) was obtained as a fluorescein conjugate containing 20 ug of immunoglobulin in 2 ml of PBS (Becton Dickinson Immunocytometry Systems, Mountain View, CA). This monoclonal antibody reacts with the same molecule as Anti-Tac-1 [84,87].

Mouse IgG\textsubscript{1} control (lot # J0524 and J0838) was obtained as a fluorescein conjugate containing 1 ug of IgG per 20 ul (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Psoralen compounds

8-Methoxypsoralen (8-MOP) (MW 216.18) (Elder Pharmaceuticals, Bryan, OH) was dissolved in 100% ethanol to make a stock concentration of 3 mg/ml. The stock solution was stored in the dark at room temperature, and was stable for at least 1 month. This 8-MOP solution was subsequently diluted in complete cell culture medium immediately prior to use. The final ethanol concentration within the cultures never exceeded 1%.

5-Methoxypsoralen (5-MOP) (M.W. 216.18) (lot # T032681), 5-methylisopsoralen (5-MIP) (M.W. 200.20) (lot # H030182) and 3-carbethoxypsoralen (3-CEP) (M.W. 258.23) (lot # T032681) were obtained from Midwest Research Institute, Kansas City, MO and were all dissolved
in 100% ethanol to make 0.5 mg/ml stock solutions. 5-MOP and 5-MIP were stored in the dark at room temperature and were stable for at least 1 month. The 3-CEP solution was used on the day it was prepared since it is readily photodegraded in alcohol solutions. These psoralens were also dissolved in complete cell culture medium prior to use and the final ethanol concentration never exceeded 1%.

B. METHODS

Lymphocyte proliferation assay

Proliferation of lymphocytes was detected by \(^{3}H\)-TdR incorporation [116]. PBMC were obtained as previously described and resuspended at a concentration of \(1 \times 10^6\) viable cells/ml. Either mitogen alone or mitogen plus other agents was added in triplicate to a flat-bottomed 96-well microtiter plate (Falcon 3072, Becton Dickinson & Co., Oxnard, CA) in a total volume of 100 ul of medium. One hundred ul of cell suspension (\(1 \times 10^5\) cells/well) was added to each well to provide a final volume of 200 ul/well. After 72 hours of culture at 37°C (humidified, 5% CO\(_2\) incubator), 10 ul (0.5 uCi) of \(^{3}H\)-TdR was added to each microtiter well using a sterile repeating Hamilton syringe. Cells were incubated for an additional 16 hours (88 hour total incubation) and then harvested onto 934-AH glass fiber filters (Whatman Inc., Clifton, N.J.) using an
automated cell harvester. Approximately 2.5 ml of ScintiVerse II liquid scintillation cocktail (Fisher Scientific, Cincinnati, OH) was added along with each filter to a mini-scintillation vial (Kew Scientific, Columbus, OH). The amount of $^3$H-TdR incorporated into newly synthesized cellular DNA was detected by using a Beckman LS 6800 liquid scintillation counter. Results were expressed as the mean counts per minute (cpm) ± standard error of the mean (sem) after subtracting the background cpm (cells without mitogen).

**Mixed lymphocyte culture reaction (MLR)**

Two separate populations of PBMC (denoted as A and B) were isolated from the peripheral blood of two healthy adult volunteers. Each population was adjusted to a final concentration of $1 \times 10^6$ viable cells/ml in complete medium. Each population was then further subdivided and placed into two tissue culture centrifuge tubes (denoted as A and A or B and B). $A_x$ and $B_x$ were then irradiated at 2500 Rads by placing the centrifuge tubes in a $^{137}$cesium source gamma irradiator (Cammacell 40, Atomic Energy of Canada, Ltd.) for approximately 16.7 minutes. A similarly treated third population of cells, LCL, served as a strong positive control in the MLR. LCL denotes a lymphoblastoid cell line (LCL) which was derived from Epstein-Barr virus-infected human PBMC (provided by
Dr. Charles Orosz, The Ohio State University College of Medicine). One hundred ul of each cell suspension (1 x 10^5 cells/well) was added in triplicate to a 96-well microtiter plate to provide a final volume of 200 ul/well. After 5 days of culture at 37°C (humidified, 5% CO₂ incubator), 20 ul (1 uCi) of ^3H-TdR was added to each microtiter well as previously described. Cells were incubated for an additional 16 hours (136 hour total incubation) and then harvested and counted as before. Each treatment group required the following combination of cells in culture:

R + medium
R + Rx
R + Sx
R + LCLx

Since each volunteer's PBMC can serve as both responder (R) and stimulator (S) cells, two replicate plates (or MLRs) were established.

Supernatant assay for the detection of IL-2 activity and inhibitory activity

Cell culture supernatants were assayed for IL-2 activity according to the method of Gillis et al. [76]. PBMC were established in culture exactly as they were for the lymphocyte proliferation assay. After either 24 or 48 hours of culture, 100 ul of supernatant was carefully
removed from each of the triplicate treatment wells and pooled together in a sterile 12 mm x 75 mm culture tube. One hundred and fifty ul of each pooled sample was then transferred to the second and eighth wells of a 96-well microtiter plate; the first and seventh wells were for medium controls. Serial (1/3) dilutions of 50 ul of sample and 100 ul of complete medium were performed across the remaining halves of the microtiter plate. CTLL-20 cells were washed and resuspended in complete medium at 2 x 10^5/ml. Each well received 50 ul of CTLL-20 cells (1 x 10^4 cells/well). One set of sample dilutions received 50 ul of complete medium (IL-2 activity assay wells). The other set of sample dilutions received 50 ul of a 1/5 dilution of MLA-144 supernatant (as a source of exogenous IL-2) to give a final concentration of 5%/well (inhibitory activity assay wells). Therefore, the total volume per well was 200 ul. CTLL-20 cells were cultured for 16 hours and then pulsed with ^3H-TdR (1 uCi/well) for an additional 8 hours (24 hour total incubation) before harvesting as usual. The amount of IL-2 which had accumulated in the original culture supernatant was expressed as the amount of ^3H-TdR incorporated by CTLL-20 cells in the absence of 5% MLA supernatant. The presence of activity in culture supernatants which could interfere with the IL-2 bioassay was detected as an
inability of CTLL-20 cells to incorporate $^3$H-TdR in the presence of 5% MLA supernatant.

Direct immunofluorescence staining and cytofluorometric analysis of IL-2 receptors

(a) Reagents

Staining medium was Dulbecco's PBS (contains calcium and magnesium) containing 0.1% sodium azide and 2% FBS.

Washing buffer was Dulbecco's PBS containing 0.1% sodium azide.

2% Paraformaldehyde solution was prepared by adding 2 g of paraformaldehyde (Fisher Scientific) to 100 ml of 0.85% saline. This was heated at 70°C in a fume hood until the paraformaldehyde went into solution, allowed to cool and adjusted to pH 7.4. All solutions were stored at 4°C.

(b) Procedure

PHA alone or in combination with other agents was added to duplicate wells of a 24-well tissue culture plate (Corning Glass Works, Corning, N.Y.) in a total volume of 750 ul of complete medium. Normal human PBMC were isolated as usual and resuspended at a final concentration of $4 \times 10^6$/ml. Two hundred and fifty ul of cell suspension was added to each well ($1 \times 10^6$ cells/well)
to bring the total volume to 1 ml/well. The plate was incubated at 37°C as usual. Cells plus medium were removed from the replicate wells after various incubation times and transferred to separate 12 mm x 75 mm plastic culture tubes for staining with either anti-IL-2 receptor or IgG control. The cells were centrifuged at 300 x g for 10 minutes at 2-8°C. The supernatant was aspirated and the remaining cell pellet was resuspended gently with 50 ul of staining medium and placed on ice. Both monoclonal reagents were diluted with staining medium immediately prior to use (0.3 ug protein/50 ul) and kept on ice. Fifty ul of either reagent was added to the cell suspension with gentle mixing and incubated on ice (in the dark) for 30 minutes. Two ml of cold staining medium was added and the tube was centrifuged at 300 x g for 10 minutes at 2-8°C. The supernatant was aspirated gently and the cell pellet was washed twice with washing buffer. Paraformaldehyde fixation of the cells was performed by a modification of a procedure described previously [117]. The final cell pellet was resuspended in 0.5 ml of cold paraformaldehyde solution and vortexed immediately to prevent clumping. The fixed cells were stored at 4°C in the dark. Antibody binding was quantitated using a Coulter Epics V Flow Cytometer and Multiparameter Data Acquisition and Display System (Hialeah, FL). Measurement
of the forward angle light scatter (FALS) and the right angle light scatter (90°LS) (which indicate the size and granularity of the cells, respectively) allowed for the gating out of debris and analysis of the leukocyte population. A total of $10^4$ cells were analyzed and a comparison was made of the log integrated green fluorescence (LIGFL) histograms from matched anti-IL-2 receptor-stained and IgG₁ control-stained samples. The percentage of cells which stained positive for the IL-2 receptor (Tac⁺) was determined by calculating the percentage of cells which sorted into channels to the right of the negative IgG₁ control peak minus the percentage of false positive cells (observed as an overlapping of the nonspecific IgG₁ control peak into the anti-IL-2 receptor peak). Logarithmic mean channel numbers (from a 256 channel, 3-decade amplifier) were converted to arbitrary fluorescence units (AFU) as previously described [118] using the following equation:

$$\text{AFU} = 10^{(\log \text{mean channel } \# / 85) - 1}$$

III. BIOCHEMICAL ANALYTICAL TECHNIQUES

A. PROTEIN ASSAY

The protein assay was performed by a modification of the method of Lowry et al. [119].
(1) Reagents

Reagent A was 2% Na$_2$CO$_3$ in 0.10 N NaOH.

Reagent B was 0.5% CuSO$_4$·5H$_2$O in 1% Na/K tartrate.

Reagent C was made by combining 50 parts of reagent A and 1 part of reagent B.

Reagent D was phenol reagent (Folin-Ciocalteau reagent) and was titrated against NaOH and adjusted to 1 N (The Ohio State University Chemical Stores).

Bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO) was used as the protein standard. A 1 mg/ml stock solution was aliquoted and stored at -20°C.

(2) Preparation of the standards and samples

Four hundred and eighty ul of the BSA stock was diluted with 720 ul of 0.1 N NaOH to yield 80 ug/200 ul. Using a serial 1/2 dilution with 0.1 N NaOH, a six point standard curve was prepared, including a NaOH blank which was used to zero the photometer. Absorbance was linearly proportional to the amount of protein (5-80 ug per assay tube). Samples were either solubilized or diluted in 0.1 N NaOH prior to assay.

(3) Assay procedure

Two hundred ul of either the standards or samples was added to duplicate 12 mm x 75 mm glass tubes. Two ml of
reagent C was added, and the tubes were vortexed and incubated for 10 minutes at room temperature. One hundred ul of reagent D was added while vortexing, and the tubes were incubated for an additional 30 minutes at room temperature. Absorbance was measured at 580 nm using a spectrophotometer.

B. RADIOIODINATION OF CYCLIC NUCLEOTIDES

Adenosine 3':5'-cyclic monophosphate (cAMP) and guanosine 3':5'-cyclic monophosphate (cGMP) were radiolabeled with $^{125}$I using a modification of a procedure previously described [120,121].

(1) Reagents

$O^{2'}$-monosuccinyl cAMP tyrosyl methyl ester,
$O^{2'}$-monosuccinyl cGMP tyrosyl methyl ester,
chloramine T, sodium metabisulfite and BSA were obtained from Sigma, St. Louis, MO.

Sephadex G-10 was obtained from Pharmacia, Piscataway, N.J.

$^{125}$I was obtained as sodium iodide in dilute NaOH solution, pH 7-11 (Amersham Corp., Arlington Heights, Il).

Potassium phosphate buffer was prepared at 0.25 M and pH 7.55.
PBS was prepared at pH 7.5 in distilled water as follows:

\[
\begin{align*}
\text{NaCl} & \quad 10.60 \text{ g/L} \\
\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O} & \quad 7.36 \text{ g/L} \\
\text{KH}_2\text{PO}_4 & \quad 1.36 \text{ g/L}
\end{align*}
\]

(2) Procedure

Approximately 5 g of sephadex G-10 was washed and allowed to swell in 200 ml of PBS for 3 hours. The PBS was decanted and the sephadex was washed once more with PBS. The sephadex was poured carefully into a column at room temperature. A 10 ml plastic pipet served as a good column when fitted with glass wool, rubber tubing and a clamp. The column was washed with 100 ml of PBS (25°C), loaded with 1 ml of 3% BSA (in PBS) and washed with an additional 30 ml of PBS. Forty ul of 0.25 M phosphate buffer was added to a vial containing either succinylated cAMP or cGMP (2 ug in 20 ul PBS). Ten ml of 0.25 M phosphate buffer was added to one tube containing 35 mg of chloramine T and another tube containing 24 mg of sodium metabisulfite. The cAMP or cGMP was transferred to a vial containing 1 mCi of \(^{125}\text{I}\) and mixed thoroughly. Chloramine T (50 ul) was added to the vial to start the reaction. The reaction was terminated after either 20 (cAMP) or 25 (cGMP) seconds by the addition of 100 ul of sodium metabisulfite. The reaction mixture was applied to the column and was eluted with PBS. Fractions
(approximately 40) were collected at 5 minute intervals, counted in a Beckman Gamma 7000 spectrometer and assayed for absolute binding in the cyclic nucleotide radioimmunoassay.

C. PREPARATION AND TREATMENT OF MOLT-4 CELLS FOR CYCLIC NUCLEOTIDE EXPERIMENTS

MOLT-4 cells were obtained from cultures during the log phase of the growth cycle (approximately two days after subculture). The cells were washed free of the culture medium and resuspended at a concentration of 4 x 10^6 viable cells/ml in HBSS. The cell viability was always greater than 90%. Five hundred ul of cell suspension (2 x 10^6 cells) was transferred to duplicate 12 mm x 75 mm plastic culture tubes and allowed to preincubate for 30 minutes in a 37°C water bath. The experiment was initiated by the addition of 500 ul of drug (in HBSS) to the cells in the 37°C water bath. The incubation period was terminated by the addition of 500 ul of 30% trichloroacetic acid (TCA), which effectively halted further cyclic nucleotide metabolism within these cells. The cell suspension was sonicated, transferred to a polypropylene microcentrifuge tube and centrifuged at 13,000 x g for 10 minutes. The resulting supernatant was transferred to a 16 mm x 125 mm glass tube in preparation for the cyclic nucleotide radioimmunoassay. The TCA
precipitated cellular pellet was solubilized with 1 ml of 0.1 N NaOH and assayed for protein as described above.

D. CYCLIC NUCLEOTIDE RADIOIMMUNOASSAY (RIA)

The cell culture samples were analyzed for cAMP and cGMP content by a modification of the RIA procedure of Steiner et al. [121].

(1) Reagents

Antisera - the cAMP and cGMP antisera were developed in this laboratory and are highly specific and sufficiently sensitive to measure 10 femtomoles of either nucleotide. The antisera were diluted in 50 mM sodium acetate buffer, pH 6.3, with 0.2% BSA to provide a trace binding of 25-35%.

Radiolabeled nucleotides - prepared as described above.

Bovine gamma-globulin - 0.25% in 0.05 M sodium acetate buffer.

60% saturated ammonium sulfate - prepared by adding 780 g of ammonium sulfate to 2 L of distilled water.

Ethyl ether (water saturated), acetic anhydride and triethylamine

(2) Procedure

Samples for cyclic nucleotide analysis were extracted three times with a 1:3 (v:v) ratio of sample to
water-saturated ethyl ether in order to remove the TCA. The remaining ether was evaporated during a 30 minute incubation in a 57°C water bath. The pH of the samples was adjusted to approximately 6.3 by the addition of 1 M sodium acetate buffer pH 6.5 at a 1:10 (v:v) ratio of buffer to sample. An eleven point standard curve which provided a sensitivity of approximately 10-10,000 fmol/sample was constructed by performing serial 1/2 dilutions (with HBSS) from 0.1 uM stock solutions of either cAMP or cGMP. Either 100 ul (for cAMP assay) or 300 ul (for cGMP assay) of standards and samples was aliquoted into duplicate 12 mm x 75 mm glass tubes. Additional tubes were prepared for the determination of 100% binding (B_o) and nonspecific binding (blank). B_o tubes contained HBSS and antibody; blank tubes contained HBSS without antibody. To increase the sensitivity of the RIA, 10 ul of a 2:5 (v:v) mixture of acetic anhydride and triethylamine was added to all the tubes while vortexing [122]. The tubes were incubated at room temperature for 10 minutes and 50 ul of an appropriate antiserum dilution was added to each tube (except blanks). Radiolabeled cAMP or cGMP was diluted with 0.25% bovine gamma-globulin and added (50 ul/tube) to give 20,000 cpm per tube. Two tubes received 50 ul of radiolabeled nucleotide only and served as a control for the total cpm added to the assay. The
tubes were vortexed and incubated for 18 hours at 4°C. The bound and free fractions were separated by a 20 minute incubation with 60% saturated ammonium sulfate at room temperature. After a 30 minute centrifugation at 1900 x g, the supernatant fraction was decanted and the remaining precipitate was counted in a Beckman Gamma 7000 spectrometer. The cyclic nucleotide concentrations were determined using the standard curve, which was plotted on logit-log paper as %B<sub>o</sub> versus concentration. Equations used in the RIA analysis were as follows:

\[ \text{absolute binding} = \frac{B_o - \text{blank}}{\text{total} - \text{blank}} \]  

(1)

\[ \%B_o = \frac{\text{sample} - \text{blank}}{B_o - \text{blank}} \]  

(2)

E. PREPARATION OF MOLT-4 PHOSPHODIESTERASE (PDE)

Approximately 7 x 10<sup>8</sup> MOLT-4 cells were obtained from cultures in the log phase of the growth cycle. The cells were washed, resuspended in 10 ml of cold 50 mM glycylglycine buffer pH 7.6 (GCG) (containing 5 mM dithiothreitol) and sonicated on ice. The suspension of cellular material was centrifuged in a Beckman L2-65B ultracentrifuge at 100,000 x g for 1 hour at 4°C. The resulting supernatant fraction contained the soluble enzyme, PDE, and was in the glycylglycine buffer, which is
compatible with the PDE assay system. The protein content of this crude PDE preparation was assayed as described previously.

F. CYCLIC AMP PHOSPHODIESTERASE (PDE) ASSAY

The activity of cAMP PDE was assayed by a modification of the method of Fertel and Weiss [123].

(1) Principles of the assay

PDE converts cAMP to 5'-AMP. Myokinase (EC 2.7.4.3) converts 5'-AMP in the presence of very low concentrations of ATP (which acts as a phosphate donor) to ADP. Pyruvate kinase (EC 2.7.1.40) converts ADP to ATP in the presence of the phosphate donor phosphoenol pyruvate (PEP). The resulting ATP concentration is directly proportional to the 5'-AMP produced by PDE. ATP is then determined by the firefly luciferin-luciferase reaction by measurement of phospholuminescence. These reactions are summarized in the equations below:

\[
\begin{align*}
\text{CAMP} & \xrightarrow{\text{PDE}} 5'-\text{AMP} \quad (1) \\
5'-\text{AMP} + \text{ATP} & \xleftarrow{\text{myokinase}} \rightarrow (2)\text{ADP} \quad (2) \\
(2)\text{ADP} + (2)\text{PEP} & \xleftarrow{\text{pyruvate kinase}} \rightarrow (2)\text{ATP} + (2)\text{pyruvate} \quad (3) \\
\text{ATP} + \text{ luciferin} + \text{O}_2 & \xrightarrow{\text{luciferase}} \rightarrow \text{AMP} + \text{PP}_i + \text{oxyluciferin} + \text{CO}_2 + \text{light} \quad (4)
\end{align*}
\]
(2) Reagents

Myokinase from rabbit muscle was obtained (Sigma) as a suspension in 3.2 M \((\text{NH}_4)_2\text{SO}_4\) solution, containing 1 mM EDTA at pH 6 with an activity of 1,480 units/mg protein. A unit is defined as follows: one unit will convert 2 umoles of ADP to ATP + AMP per minute at pH 7.6 and 37°C.

Pyruvate kinase from rabbit muscle was obtained (Sigma) as a suspension in 2.2 M \((\text{NH}_4)_2\text{SO}_4\) solution at pH 6 with an activity of 465 units/mg protein. One unit will convert 1 umole of phosphoenol pyruvate to pyruvate per minute at pH 7.6 and 37°C.

Both myokinase and pyruvate kinase were centrifuged at 1000 x g for 10 minutes at 4°C. The resulting precipitate, which contained the enzyme, was dissolved in reagent A.

Luciferin-luciferase was obtained from Sigma (L-0633) and contained luciferin, luciferase, EDTA, glycine buffer salts and human albumin as a protein base.

Reagent A:

150 mM glycyglycine buffer, pH 7.6 (GCG)
75 mM ammonium acetate
9 mM magnesium chloride
0.03 mM calcium chloride
0.78 mM phosphoenol pyruvate
15 mM dithiothreitol
3 nM ATP

Reagent A is stable for several months at -20°C.
Reagent B:

1 ml Reagent A
12.5 µl 3% BSA
12.5 U myokinase (in 12.5 µl reagent A)
6.25 U pyruvate kinase (in 12.5 µl reagent A)
3.75 µl 10 mM calcium chloride

Reagent B is made on the day of each assay.

Morpholinopropane sulfonic acid (MOPS) buffer:

10 mM MOPS, pH 7.8
10 mM MgSO₄
1 mM dithiothreitol

This reagent is stable for several months at 4°C.

Luciferin-luciferase reagent:

1 ml MOPS buffer
7.5 mg luciferin-luciferase
10 mg BSA

This reagent is made in a plastic tube 1 hour prior to use
and stored in the dark at 4°C.

(3) Procedure

The assay was performed in 2 steps. In the first
step, the following components were added to 6 mm x 50 mm
glass tubes:

Standard curve

50 µl GCG
50 µl reagent B
50 µl distilled water (or drug)
50 µl of various concentrations of 5'-AMP (in water to
provide 0.06-4 nmoles/assay tube)
**Sample tubes**

50 ul PDE preparation  
50 ul reagent B  
50 ul distilled water (or drug)  
50 ul cAMP (in water)  

The reaction was initiated by adding the 5'-AMP (standards) or cAMP (samples) and then incubating the tubes in a 37°C water bath. The reaction was terminated by placing the tubes in a boiling water bath for 10 minutes. In the final step, the tubes were cooled and placed in an integrating photometer (SAI Technology Co., Model 3000) which was modified to accept a repeating Hamilton syringe for microinjection. A plastic 1 ml syringe was used in place of a glass syringe in order to eliminate the time dependent degradation of the light generating system. Twenty ul of firefly reagent was added to each tube and the concentration of ATP was determined by measuring the subsequent light generated.

G. **STATISTICAL ANALYSIS**

Results were analyzed using analysis of variance, Student's t-test, and Dunnett's t-test [124]. Statistical significance was determined at P<0.05 and P<0.01.
RESULTS

A. THE EFFECT OF LECTIN MITOGENS ON LYMPHOCYTE PROLIFERATION

Initial experiments were designed to determine the optimal and suboptimal mitogen concentrations for lymphocyte proliferation. Figure 2 shows a typical dose-response curve observed with increasing concentrations of Con A. A concentration of 10 ug/ml of Con A provided the optimal (maximum) $^3$H-TdR uptake into PBMC. As expected, supraoptimal concentrations of lectin decreased lymphocyte responsiveness. A concentration of 2.5 ug/ml of Con A was chosen to provide a suboptimal (submaximum) response. A titration of PHA demonstrated a similar dose-dependent initial increase, and then decrease, in $^3$H-TdR uptake into PBMC (Figure 3). The concentration of PHA which provided an optimal response was 1% (v:v). The concentration chosen to provide a suboptimal response was 0.05% (v:v). Time-response experiments demonstrated that a 3 day incubation of PBMC with either mitogen, followed by a 16 hour terminal pulse with $^3$H-TdR (chosen for convenience), provided a proliferative response which was close to optimal (data not shown).
FIGURE 2. THE EFFECT OF CON A ON LYMPHOCYTE PROLIFERATION.

Various concentrations of Con A were added to cultures containing 1 x 10⁷ PBMC. Lymphocyte proliferation was detected as described in methods after a 72 hour incubation and a 16 hour terminal pulse with H-TdR. Each value represents the mean ± sem of triplicate cultures from one representative experiment.
FIGURE 3. THE EFFECT OF PHA ON LYMPHOCYTE PROLIFERATION.

Various concentrations of PHA were added to cultures containing $1 \times 10^7$ PBMC. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one representative experiment.
B. THE EFFECT OF 8-MOP ON LYMPHOCYTE PROLIFERATION

In order to determine the effect of 8-MOP on lymphocyte proliferation, PBMC were incubated with either an optimal or a suboptimal concentration of PHA and various concentrations of 8-MOP (dissolved in ethanol). Proliferation was detected as described in methods following a total incubation time of 88 hours. The presence of 8-MOP in cell culture impaired the mitogen-induced proliferation of normal human lymphocytes. As shown in Figure 4, approximately 2 to 140 uM (0.4-30 ug/ml) 8-MOP caused a dose-dependent decrease of PHA-induced lymphocyte proliferation. The suppressive effect of 8-MOP was most apparent (96% reduction in proliferation) when a suboptimal (0.05%) concentration of PHA was used to stimulate lymphocyte proliferation. Less suppression (52% reduction in proliferation) was apparent at the optimal (1%) PHA concentration. As shown in Figure 5, higher concentrations of PHA were unable to overcome the suppressive effect of 8-MOP. In fact, 8-MOP (approximately 140 uM) suppressed the entire PHA titration.

8-MOP also impaired the proliferation of lymphocytes stimulated by two concentrations of another mitogenic lectin, Con A (Figure 6).
FIGURE 4. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^7$ PBMC plus 1% PHA (●) or 0.05% PHA (○). Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of four experiments. The value from each experiment is the mean of triplicate cultures. Analysis by Dunnett's t-test indicated that cells treated with 8-MOP are statistically different from control cells (* $P<0.05$ and ** $P<0.01$).
FIGURE 5. THE EFFECT OF PHA ON LYMPHOCYTE PROLIFERATION IN THE PRESENCE OF 8-MOP.

Various concentrations of PHA were added to cultures containing 1 x 10^5 PBMC plus no 8-MOP (•) or 140 uM 8-MOP (○). Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments. Analysis by Student's t-test indicated that cells treated with 8-MOP are statistically different from control cells (P<0.01 for all values).
FIGURE 6. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON CON A-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^5$ PBMC plus 10 ug/ml Con A (●) or 2.5 ug/ml Con A (○). Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of two experiments. The value from each experiment is the mean of triplicate cultures. Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells (* $P<0.05$ and ** $P<0.01$).
Since the 8-MOP was dissolved in ethanol, an experiment was performed to eliminate the possibility of an inhibitory or stimulatory effect of ethanol alone on mitogen-induced lymphocyte proliferation. Figure 7 demonstrates that concentrations of ethanol (0.25% to 1%) expected to be present in the 8-MOP solution did not significantly influence the lymphocyte proliferation induced by either an optimal or a suboptimal concentration of PHA. The proliferation induced by an optimal concentration of PHA remained unaffected by ethanol concentrations of up to 4%, while the proliferation induced by a suboptimal concentration of PHA appeared to be significantly inhibited by 4% ethanol.

To determine if the observed suppression of lymphocyte proliferation was due to 8-MOP killing the cells in culture, PBMC were collected and stained with trypan blue following a typical incubation with PHA and various concentrations of 8-MOP. As shown in Figure 8, the 8-MOP suppression was not the result of direct cytotoxicity, since cells exposed to 8-MOP were more than 90% viable at the end of the incubation period.

Figure 8 also demonstrates directly that 8-MOP suppresses lymphocyte proliferation. 8-MOP caused a dose-dependent decrease of PBMC accumulation in culture medium following an 88 hour incubation with PHA.
**FIGURE 7.** THE EFFECT OF ETHANOL ON PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of ethanol were added to cultures containing $1 \times 10^7$ PBMC plus 1% PHA (•) or 0.05% PHA (○). Cultures were incubated and harvested as in Figure 2. Each value is the mean ± sem of two experiments. The value from each experiment is the mean of triplicate cultures. Analysis by Dunnett's t-test indicated that ethanol treated cells (up to 2%) are not statistically different from control cells at P<0.05. (** P<0.01)
FIGURE 8. THE EFFECT OF 8-MOP ON CELL VIABILITY.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^7$ PBMC plus 0.05% PHA. After 88 hours of incubation, the cells were stained with trypan blue and counted as described in methods. Each value represents cell viability (•) or cell density (○) from one representative experiment. Control cultures (no PHA or 8-MOP) were 94% viable, and contained $0.94 \times 10^6$ cells per ml of medium, after 88 hours of incubation.
To determine the time-dependence of the observed suppression, replicate cultures of human PBMC were stimulated with a suboptimal (0.05%) concentration of PHA and 140 uM 8-MOP was added to individual cultures at various times thereafter. All cultures were pulsed with $^3$H-TdR at 72 hours and harvested after an additional 16 hours of incubation. Table 1 demonstrates that 8-MOP was most efficient at suppressing PHA-induced proliferation when added within the first 24 hours of culture. However, significant suppression was still evident even when 8-MOP was added along with the $^3$H-TdR at the 72nd hour of incubation.

The mixed lymphocyte culture reaction (MLR) utilizes alloantigens, instead of mitogen, as a stimulus for lymphocyte proliferation. Accordingly, lymphocytes respond with specific clonal proliferation (alloantigens), rather than, nonspecific polyclonal proliferation (mitogens). Therefore, the MLR represents a model system which is perhaps a better in vitro correlate of the cellular immune response. In order to determine the effect of 8-MOP on lymphocyte proliferation under these conditions, PBMC from two volunteers were cultured together along with various concentrations of 8-MOP as described in methods. As shown in Figure 9 (upper and lower panels), 1.7 to 140 uM 8-MOP caused a dose-dependent
TABLE 1. THE EFFECT OF 8-MOP ADDITION TIME ON THE INHIBITION OF PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Replicate cultures were stimulated with 0.05% PHA as described in methods and at various times thereafter 140 μM 8-MOP was added to individual cultures. Control cultures had no 8-MOP added at 0 hour. Data represents the mean ± sem of six replicate cultures from two representative experiments. Analysis by Dunnett's t-test indicated that 8-MOP treated cells were statistically different from control cells at all incubation times examined (P<0.01).
TABLE 1. THE EFFECT OF 8-MOP ADDITION TIME ON THE INHIBITION OF PHA-INDUCED LYMPHOCYTE PROLIFERATION.

<table>
<thead>
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<th>Hour of 8-MOP addition</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10^{-3}</td>
<td>cpm x 10^{-3}</td>
</tr>
<tr>
<td>0</td>
<td>9.3±1.1</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>12</td>
<td>22.3±2.4</td>
<td>4.3±0.3</td>
</tr>
<tr>
<td>24</td>
<td>29.0±3.0</td>
<td>4.3±0.5</td>
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<tr>
<td>48</td>
<td>76.0±3.6</td>
<td>15.0±1.8</td>
</tr>
<tr>
<td>72</td>
<td>109.0±3.4</td>
<td>54.7±3.3</td>
</tr>
<tr>
<td>No 8-MOP</td>
<td>151.1±2.3</td>
<td>88.4±4.0</td>
</tr>
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</table>

% Inhibition

<table>
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<tr>
<th>Hour of 8-MOP addition</th>
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<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(93.9)</td>
<td>(93.1)</td>
</tr>
<tr>
<td>12</td>
<td>(85.2)</td>
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<td>(0)</td>
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</table>
FIGURE 9. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON ALLOANTIGEN-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^7$ responder PBMC and $1 \times 10^5$ stimulator PBMC. Lymphocyte proliferation was detected as described in methods after a 5 day incubation and a 16 hour terminal pulse with H-TdR. Each value represents the mean ± sem of triplicate cultures from either Subject 1 + Subject 2 (upper panel) or Subject 2 + Subject 1 (lower panel). HLA histocompatibility was as follows:

Subject 1 - A2, A30, B7, B13, DR2, DR6
Subject 2 - A29, A30, B51, B13, DR5, DR7

Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells. (* P<0.05 and ** P<0.01). Responder cells incubated with irradiated LCL cells in the absence of 8-MOP (positive control) had approximately 70,000 cpm (subject 1) and 120,000 cpm (subject 2).
FIGURE 9. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON ALLOANTIGEN-INDUCED LYMPHOCYTE PROLIFERATION.
decrease of alloantigen-induced lymphocyte proliferation.

C. THE EFFECT OF 8-MOP ON LYMPHOCYTE PROLIFERATION IN THE ABSENCE OF ULTRAVIOLET (UV) IRRADIATION

Previous results from this laboratory indicated that UV irradiation of PBMC was not required for the biochemical effects of 8-MOP [17]. Analysis of laboratory ambient light using Blak-Ray Ultraviolet Meters J-221 and J-225 (Ultra-Violet Products, San Gabriel, CA) indicated that no measurable UV light was present. However, since the lower limit of meter sensitivity is 2 uW/cm², the possibility of some UV light interaction was not entirely eliminated. Therefore, experiments were performed in which mitogen-stimulated human PBMC were incubated in total darkness and were exposed to a low light level (Kodak Safelight Filter 1A) only during the addition of drug and ³H-TdR. As shown in Figure 10, 2 to 140 μM 8-MOP caused a dose-dependent decrease of suboptimal PHA-induced lymphocyte proliferation even under these rigidly controlled conditions. 8-MOP also decreased optimal PHA-induced proliferation in the dark (data not shown). All subsequent experiments were performed in ambient light, since this condition did not significantly influence the experimental results. These experiments indicate that 8-MOP without UV irradiation is immunosuppressive in vitro.
**FIGURE 10.** THE DOSE-DEPENDENT EFFECT OF 8-MOP ON PHA-INDUCED LYMPHOCYTE PROLIFERATION IN THE DARK.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^5$ PBMC plus 0.05% PHA. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments. Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells. (** $P<0.01$)
D. THE EFFECT OF OTHER PSORALENS ON LYMPHOCYTE PROLIFERATION

Experiments were performed to determine if other psoralen analogues in addition to 8-MOP could impair lectin-induced lymphocyte proliferation. As shown in Figure 11, 5-MOP, 5-MIP and 3-CEP all caused a dose-dependent impairment of lymphocyte proliferation induced by a suboptimal concentration of PHA. In addition, 4,5',8-trimethylpsoralen also impaired PHA-induced lymphocyte proliferation (data not shown). The fact that other psoralen compounds, which are relatively incapable of forming photoadducts with DNA (5-MIP and 3-CEP), can impair lymphocyte proliferation, further suggests that the effect of 8-MOP is independent of its interaction with UV radiation.

E. THE EFFECT OF 8-MOP ON THE KINETICS OF LYMPHOCYTE PROLIFERATION

Two ways by which 8-MOP could cause a decrease in mitogen-induced lymphocyte proliferation, when measured at a single time of incubation, are by delaying the proliferative response or by completely inhibiting the proliferative response. In fact, 8-MOP had both effects, depending on the concentration of PHA used to stimulate lymphocyte proliferation.
FIGURE 11. THE DOSE-DEPENDENT EFFECT OF PSORALEN ANALOGUES ON PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of 8-MOP (○), 5-MOP (■), 3-CEP (△), or 5-MIP (△) were added to cultures containing 1 x 10⁶ PBMC plus 0.05% PHA. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
In order to determine the effect of 8-MOP on the time-dependence of lymphocyte proliferation (hereafter referred to as "kinetics of lymphocyte proliferation"), replicate microtiter plates were prepared containing PBMC and an optimal or a suboptimal concentration of PHA in the presence or absence of 8-MOP. Individual plates were incubated for various times and proliferation was assessed by $^3$H-TdR uptake. As shown in Figure 12, when cultures were stimulated with an optimal concentration of PHA in the absence of 8-MOP the peak proliferative response (approximately 160,000 cpm) occurred at 96 hours. In the presence of 140 uM 8-MOP, however, the peak proliferative response was delayed until 144 hours. As shown in Figure 13, cultures stimulated with a suboptimal concentration of PHA in the absence of 8-MOP also developed a peak proliferative response (approximately 95,000 cpm) at 96 hours. However, 140 uM 8-MOP caused a complete inhibition of, rather than a delay in, the proliferative response. Similar results were observed using 70 uM 8-MOP with an optimal and a suboptimal concentration of PHA (data not shown). Therefore, 8-MOP has two effects on mitogen-induced lymphocyte proliferation. 8-MOP causes a delay in the proliferation induced by an optimal mitogen concentration and 8-MOP causes a complete inhibition of
FIGURE 12. THE EFFECT OF 8-MOP ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate culture plates containing 1 x 10^5 PBMC plus 1% PHA received no 8-MOP (■) or 140 μM 8-MOP (●). Individual plates were harvested at various incubation times, following a 24 terminal pulse with ^3H-TdR. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
FIGURE 13. THE EFFECT OF 8-MOP ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate culture plates containing $1 \times 10^5$ PBMC plus 0.05% PHA received no 8-MOP (■) or 140 μM 8-MOP (○). Individual plates were incubated and harvested as in Figure 12. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
the proliferation induced by a suboptimal mitogen concentration.

F. THE EFFECT OF 8-MOP ON INTERLEUKIN-2 (IL-2) ACCUMULATION

Lectin mitogen-induced lymphocyte proliferation is dependent on the endogenous production and secretion of mitogenic lymphokines and the expression of lymphokine receptors [66,68]. Accordingly, 8-MOP may affect lymphocyte proliferation by interfering with IL-2 production and/or IL-2 receptor expression. To determine the effect of 8-MOP on the PHA-induced accumulation of IL-2, PBMC were incubated with an optimal concentration of PHA and various concentrations of 8-MOP. Culture supernatants were collected after 24 and 48 hours of incubation, and IL-2 was detected as described in methods. As shown in Figures 14 (upper panel) and 15, 8-MOP caused a dose-dependent decrease of IL-2 accumulation during the first 24 hours of culture. However, 8-MOP did not influence the accumulation of IL-2 by the 48th hour of culture; similar levels of IL-2 had accumulated regardless of the presence of 8-MOP (Figures 14, lower panel and 15). Therefore, 8-MOP appeared to cause a delay in IL-2 accumulation.

Before concluding that a decrease of IL-2 accumulation had occurred, it was necessary to eliminate
FIGURE 14. THE EFFECT OF 8-MOP ON THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH AN OPTIMAL CONCENTRATION OF PHA.

Triplicate cultures containing $1 \times 10^5$ PBMC plus 1% PHA received no 8-MOP (●), 1.4 μM 8-MOP (○), 14 μM 8-MOP (▲) or 140 μM 8-MOP (■). After 24 hours (upper panel) or 48 hours (lower panel) of incubation, the culture supernatants were collected and titered into replicate cultures containing $1 \times 10^4$ CTLL-20 cells. The presence of IL-2 in the supernatant was detected as $^3$H-TdR uptake by the CTLL-20 cells after a 24 hour incubation period which included an 8 hour terminal pulse with $^3$H-TdR. Cultures of PBMC which contained no PHA or 8-MOP (□) were established as controls for basal IL-2 activity. Each value represents the mean of duplicate cultures from one of two representative experiments.
FIGURE 14. THE EFFECT OF 8-MOP ON THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH AN OPTIMAL CONCENTRATION OF PHA.
FIGURE 15. 8-MOP DELAYS THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH AN OPTIMAL CONCENTRATION OF PHA.

This figure represents the data obtained at a 1/6 dilution of 24 (○) or 48 (●) hour culture supernatants from Figure 14. Each value represents the mean ± sem of duplicate cultures. Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells (** P<0.01).
the possibility that 8-MOP directly interfered with the IL-2 bioassay or induced a factor that suppressed IL-2 detection. To test this, PBMC were stimulated with an optimal concentration of PHA in the presence or absence of 140 uM 8-MOP. After 24 hours the culture supernatants were collected and tested for both IL-2 activity and inhibitory activity as described in methods. As shown in Figure 16 (upper panel), the 24-hour culture supernatants from PBMC stimulated with PHA in the absence of 8-MOP contained readily detectable levels of IL-2. Addition of 8-MOP to PHA-stimulated PBMC significantly reduced the amount of IL-2 accumulated during the first 24 hours of culture. When 24-hour culture supernatants from 8-MOP treated PBMC were tested for inhibitory activity, they demonstrated little inhibitory activity except at the lowest supernatant dilution tested, where PHA alone had a similar inhibitory affect on CTLL-20 ³H-TdR uptake (Figures 16, lower panel and 17). In summary, 8-MOP causes a delay in IL-2 accumulation in culture supernatants of PBMC by delaying the production or secretion of IL-2, rather than by directly or indirectly interfering with the IL-2 bioassay. This delay may, therefore, explain the mechanism by which 8-MOP impairs the lymphocyte proliferation induced by an optimal concentration of PHA.
FIGURE 16. CULTURE SUPERNATANTS FROM PBMC INCUBATED WITH AN OPTIMAL CONCENTRATION OF PHA AND 8-MOP DO NOT INHIBIT THE DETECTION OF IL-2 ACTIVITY.

Triplicate cultures containing $1 \times 10^5$ PBMC + 1% PHA ($\blacksquare$), PBMC + 1% PHA + 140 uM 8-MOP ($\bullet$), PBMC + 140 uM 8-MOP ($\Diamond$) or PBMC alone ($\times$) were incubated for 24 hours. The culture supernatants were collected and tested for IL-2 activity (upper panel) or for inhibitory activity (lower panel). To test for IL-2 activity, the supernatants were treated as in Figure 14. To test for inhibitory activity, the supernatants were titered into replicate cultures containing $1 \times 10^4$ CTLL-20 cells plus 5% MLA supernatant. Cultures were harvested after a 24 hour incubation period which included an 8 hour terminal pulse with $^3$H-TdR. Each value represents the mean of duplicate cultures from one of two representative experiments.
FIGURE 16. CULTURE SUPERNATANTS FROM PBMC INCUBATED WITH AN OPTIMAL CONCENTRATION OF PHA AND 8-MOP DO NOT INHIBIT THE DETECTION OF IL-2 ACTIVITY.
FIGURE 17. 8-MOP DOES NOT INHIBIT THE DETECTION OF IL-2 ACTIVITY.

This figure represents the data obtained at a 1/6 dilution of culture supernatant from Figure 16. Each value is the mean of duplicate cultures from one of two representative experiments.
Using the same approach as above, experiments were performed to determine the effect of 8-MOP on the accumulation of IL-2 from PBMC which were stimulated with a suboptimal concentration of PHA. As shown in Figures 18 (upper panel) and 19, 8-MOP caused a dose-dependent decrease of IL-2 accumulation during the first 24 hours of culture. 8-MOP also caused a dose-dependent decrease of IL-2 accumulation during the following 24 hours of culture (48 hour incubation) (Figures 18, lower panel and 19).

To determine if 8-MOP interfered with the IL-2 bioassay under these experimental conditions, 48-hour culture supernatants were collected and tested for both IL-2 activity and inhibitory activity as before. The 48-hour culture supernatants from 8-MOP treated PBMC did not demonstrate inhibitory activity in the CTLL-20 bioassay (data not shown). In summary, 8-MOP causes a decrease in IL-2 accumulation in culture supernatants of PBMC by inhibiting the production or secretion of IL-2, rather than by interfering with the IL-2 bioassay. This inhibition may, therefore, explain the mechanism by which 8-MOP impairs the lymphocyte proliferation induced by a suboptimal concentration of PHA.
FIGURE 18. THE EFFECT OF 8-MOP ON THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH A SUBOPTIMAL CONCENTRATION OF PHA.

Triplicate cultures containing $1 \times 10^5$ PBMC plus 0.05% PHA received no 8-MOP (♦), 1.4 uM 8-MOP (◆), 14 uM 8-MOP (▲) or 140 uM 8-MOP (■). After 24 hours (upper panel) or 48 hours (lower panel) of incubation, the culture supernatants were collected and treated as in Figure 14. Cultures of PBMC which contained no PHA or 8-MOP (▲) were established as controls for basal IL-2 activity. Each value represents the mean of duplicate cultures from one of two representative experiments.
FIGURE 18. THE EFFECT OF 8-MOP ON THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH A SUBOPTIMAL CONCENTRATION OF PHA.
FIGURE 19. 8-MOP INHIBITS THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF PBMC INCUBATED WITH A SUBOPTIMAL CONCENTRATION OF PHA.

This figure represents the data obtained at a 1/2 dilution of 24 (o) or 48 (●) hour culture supernatants from Figure 18. Each value represents the mean ± sem of duplicate cultures. Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells (* P<0.05 and ** P<0.01).
G. THE EFFECT OF EXOGENOUS INTERLEUKIN-2 (IL-2) ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF LYMPHOCYTE PROLIFERATION

Initial studies were designed to determine the dose-dependence for IL-2's effect on lymphocyte proliferation. PBMC were incubated without PHA in the presence or absence of 8-MOP and various concentrations of IL-2 (3 to 80 U/ml). Proliferation was quantitated after a 3 day incubation and a 16 hour terminal pulse with \(^{3}\text{H}-\text{TdR}\). As shown in Figure 20, exogenous IL-2 increased the proliferation of nonmitogen-stimulated cells in the presence and absence of 8-MOP. However, IL-2's potency was decreased in the presence of both a low (14 uM) and high (70 uM) dose of 8-MOP. Additionally, 8-MOP caused a dose-dependent decrease of IL-2-stimulated lymphocyte proliferation (Figure 21). This provided the first indication that 8-MOP had an effect on lymphocytes in addition to its ability to impair IL-2 accumulation.

If a delay in IL-2 production or secretion is the only mechanism by which 8-MOP impairs the lymphocyte proliferation induced by an optimal concentration of mitogen, then addition of exogenous IL-2 at the initiation of culture should overcome the effect of 8-MOP. To test this, cultures of PBMC were incubated with an optimal concentration of PHA and 80 U/ml of IL-2 in the presence
FIGURE 20. THE DOSE-DEPENDENT EFFECT OF EXOGENOUS IL-2 ADDITION ON LYMPHOCYTE PROLIFERATION.

Various concentrations of exogenous IL-2 were added to cultures containing 1 x 10^5 PBMC plus no 8-MOP (■), 14 μM 8-MOP (●) or 70 μM 8-MOP (○) in the absence of PHA. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
**FIGURE 21.** THE DOSE-DEPENDENT EFFECT OF 8-MOP ON IL-2-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^5$ PBMC plus 80 U/ml of exogenous IL-2. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one experiment. Analysis by Dunnett's t-test indicated that cells treated with 8-MOP are statistically different from control cells. (**) P<0.01.)
and absence of 140 μM 8-MOP. As shown in Figure 22, 8-MOP caused a delay in the proliferative response of lymphocytes regardless of the presence of exogenous IL-2. Similar results were observed using 70 μM 8-MOP (data not shown). This data indicates that although 8-MOP delays the production or secretion of IL-2 by lymphocytes stimulated with an optimal concentration of PHA, this is not the only mechanism by which 8-MOP impairs lymphocyte proliferation, since exogenous IL-2 did not reverse the effect of 8-MOP.

Similarly, if an inhibition of the production or secretion of IL-2 is the only mechanism by which 8-MOP impairs the lymphocyte proliferation induced by a suboptimal concentration of mitogen, then addition of exogenous IL-2 should overcome the effect of 8-MOP. To test this, various concentrations of exogenous IL-2 (3 to 80 U/ml) were added to cultures of PBMC incubated with a suboptimal concentration of PHA in the presence or absence of 8-MOP. IL-2 augmented the proliferation of these cells both in the presence and absence of 8-MOP. However, the highest concentration of IL-2 tested (80 U/ml) could not completely overcome the inhibition observed with either a low (14 μM) or high (70 μM) dose of 8-MOP (Figure 23). In a more rigorous experiment, cultures of PBMC were incubated with a suboptimal concentration of PHA and 80
FIGURE 22. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate culture plates containing $1 \times 10^5$ PBMC plus 1% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 uM 8-MOP (●). Individual plates were incubated and harvested as in Figure 12. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
FIGURE 23. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of exogenous IL-2 were added to cultures containing $1 \times 10^6$ PBMC plus no 8-MOP (■), 14 uM 8-MOP (●) or 70 uM 8-MOP (○) in the presence of 0.05% PHA. Cultures were incubated and harvested as in Figure 2. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
U/ml of IL-2 in the presence and absence of 140 uM 8-MOP. As shown in Figure 24, IL-2 restored the magnitude of the proliferative response. However, it also resulted in the delayed proliferative response which was typical of the effect of 8-MOP on the lymphocyte proliferation induced by an optimal concentration of PHA. Similar results were obtained using 70 uM 8-MOP (data not shown). This data suggests that an inhibition of IL-2 production or secretion is one mechanism by which 8-MOP impairs the proliferation of lymphocytes stimulated with a suboptimal concentration of mitogen, but that other mechanisms may also be important.

H. THE EFFECT OF 8-MOP ON INTERLEUKIN-2 RECEPTOR EXPRESSION

As mentioned above, another possible mechanism by which 8-MOP could impair PHA-induced lymphocyte proliferation is by interfering with the expression of IL-2 receptors. To examine the effect of 8-MOP on IL-2 receptor expression, PBMC were incubated with an optimal or a suboptimal concentration of PHA in the presence or absence of 8-MOP. Individual cultures were incubated for various times and the cells were analyzed by cytofluorometry following immunofluorescence staining of their cell surfaces as described in methods.
FIGURE 24. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate culture plates containing $1 \times 10^5$ PBMC plus 0.05% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 μM 8-MOP (●). Individual plates were incubated and harvested as in Figure 12. Each value represents the mean ± sem of triplicate cultures from one of two representative experiments.
As shown in Figure 25, when cultures were stimulated with an optimal concentration of PHA in the absence of 8-MOP the maximum expression of cells containing the IL-2 receptor (approximately 65% Tac+ cells) occurred at 96 hours. In the presence of 140 uM 8-MOP, however, the maximum expression of Tac+ cells was delayed until approximately 120 hours. The relative density of IL-2 receptors per cell (the mean fluorescence of anti-Tac binding) for 8-MOP treated cultures was lower than that of control cultures during the first 48 to 72 hours of incubation. This was followed by an increase in the mean fluorescence of anti-Tac binding for 8-MOP treated cultures compared to that of control cultures (Figure 26). Results from a parallel proliferation experiment (data not shown) correlated with the results obtained in Figure 25.

As shown in Figure 27, approximately 1.5 to 140 uM 8-MOP caused a dose-dependent decrease of the expression of Tac+ cells in cultures of lymphocytes which were stimulated with a suboptimal concentration of PHA and incubated for 96 hours. The highest concentration of 8-MOP tested (140 uM) produced approximately a 95% reduction in the expression of Tac+ cells. The percentage of Tac+ cells was reduced from 37% in the absence of 8-MOP to 2% in the presence of 8-MOP. In a more rigorous experiment, replicate cultures were
FIGURE 25. THE EFFECT OF 8-MOP ON THE EXPRESSION OF TAC-POSITIVE CELLS INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 1% PHA received no 8-MOP (■) or 140 μM 8-MOP (●). Individual cultures were harvested at various incubation times, and stained with either FITC-conjugated anti-IL-2 receptor (anti-Tac) or FITC-conjugated mouse IgG, control. The binding of either monoclonal reagent was detected by cytofluorometry as described in methods. Each value represents the percentage of cells expressing the IL-2 receptor, based on the specific binding of anti-Tac to a single culture from one experiment. Control cells (no PHA or 8-MOP) were less than 3% Tac$^+$ between 48 and 72 hours of incubation in this and subsequent IL-2 receptor experiments.
Replicate cultures containing $1 \times 10^6$ PBMC plus 1% PHA received no 8-MOP (■) or 140 uM 8-MOP (●). Individual cultures were incubated and harvested as in Figure 25. The binding of anti-Tac was detected by cytofluorometry as described in methods. Each value represents the mean fluorescence (in arbitrary fluorescence units) of anti-Tac binding to a single culture from one experiment. Control cells (no PHA or 8-MOP) had a mean fluorescence of anti-Tac binding of less than 5 arbitrary fluorescence units (AFU) between 48 and 72 hours of incubation in this and subsequent IL-2 receptor experiments.
FIGURE 27. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON THE EXPRESSION OF TAC-POSITIVE CELLS INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Various concentrations of 8-MOP were added to cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA. Cultures were incubated for 96 hours and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
incubated for various times with a suboptimal concentration of PHA in the presence and absence of 8-MOP and then analyzed for IL-2 receptor expression. As shown in Figure 28, control cultures again developed the maximum expression of Tac$^+$ cells (approximately 12% Tac$^+$) at 96 hours. The presence of 140 uM 8-MOP, however, caused a complete inhibition of, rather than a delay in, the expression of Tac$^+$ cells. The mean fluorescence of anti-Tac binding for 8-MOP treated cultures was lower than that of control cultures during the first 48 to 96 hours of incubation. This was followed by an equivocal increase in the mean fluorescence of anti-Tac binding for 8-MOP treated cultures compared to that of control cultures (Figure 29). Results from a parallel proliferation experiment (data not shown) correlated with the results obtained in Figure 28.

I. THE EFFECT OF EXOGENOUS INTERLEUKIN-2 (IL-2) ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF IL-2 RECEPTOR EXPRESSION

Since IL-2 is known to regulate the expression of its own receptor [125-128], the effects of 8-MOP on IL-2 receptor expression in the presence of exogenous IL-2 were examined. Cultures of PBMC were incubated with an optimal concentration of PHA and 80 U/ml of IL-2 in the presence and absence of 140 uM 8-MOP. As shown in Figure 30, 8-MOP
FIGURE 28. THE EFFECT OF 8-MOP ON THE EXPRESSION OF TAC-POSITIVE CELLS INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA received no 8-MOP (■) or 140 μM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
**FIGURE 29.** THE EFFECT OF 8-MOP ON THE MEAN FLUORESCENCE OF ANTI-TAC BINDING INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA received no 8-MOP (■) or 140 µM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 26. Each value represents the mean fluorescence of anti-Tac binding to a single culture from one experiment.
caused a delay in the expression of Tac\(^+\) cells regardless of the presence of exogenous IL-2. There was no consistent change in the mean fluorescence of anti-Tac binding between 8-MOP treated cultures and control cultures throughout the incubation times examined (Figure 31). Results from a parallel proliferation experiment (data not shown) correlated with the results obtained in Figure 30. The inability of exogenous IL-2 to overcome this effect was consistent with its inability to restore the kinetics of lymphocyte proliferation induced by 8-MOP plus an optimal concentration of PHA (see Figure 22). These data collectively suggest that an additional mechanism by which 8-MOP impairs the proliferation of lymphocytes stimulated with an optimal concentration of mitogen is by delaying the expression of Tac\(^+\) cells.

Using the same approach as above, cultures of PBMC were incubated with a suboptimal concentration of PHA and 80 U/ml of IL-2 in the presence and absence of 140 uM 8-MOP. As shown in Figure 32, IL-2 enhanced the expression of Tac\(^+\) cells in cultures incubated with and without 8-MOP. However, it also resulted in the delayed expression of Tac\(^+\) cells which was typical of the effect of 8-MOP on the expression of Tac\(^+\) cells in cultures of lymphocytes stimulated with an optimal concentration of PHA (see Figures 25 and 30). In contrast to the results
FIGURE 30. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF THE EXPRESSION OF TAC-POSITIVE CELLS INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 1% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 uM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
FIGURE 31. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF THE MEAN FLUORESCENCE OF ANTI-TAC BINDING INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 1% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 uM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 26. Each value represents the mean fluorescence of anti-Tac binding to a single culture from one experiment.
FIGURE 32. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF THE EXPRESSION OF TAC-POSITIVE CELLS INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing 1 x 10⁶ PBMC plus 0.05% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 μM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
obtained with an optimal concentration of PHA (see Figures 26 and 31), at all incubation times examined, 8-MOP treated cultures demonstrated a higher level of mean fluorescence of anti-Tac binding than control cultures (Figure 33). Results from a parallel proliferation experiment (data not shown) correlated with the results obtained in Figure 32. The ability of exogenous IL-2 to restore the percentage of Tac+ cells, and to induce a delay in this response, was consistent with its effects on the kinetics of lymphocyte proliferation induced by 8-MOP plus a suboptimal concentration of PHA (see Figure 24). These data collectively suggest that there are two ways by which 8-MOP impairs the proliferation of lymphocytes stimulated with a suboptimal concentration of mitogen. 8-MOP inhibits IL-2 production or secretion and also delays the expression of Tac+ cells.

J. THE EFFECT OF 8-MOP ON LYMPHOCYTE CELL LINES

The ability of 8-MOP to impair normal human lymphocyte proliferation in vitro has been well characterized, and immunological mechanisms have been demonstrated to account for this impairment. However, the biochemical basis for the effects of 8-MOP on lymphocyte function remain to be elucidated. A previous report from this laboratory demonstrated that 8-MOP can elevate the concentration of cAMP in several cell types in vitro,
FIGURE 33. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE 8-MOP-MEDIATED IMPAIRMENT OF THE MEAN FLUORESCENCE OF ANTI-TAC BINDING INDUCED BY A SUBOPTIMAL CONCENTRATION OF PHA.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA and 80 U/ml of exogenous IL-2 received no 8-MOP (■) or 140 μM 8-MOP (○). Individual cultures were incubated and harvested as in Figure 26. Each value represents the mean fluorescence of anti-Tac binding to a single culture from one experiment.
including normal human PBMC [17]. Additionally, several laboratories have proposed a role for cyclic nucleotides in the regulation of lymphocyte proliferation [129]. Therefore, preliminary experiments were designed to establish an in vitro model system which could be used to correlate lymphocyte function with alterations in cyclic nucleotide metabolism. Normal human PBMC were eliminated as a model system for several reasons. In particular, PBMC represent a heterogeneous population of cells, each of which may individually influence both cyclic nucleotide metabolism and lymphocyte proliferation. Furthermore, the procedure for isolating PBMC appeared to disturb intracellular cyclic nucleotide metabolism and resulted in inconsistent cyclic nucleotide measurements.

The JURKAT cell line is a homogeneous population of human leukemic cells which morphologically resembles normal resting lymphocytes [130] and can be stimulated with PHA (like normal lymphocytes) to produce IL-2 [113]. To determine the growth characteristics of these cells, JURKAT cells were cultured in a T-75 flask at an initial cell density of \(8 \times 10^4\) viable cells/ml and were examined every 24 hours for cell growth and viability. Figure 34 demonstrates that JURKAT cells had a population doubling time of approximately 24-30 hours and reached maximum cell density (approximately \(7 \times 10^5\)/ml) by the
FIGURE 34. THE GROWTH CYCLE OF JURKAT CELLS IN CULTURE.

JURKAT cells were incubated in an upright T-75 cm$^2$ flask at an initial (day 0) density of $8 \times 10^6$ viable cells/ml in complete cell culture medium as described in methods. At 24-hour intervals, a 100 ul aliquot of cell suspension was removed from the culture flask, stained with trypan blue and counted. Each value represents the cell density (o) or cell viability (£) from a single culture flask from one representative experiment.
4th day of culture. Both cell density and cell viability decreased dramatically after 4 days in culture. This data indicates that JURKAT cells should be subcultured on day 3 and should be used between days 2 and 3 of culture (during the log phase of the growth cycle) when the growth rate is high and cell sampling is the most consistent. This, however, does not imply that the cells are all in the same phase of the cell cycle.

To examine the effect of 8-MOP on the PHA-induced accumulation of IL-2 from JURKAT cells, JURKAT cells were incubated with various concentrations of PHA in the presence or absence of 140 uM 8-MOP. Culture supernatants were collected after 24 hours of incubation and IL-2 activity was detected as described previously. As shown in Figure 35, PHA caused dramatic increases in IL-2 accumulation in the absence of 8-MOP. The presence of 140 uM 8-MOP appeared to cause a maximal decrease of only 20-30% in the PHA-induced IL-2 accumulation. Before concluding that a decrease of IL-2 accumulation had occurred, it was again necessary to eliminate the possibility that PHA and/or 8-MOP directly or indirectly interfered with the IL-2 bioassay. To test this, JURKAT cells were incubated with various concentrations of PHA in the presence or absence of 140 uM 8-MOP. Culture supernatants were collected after 24 hours of incubation.
FIGURE 35. THE EFFECT OF 8-MOP ON THE ACCUMULATION OF IL-2 ACTIVITY IN CULTURES OF JURKAT CELLS INCUBATED WITH PHA.

Triplicate cultures containing $2 \times 10^5$ JURKAT cells received various concentrations of PHA in the absence ($) or presence ($) of 140 µM 8-MOP. After 24 hours of incubation, the culture supernatants were collected and tested for IL-2 activity as in Figure 14. Each value represents the mean of duplicate cultures from one experiment and was obtained at a 1/6 dilution of culture supernatant.
and inhibitory activity was detected as described previously. The addition of 8-MOP to PHA stimulated cultures did not demonstrate inhibitory activity except at the lowest (1/2) supernatant dilution tested (data not shown). In summary, 8-MOP caused only a small decrease of IL-2 production or secretion from PHA-stimulated JURKAT cells during the first 24 hours of incubation.

Since 8-MOP caused only a small decrease in IL-2 production, JURKAT cells were not considered to be a reliable model system for studying the inhibitory effects of 8-MOP on lymphocyte function. Therefore, the effect of 8-MOP on the MOLT-4 T-lymphoblastoid cell line was examined. As for the JURKAT cell line, preliminary experiments were designed to determine the growth characteristics of the MOLT-4 cells. MOLT-4 cells were cultured in a T-75 flask at an initial cell density of 5 x 10^5 viable cells/ml and were examined every 24 hours for cell growth and viability. Figure 36 demonstrates that MOLT-4 cells had a population doubling time of approximately 36 hours and reached maximum cell density (approximately 1.7 x 10^6/ml) by the 3rd day of culture. Both cell density and cell viability decreased after 3 days in culture. This data indicates that MOLT-4 cells should be used between days 2 and 3 of culture (during the log phase of the growth cycle) when the growth rate is
FIGURE 36. THE GROWTH CYCLE OF MOLT-4 CELLS IN CULTURE.

MOLT-4 cells were incubated in an upright T-75 cm² flask at an initial (day 0) density of 5 x 10⁵ viable cells/ml in complete cell culture medium as described in methods. At 24-hour intervals, a 100 ul aliquot of cell suspension was removed from the culture flask, stained with trypan blue and counted. Each value represents the cell density (•) or cell viability (o) from a single culture flask from one representative experiment.
high and cell sampling is the most consistent. Once again, this does not imply that the cells are all in the same phase of the cell cycle.

To determine the effect of 8-MOP on the proliferation of MOLT-4 cells, replicate cultures received various concentrations of 8-MOP and were incubated for a 30 hour period, which included an 8 hour terminal pulse with $^3$H-TdR. Cells were harvested and proliferation was detected as usual. As shown in Figure 37, approximately 9 to 140 uM 8-MOP caused a dose-dependent decrease of MOLT-4 spontaneous proliferation. The highest concentration of 8-MOP tested (140 uM) caused an approximate 40% decrease of proliferation. It remains to be determined whether this decrease of proliferation is the result of a delay in the proliferative response or an inhibition of proliferation. The effect of 8-MOP was not, however, the result of decreased cell viability. Cells were more than 90% viable at the end of the incubation period (data not shown).

K. THE EFFECT OF 8-MOP ON THE CYCLIC NUCLEOTIDE CONCENTRATION OF MOLT-4 LYMPHOBLASTS

To determine the effect of 8-MOP on the concentration of cAMP within MOLT-4 cells, cells were incubated for various times in the presence or absence of 140 uM 8-MOP. Figure 38 demonstrates the time-dependence of 8-MOP's
FIGURE 37. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON THE SPONTANEOUS PROLIFERATION OF MOLT-4 CELLS.

Various concentrations of 8-MOP were added to cultures containing 1 x 10^6 MOLT-4 cells. Cells were harvested after a 24-hour incubation and an 8-hour terminal pulse with ^3H-TdR. Each value represents the mean ± sem of four experiments. The value from each experiment is the mean of triplicate cultures. Analysis by Dunnett's t-test indicated that cells treated with 8-MOP are statistically different from control cells. (* P<0.05 and ** P<0.01)
FIGURE 38. THE TIME-DEPENDENT EFFECT OF 8-MOP ON THE CYCLIC AMP CONCENTRATION OF MOLT-4 CELLS.

MOLT-4 cells (2 x 10^6) were incubated with 140 μM 8-MOP (•) or buffer only (○) as described in methods. At the times indicated, TCA was added to inhibit further cyclic nucleotide metabolism. The concentration of cAMP was determined by RIA and protein measurements were made as described in methods. Each value represents the mean ± sem of three experiments. The value from each experiment is the mean of duplicate samples. Analysis by Dunnett's t-test indicated that 8-MOP treated samples incubated for 10 to 120 minutes were statistically different from 8-MOP treated samples incubated for 0 minutes (* P<0.05 and ** P<0.01).
effect on MOLT-4 cAMP concentration. 8-MOP caused an approximate 7-fold increase of intracellular cAMP concentration within 15 minutes of incubation. This dramatic increase (from approximately 7 to 50 pmol/mg protein) of cAMP was sustained for at least 2 hours. Control cells did not demonstrate any change of intracellular cAMP concentration throughout the 2 hour experiment. To further investigate this effect, cells were incubated with various concentrations of 8-MOP for 1 hour. As shown in Figure 39, approximately 4 to 140 μM 8-MOP caused a dose-dependent increase of MOLT-4 intracellular cAMP concentration. The highest 8-MOP concentration tested (140 μM) caused an approximate 6-fold increase of cAMP concentration relative to control (from 11 to 68 pmol/mg protein). Figure 39 also demonstrates that ethanol alone did not contribute to the observed increase of intracellular cAMP.

As shown in Figure 40, 140 μM 8-MOP did not cause a time-dependent change of MOLT-4 cGMP concentration. Similarly, concentrations of 9 to 140 μM 8-MOP did not cause a dose-dependent change of MOLT-4 cGMP concentration (Figure 41). This data indicates that 8-MOP alters the cyclic nucleotide metabolism of MOLT-4 cells in vitro by selectively increasing the intracellular concentration of cAMP. The ability of 8-MOP to cause a dramatic and
FIGURE 39. THE DOSE-DEPENDENT EFFECT OF 8-MOP ON THE CYCLIC AMP CONCENTRATION OF MOLT-4 CELLS.

MOLT-4 cells (2 x 10^6) were incubated with various concentrations of 8-MOP (●) or buffer only (○). After 1 hour TCA was added to inhibit further cyclic nucleotide metabolism. The concentration of cAMP and protein was determined as in Figure 38. Each value represents the mean ± sem of two experiments. The value from each experiment is the mean of duplicate samples. Analysis by Dunnett's t-test indicated that cells treated with 8-MOP are statistically different from control cells (**) P<0.01. Analysis by Student's t-test indicated that cells treated with ethanol vehicle only are not statistically different from cells treated without ethanol vehicle.
FIGURE 40. THE EFFECT OF 8-MOP ON THE CYCLIC GMP CONCENTRATION OF MOLT-4 CELLS.

MOLT-4 cells (2 x 10⁶) were incubated with 140 μM 8-MOP (●) or buffer only (○) as described in methods. At the times indicated, TCA was added to inhibit further cyclic nucleotide metabolism. The concentration of cGMP and protein was determined as in Figure 38. Each value represents the mean ± sem of duplicate samples from one experiment.
FIGURE 41. THE EFFECT OF 8-MOP ON THE CYCLIC GMP CONCENTRATION OF MOLT-4 CELLS.

MOLT-4 cells (2 x 10^6) were incubated with various concentrations of 8-MOP (•) or buffer only (○). After 1 hour TCA was added to inhibit further cyclic nucleotide metabolism. The concentration of cGMP and protein was determined as in Figure 38. Each value represents the mean ± sem of duplicate samples from one experiment.
sustained increase of cAMP in MOLT-4 cells, and possibly normal human PBMC, may be one biochemical mechanism by which 8-MOP impairs lymphocyte proliferation and function.

L. THE EFFECT OF 8-MOP ON PHOSPHODIESTERASE (PDE)

One mechanism by which 8-MOP could cause an increase of intracellular cAMP is by inhibiting cAMP phosphodiesterase. The ability of 8-MOP to inhibit the activity of phosphodiesterase has already been proposed as an alternative mechanism of action of 8-MOP [17,131].

Initial experiments were designed to partially characterize the enzyme activity present in the relatively crude MOLT-4 PDE preparation. Figure 42 demonstrates the time- and dose-dependence of the preparation in the PDE assay system. MOLT-4 PDE activity was linearly proportional to time, up to at least 2 hours of incubation (Figure 42, upper panel). Similarly, PDE activity was linearly proportional to the amount of MOLT-4 PDE preparation added, up to at least 34 ug protein/assay tube (Figure 42, lower panel). Figure 42 also demonstrates that there was considerable enzyme activity produced when the MOLT-4 PDE preparation was incubated without cAMP substrate. Since this activity did not increase with time, it may have been due to the presence of ATP (rather than 5'-AMP or cAMP) in the enzyme preparation. Regardless of the nature of this contaminant, the high
FIGURE 42. THE TIME- AND PROTEIN-DEPENDENCE OF MOLT-4 PDE ACTIVITY.

Samples containing 34 ug of MOLT-4 PDE protein and either 100 uM cAMP (•) or 0 uM cAMP (○) were incubated for various times (upper panel). Alternatively, samples containing various amounts of MOLT-4 PDE protein and either 100 uM cAMP (•) or 0 uM cAMP (○) were incubated for 1 hour (lower panel). The enzyme activity of the MOLT-4 PDE preparation was assayed as described in methods. Each value represents the mean ± sem of triplicate samples from one of two representative experiments.
FIGURE 42. THE TIME- AND PROTEIN-DEPENDENCE OF MOLT-4 PDE ACTIVITY.
level of basal PDE activity was always subtracted from the level of activity produced in the presence of known concentrations of substrate.

To further characterize the properties of the MOLT-4 PDE, a constant amount of enzyme preparation was incubated with various concentrations of cAMP substrate. Figure 43 demonstrates that the activity of the MOLT-4 PDE enzyme followed Michaelis-Menten kinetics. The apparent $K_M$ was determined from the Hofstee plot and was found to be approximately 0.8 uM (Figure 43, insert). The apparent $V_{\text{max}}$ was approximately 7 nmol/mg protein/hr.

The ability of 8-MOP to inhibit PDE activity was determined by incubating a constant amount of the PDE preparation with various concentrations of 8-MOP. As shown in Figure 44, 8-MOP concentrations of approximately 9 to 70 uM caused a dose-dependent decrease of MOLT-4 PDE activity using a 0.4 uM concentration of cAMP substrate. Analysis by the method of Dixon [132] indicated that 8-MOP was a competitive inhibitor of MOLT-4 PDE and had an apparent $K_i$ of approximately 30 uM (Figure 45). Table 2 compares the apparent $K_i$ of 8-MOP with that determined for two other known PDE inhibitors, papaverine and isobutylmethylxanthine (IBMX). 8-MOP was more potent than IBMX, but less potent than papaverine, in its ability to inhibit MOLT-4 PDE.
Figure 43. The substrate-dependence of MOLT-4 PDE activity.

Samples containing 34 ug of MOLT-4 PDE protein and various concentrations of cAMP were incubated for 2 hours. PDE activity was assayed as described in methods. Each value represents the mean ± sem of triplicate samples from one of two representative experiments.
FIGURE 44. THE INHIBITORY EFFECT OF 8-MOP ON MOLT-4 PDE ACTIVITY.

Various concentrations of 8-MOP were added to samples containing 34 ug of MOLT-4 PDE protein and 0.4 uM cAMP. PDE activity was assayed as described in methods following a 2 hour incubation. Each value represents the mean ± sem of three experiments. The value from each experiment is the mean of triplicate samples. Analysis by Dunnett's t-test indicated that 8-MOP treated samples are statistically different from control samples (* P<0.05 and ** P<0.01).
FIGURE 45. THE DETERMINATION OF $K_i$ FOR 8-MOP.

Samples containing 34 μg of MOLT-4 PDE protein and either 0.4 μM cAMP (■), 0.8 μM cAMP (▲), 2.5 μM cAMP (●) or 10 μM cAMP (○), were incubated with various concentrations of 8-MOP for 2 hours. PDE activity was assayed as described in methods. Each value represents the mean of two or three experiments. The value from each experiment is the mean of triplicate samples.
### TABLE 2. COMPARISON OF Kᵢ VALUES FOR 8-MOP AND OTHER KNOWN PDE INHIBITORS.

Samples containing 34 ug of MOLT-4 PDE protein and either 0.4 uM, 0.8 uM, 2.5 uM or 10 uM cAMP, were incubated with various concentrations of either 8-MOP, papaverine or IBMX for 2 hours. PDE activity was assayed as described previously. Kᵢ values were determined as in Figure 45.

<table>
<thead>
<tr>
<th>PDE Inhibitor</th>
<th>Apparent Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isobutylmethylxanthine</td>
<td>100 uM</td>
</tr>
<tr>
<td>8-Methoxypsoralen</td>
<td>30 uM</td>
</tr>
<tr>
<td>Papaverine</td>
<td>13 uM</td>
</tr>
</tbody>
</table>
This data indicates that 8-MOP is a relatively potent inhibitor of MOLT-4 PDE. The ability of 8-MOP to inhibit MOLT-4 PDE, and possibly normal lymphocyte PDE, is at least one mechanism by which 8-MOP can increase the concentration of cAMP. Since cAMP is an important regulator of cellular proliferation and function, the ability of 8-MOP to inhibit PDE represents one possible biochemical mechanism by which 8-MOP impairs lymphocyte proliferation and function.

M. THE EFFECT OF PHOSPHODIESTERASE INHIBITORS ON LYMPHOCYTE PROLIFERATION AND FUNCTION

If PDE inhibition is the only biochemical mechanism of action of 8-MOP, then other known PDE inhibitors might also be expected to cause a similar impairment of lymphocyte proliferation. Neither papaverine (0.5 to 20 uM) nor IBMX (2 to 180 uM) were capable of decreasing MOLT-4 spontaneous proliferation. However, elevation of intracellular cAMP concentration with dibutyryl cAMP did cause a dose-dependent decrease of MOLT-4 proliferation (Figure 46). Additionally, papaverine (0.6 to 10 uM), IBMX (10 to 90 uM) and dibutyryl cAMP (6 to 100 uM) all caused dose-dependent decreases of normal human lymphocyte proliferation induced by a suboptimal concentration of PHA (Figure 47). It remains to be determined whether these decreases represent a delay in the proliferative response
FIGURE 46. THE DOSE-DEPENDENT EFFECT OF DIBUTYRYL CYCLIC AMP ON THE SPONTANEOUS PROLIFERATION OF MOLT-4 CELLS.

Various concentrations of dibutyryl cAMP were added to cultures containing 1 x 10^5 MOLT-4 cells. Cells were incubated and harvested as in Figure 37. Each value represents the mean ± sem of triplicate cultures from one experiment. Analysis by Dunnett's t-test indicated that 8-MOP treated cells are statistically different from control cells (** P<0.01).
FIGURE 47. THE DOSE-DEPENDENT EFFECT OF AGENTS WHICH ELEVATE CYCLIC AMP ON PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Various concentrations of papaverine (■), IBMX (●), dibutyryl cAMP (◆) or 8-MOP (♦) were added to cultures containing 1 x 10⁶ PBMC plus 0.05% PHA. Cultures were harvested after a 72-hour incubation and a 16 hour terminal pulse with ³H-Tdr. Proliferation was detected as described in methods. Each value represents the mean ± sem of triplicate cultures from one experiment.
or an inhibition of proliferation.

Papaverine was used as a representative PDE inhibitor in a preliminary examination of the effect of PDE inhibition on IL-2 receptor expression. Replicate cultures were prepared containing PBMC and a suboptimal concentration of PHA in the presence or absence of papaverine. Individual cultures were incubated for various times and the cells were stained and analyzed as before for IL-2 receptor expression. As shown in Figure 48, control cultures developed the maximum expression of Tac\(^+\) cells (approximately 12\% Tac\(^+\)) at 96 hours. The presence of 5 uM papaverine appeared to cause an inhibition of, rather than a delay in, the expression of Tac\(^+\) cells. The mean fluorescence of anti-Tac binding for papaverine treated cultures was lower than that of control cultures during the first 48 to 72 hours of incubation. This was followed by an increase in the mean fluorescence of anti-Tac binding for papaverine treated cultures compared to that of control cultures (Figure 49). Therefore, the response to papaverine was essentially analogous to that seen with 8-MOP, when a suboptimal concentration of PHA was used to stimulate lymphocytes. To determine if IL-2 could reverse these effects, cultures of PBMC were incubated with a suboptimal concentration of PHA and 80 U/ml of IL-2 in the presence and absence of
FIGURE 48. THE EFFECT OF PAPAVERINE ON THE PHA-INDUCED EXPRESSION OF TAC-POSITIVE CELLS.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA received no papaverine (■) or 5 μM papaverine (○). Individual cultures were incubated and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
FIGURE 49. THE EFFECT OF PAPAVERINE ON THE PHA-INDUCED MEAN FLUORESCENCE OF ANTI-TAC BINDING.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA received no papaverine (■) or 5 µM papaverine (●). Individual cultures were incubated and harvested as in Figure 26. Each value represents the mean fluorescence of anti-Tac binding to a single culture from one experiment.
5 uM papaverine. As shown in Figure 50, IL-2 partially restored the magnitude of the expression of Tac\(^+\) cells. However, it did not cause a delay in the expression of Tac\(^+\) cells, as it did in the case of 8-MOP and a suboptimal concentration of PHA. At all incubation times examined, papaverine treated cultures demonstrated a higher mean fluorescence of anti-Tac binding compared to that of control cultures (Figure 51).

In summary, papaverine causes an inhibition of the expression of Tac\(^+\) cells in cultures of lymphocytes stimulated with a suboptimal concentration of PHA. The ability of exogenous IL-2 to overcome this effect is consistent with the ability of IL-2 to overcome the effect of 8-MOP. However, IL-2 did not induce a delay in the expression of Tac\(^+\) cells in the presence of papaverine, as it did in the presence of 8-MOP. Since papaverine (and possibly other PDE inhibitors) appear to mimic only some of the effects of 8-MOP, PDE inhibition may not be the only biochemical mechanism by which 8-MOP impairs lymphocyte proliferation and function in vitro.

N. THE EFFECT OF ADDITIONAL 8-MOP ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY AN OPTIMAL CONCENTRATION OF PHA

One possible explanation for the 8-MOP-mediated delay of lymphocyte proliferation is that 8-MOP was metabolized
FIGURE 50. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE PAPAVERINE-MEDIATED IMPAIRMENT OF PHA-INDUCED EXPRESSION OF TAC-POSITIVE CELLS.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05\% PHA and 80 U/ml of exogenous IL-2 received no papaverine (■) or 5 uM papaverine (•). Individual cultures were incubated and harvested as in Figure 25. Each value represents the specific binding of anti-Tac to a single culture from one experiment.
FIGURE 51. THE EFFECT OF EXOGENOUS IL-2 ADDITION ON THE PAPAVERINE-MEDIATED IMPAIRMENT OF PHA-INDUCED MEAN FLUORESCENCE OF ANTI-TAC BINDING.

Replicate cultures containing $1 \times 10^6$ PBMC plus 0.05% PHA and 80 U/ml of exogenous IL-2 received no papaverine (■) or 5 uM papaverine (●). Individual cultures were incubated and harvested as in Figure 26. Each value represents the mean fluorescence of anti-Tac binding to a single culture from one experiment.
early in the incubation period and was, therefore, not present long enough to completely inhibit lymphocyte proliferation. To indirectly test this, a second dose of 8-MOP was added at 48 hours of incubation to cultures of PBMC which received an optimal concentration of PHA and 140 μM 8-MOP. As shown in Figure 52, additional 8-MOP (3 μg/well) did not appear to cause a prolongation in the delay induced by the first dose of 8-MOP. Examination of the data suggests that if there was an effect caused by the second dose of 8-MOP it probably resulted in less than an additional 24 hours of delayed proliferation. It remains to be determined if a higher second dose of 8-MOP, or addition of the second dose at a different time in the incubation, can cause an additional effect.

0. THE EFFECT OF 8-MOP ON CYCLOSPORINE-MEDIATED INHIBITION OF LYMPHOCYTE PROLIFERATION

In order to determine the effect of 8-MOP on lymphocyte proliferation in the presence of an established immunosuppressive agent, 8-MOP was added to cultures of PBMC containing cyclosporine and either an optimal or a suboptimal concentration of PHA. Cyclosporine (M.W. 1202) concentrations of approximately $2 \times 10^{-9}$ to $1 \times 10^{-6}$ g/ml (approximately 2 nM to 1 μM) caused a dose-dependent decrease of lymphocyte proliferation induced by an optimal concentration of PHA. The $ED_{50}$ for this effect was
FIGURE 52. THE EFFECT OF ADDITIONAL 8-MOP ON THE KINETICS OF LYMPHOCYTE PROLIFERATION INDUCED BY AN OPTIMAL CONCENTRATION OF PHA.

Replicate culture plates containing 1 x 10^5 PBMC plus 1% PHA received no 8-MOP (○) or 140 uM 8-MOP (○). Individual plates were incubated and harvested as in Figure 12 (solid line). Alternatively, replicate culture plates containing 1 x 10^5 PBMC plus 1% PHA received no 8-MOP (○) or 140 uM 8-MOP (○) at the initiation of incubation (0 hour). At 48 hours of incubation, cultures received either 100 ul of PBS containing no 8-MOP (○) or 100 ul of PBS containing 3 ug of 8-MOP (○) to bring the final volume to 300 ul/well (dashed line). Individual plates were incubated and harvested as in Figure 12. Each value represents the mean ± sem of triplicate cultures from one experiment.
Concentrations of 8-MOP up to 50 μM did not affect the ED$_{50}$ value (data not shown). However, when lymphocytes were stimulated with a suboptimal concentration of PHA, cyclosporine concentrations of $2 \times 10^{-10}$ g/ml to $1 \times 10^{-7}$ g/ml (approximately 0.2 nM to 80 nM) caused a dose-dependent decrease of proliferation (Figure 53). The ED$_{50}$ for this effect was approximately $6 \times 10^{-9}$ g/ml (5 nM). 8-MOP concentrations alone (approximately 1.5 to 14 μM) decreased lymphocyte proliferation. The combination of both cyclosporine and 8-MOP resulted in a lower ED$_{50}$ (approximately $2 \times 10^{-9}$ g/ml or 1.7 nM), suggesting that under certain conditions 8-MOP enhances the immunosuppressive effect of cyclosporine.
FIGURE 53. THE EFFECT OF 8-MOP ON CYCLOSPORINE-MEDIATED INHIBITION OF PHA-INDUCED LYMPHOCYTE PROLIFERATION.

Cultures containing $1 \times 10^5$ PBMC and 0.05% PHA received various concentrations of cyclosporine plus no 8-MOP (●), 1.5 µM 8-MOP (■), 4.5 µM 8-MOP (○) or 14 µM 8-MOP (△). Cultures were harvested after a 72 hour incubation and a 16 hour terminal pulse with $^3$H-TdR. Each value represents the mean ± sem of triplicate cultures from one experiment.
A. THE USE OF LECTINS TO STUDY LYMPHOCYTE PROLIFERATION

Assays of lymphocyte transformation typically utilize either lectin mitogens, specific antigens or allogeneic cells as the stimuli for lymphocyte proliferation. These assays provide the simplest, fastest, most reproducible and widely used in vitro correlate of cell mediated immunity [116]. They have been used in both clinical and basic applications, including the evaluation of patient immunocompetence, histocompatibility typing, the elucidation of immunoregulatory mechanisms, and the evaluation of the effects of biological and chemical agents on immune cell function. Lymphocyte proliferation is typically assessed by measuring the incorporation of radiolabeled thymidine into newly synthesized DNA. A linear relationship has been reported between $^3$H-TdR incorporation by Con A-stimulated human peripheral blood lymphocytes and their proliferation assessed by other methods [133]. In general, however, incorporation of $^3$H-TdR is only an estimate of proliferation, since cells synthesizing DNA in the S phase of the cell cycle do not necessarily go on to divide. Furthermore, this method of
assaying lymphocyte proliferation has been criticized on the basis that it does not take into account alterations in the pool size of unlabeled intracellular thymidine or changes in the activities of thymidine kinase and the plasma membrane thymidine carrier [134]. Nevertheless, radiolabeled thymidine incorporation remains the most widely used method for assaying the response to mitogens, not only in lymphocytes but in other types of cultured cells.

The use of lectins as the stimuli for lymphocyte transformation in these experiments was based primarily on their ability to stimulate lymphocytes irrespective of their antigen specificity. Although cultured B- and T-cells can be induced to proliferate in vitro by specific antigens and allogeneic cells [135], studies are often complicated by the fact that only a small percentage of cells actually respond to these stimuli [136,137]. In contrast, lectins are both polyfunctional and polyclonal mitogens and, therefore, stimulate proliferation in a large percentage (as much as 60-90%) of cells in an appropriate lymphocyte population [63,116,138]. Additionally, the response to lectins (and allogeneic cells) does not require previous sensitization of lymphocytes, as does the response to specific antigens.
Classical T-cell mitogens may activate both T- and B-cells in a heterogeneous population of cells [139]. Since PHA and Con A were used in these experiments to stimulate PBMC, it is possible that the suppressive effect of 8-MOP may not be limited to T-cell proliferation and function.

Both optimal and suboptimal lectin concentrations were used to stimulate lymphocyte proliferation. The importance of choosing a suboptimal concentration of mitogen in studies of lymphocyte responsiveness has been documented. For example, in a number of immunodeficiency states and malignancies, the proliferative response of lymphocytes to optimal concentrations of mitogen is normal, whereas impaired reactivity is detectable only when a suboptimal concentration of mitogen is used [140-143].

Results from initial time- and dose-response experiments with both lectins and alloantigens were in agreement with those previously reported. Maximum lectin responsiveness occurred between 3 to 4 days of incubation and maximum alloantigen responsiveness occurred between 5 to 6 days of incubation [116]. Both Con A and PHA demonstrated typical biphasic dose-response curves [144]. The ability of supraoptimal concentrations of lectin to cause a reduction in lymphocyte proliferation has been
explained as cytotoxicity resulting from excessive cross-linking of cell surface sites [145] and as induction of suppressor T-cell activity [146].

B. THE EFFECTS OF 8-MOP ON LYMPHOCYTE PROLIFERATION

8-MOP suppresses mitogen-induced proliferation

As pointed out in the introduction, 8-MOP plus UV irradiation (PUVA) clearly alters a number of immune cell functions. However, there is also evidence to suggest that there are effects of 8-MOP which are independent of UV-induced DNA damage. The experiments reported here were initiated to characterize more extensively the effects of 8-MOP alone in vitro. The results demonstrate that 8-MOP can impair mitogen-induced lymphocyte proliferation in vitro without UV irradiation. The fact that 8-MOP was capable of decreasing lymphocyte proliferation in the presence of very high concentrations of PHA suggests that 8-MOP was not merely a competitive inhibitor of lectin binding and activation at the cell surface. The suppressive effect of 8-MOP was more pronounced when a suboptimal concentration of Con A or PHA was used to stimulate lymphocyte proliferation. In fact, an increased sensitivity of lymphocytes to immunosuppressants at suboptimal lectin concentrations is not uncommon [147]. Interestingly, 8-MOP appeared to be more potent at a
suboptimal concentration of PHA and less potent at a suboptimal concentration of Con A in comparison to its effects at an optimal concentration of either lectin. This may be due to an effect of 8-MOP on subpopulations of lymphocytes which are differentially sensitive to Con A or PHA [146]. 8-MOP also caused a dose-dependent decrease in alloantigen-stimulated lymphocyte proliferation. The dose-response pattern under these conditions resembled that obtained with a suboptimal concentration of PHA. Therefore, 8-MOP suppressed the proliferation of antigen-restricted populations of lymphocytes, in addition to suppressing antigen nonspecific lymphocyte proliferation.

The 8-MOP-mediated suppression of PHA-induced proliferation was not due to cell death, since the cells were capable of excluding trypan blue after prolonged exposure to 8-MOP. Furthermore, the suppression detected as decreased $^3$H-TdR uptake correlated with a decrease in the total number of cells recovered from mitogen-stimulated cultures of PBMC. This observation strengthens the conclusion that 8-MOP decreased lymphocyte proliferation and supports the use of $^3$H-TdR uptake as a measure of proliferation, as well as DNA synthesis, throughout these experiments.
Suppression of lymphocyte proliferation in response to PHA required a relatively brief exposure (16 hour terminal pulse) to 8-MOP, but was most efficient after prolonged exposure, or when 8-MOP was added within the first 24 hours following PHA addition. The fact that inhibition occurred well after the time required for the lectin to initiate activation argues against 8-MOP interfering with the binding of lectin to the cell surface. Furthermore, this data suggests that both early and late events in lymphocyte activation are potential targets for 8-MOP.

Clinical relevance of the concentrations of 8-MOP used

It is difficult to relate the significance of the reported phototoxic 8-MOP concentrations observed in vivo to the non-photoactivated 8-MOP concentrations which suppress lymphocyte proliferation in vitro. However, for reference purposes, it is worth noting that several of the concentrations of 8-MOP which were used in these experiments are higher than those generally reported in vivo. To put this in perspective, the plasma or serum levels of 8-MOP in treated individuals can be quite variable. For example, human serum levels between 0.03 and 2 ug/ml (approximately 0.14 to 9.3 uM) have been reported two hours after 8-MOP ingestion [148]. Although the greatest suppression of lymphocyte proliferation
reported in these experiments occurred at a higher 8-MOP concentration than might be expected in the serum or plasma of patients given 8-MOP orally, there was suppression at concentrations of 8-MOP which have been reported in patients. Approximately 0.4 ug/ml (1.8 uM) of 8-MOP caused a 30-40% decrease in proliferation induced by both a suboptimal concentration of PHA and alloantigen. Furthermore, it is not clear that plasma levels of 8-MOP are a reliable indicator of the exposure of circulating lymphocytes to 8-MOP. For example, plasma concentrations may not accurately reflect the level of 8-MOP found in specific tissues or bound to specific binding sites on cells. In fact, the existence of specific binding sites for 8-MOP on several mammalian cells has been demonstrated [18]. Nevertheless, the clinical relevance of these findings for patients receiving PUVA therapy remains uncertain, since most of the 8-MOP concentrations tested here are much higher than the 0.2 ug/ml (0.9 uM) blood level most often reported.

**Evidence that the effect of 8-MOP is not due to an interaction with UV radiation**

Although there was no detectable UVA or UVC radiation in the laboratory environment, experiments were performed to entirely eliminate the possibility that undetected UV light contributed to the effects of 8-MOP. Accordingly,
cultures were incubated in total darkness and exposed to a red photographic safety light only during the addition of 8-MOP and \(^{3}\)H-TdR. 8-MOP caused a dose-dependent suppression of lymphocyte proliferation even under these rigidly controlled conditions.

Experiments with 5-MIP and 3-CEP, which form only monoadducts with DNA following UV irradiation, further suggested that UV-induced DNA damage was not responsible for the effects observed with 8-MOP. The limited solubility of these compounds enabled only a narrow range of concentrations to be tested. However, low concentrations of 5-MIP and 3-CEP did cause a suppression of PHA-induced proliferation in the absence of UV irradiation which was essentially identical to that seen with 8-MOP. Similarly, 5-MOP which is a C-5 substituted analogue of 8-MOP, also suppressed lymphocyte proliferation. It has been reported that there is no noncovalent binding of 3-CEP to DNA in the absence of UV light [149] and that covalent binding of 3-CEP following exposure to UV light occurs at a rate which is ten times slower than 5-MIP [149] and 5 times slower than 8-MOP [150]. Additionally, the maximum amount of covalent binding of 3-CEP to DNA is at least 30 times less than that of 8-MOP, 5-MIP and 5-MOP. The binding of 3-CEP and 5-MIP is also reversed to a greater extent than that of
5-MOP or 8-MOP following prolonged UV irradiation [149]. Therefore, any effect observed with these psoralens, especially without intentional UV irradiation, is unlikely to be the result of UV-induced DNA damage.

C. IMMUNOLOGICAL MECHANISMS OF ACTION OF 8-MOP

Differential effects of 8-MOP on proliferation induced by optimal versus suboptimal concentrations of PHA

Initial experiments determined whether the 8-MOP-mediated suppression of PHA-induced proliferation (88 hour incubation) was the result of a complete inhibition of lymphocyte responsiveness, or the result of a delay in lymphocyte responsiveness. In fact, 8-MOP had both effects on PHA-induced lymphocyte proliferation. 8-MOP caused a delay in lymphocyte proliferation induced by an optimal concentration of PHA and a complete inhibition of proliferation induced by a suboptimal concentration of PHA. This dual effect of 8-MOP may be the result of a differential expression of lymphocyte subpopulations induced by high and low concentrations of PHA, some of which are more sensitive to 8-MOP exposure than others. Alternatively, this effect may be due to a difference in the level of stimulation provided to lymphocytes by various concentrations of lectin. It is noteworthy that
Mills et al. [151] have characterized the requirements for T-cell mitogenesis by using various concentrations of PHA. They concluded that low concentrations of PHA do not stimulate the level of IL-2 production required for optimal lymphocyte responsiveness. This was apparently due to a relative failure of suboptimal concentrations of PHA to increase the intracellular concentration of free calcium. Roosnek et al. [152] also suggested that the level of IL-2 production is dependent on lectin concentration. However, they proposed that the concentration of lectin determines the accessory cell dependency of IL-2 production.

**Effect of 8-MOP on IL-2 accumulation**

Since lectin-induced lymphocyte proliferation is dependent upon IL-2 [66,67], experiments were performed to examine the effect of 8-MOP on IL-2 accumulation. 8-MOP caused a dose-dependent reduction in the accumulation of IL-2 in 24-hour culture supernatants from cells stimulated with an optimal concentration of PHA. Since IL-2 production occurs throughout the first 48 hours of incubation with lectin [153], 48-hour culture supernatants were tested for IL-2 activity. 8-MOP did not cause a reduction in the accumulation of IL-2 in 48-hour culture supernatants from cells stimulated with an optimal concentration of PHA. The suppressive effect of 8-MOP on
IL-2 accumulation in 24-hour supernatants was not due to the presence of a transient inhibitor of the IL-2 bioassay, since these supernatants did not interfere with the proliferation of CTLL-20 cells supplemented with exogenous IL-2. Therefore, 8-MOP caused a delay in the accumulation of IL-2 in cultures of cells stimulated with an optimal concentration of PHA. In contrast, 8-MOP caused a dose-dependent reduction of IL-2 accumulation in both 24- and 48-hour culture supernatants from cells stimulated with a suboptimal concentration of PHA. Therefore, 8-MOP caused an inhibition, rather than a delay, of IL-2 accumulation in cultures of cells stimulated with a suboptimal concentration of PHA.

These experiments clearly suggest that 8-MOP has an effect on IL-2 production or secretion. However, no definite distinction can be made between impaired accumulation and impaired production, since no attempt was made to determine the effect of 8-MOP on IL-2 gene transcription or translation.

Evidence for an effect of 8-MOP in addition to its effect on IL-2 accumulation

In general, the pattern of impairment of IL-2 production or secretion appeared to explain the effects of 8-MOP on PHA-induced lymphocyte proliferation. However, exogenous IL-2 did not overcome the delayed proliferative
response of cells cultured with 8-MOP and an optimal concentration of PHA. As observed previously [151], exogenous IL-2 did not increase the proliferative response of cells stimulated with an optimal concentration of PHA (in the absence of 8-MOP), and may have possibly impaired their optimal response. In contrast, the presence of exogenous IL-2 restored the magnitude of the proliferative response of cells cultured with 8-MOP and a suboptimal concentration of PHA, and therefore, appeared to overcome the suppressive effect of 8-MOP. However, the observed proliferation was not typical of control cultures, but displayed the delayed proliferative response characteristic of cells cultured with 8-MOP and an optimal concentration of PHA. Therefore, addition of exogenous IL-2 did not completely overcome the effect of 8-MOP on PHA-induced lymphocyte proliferation. It remains possible that higher concentrations of IL-2 might overcome the suppressive effects of 8-MOP. However, IL-2 alone at concentrations greater than those used (80 U/ml) had an inhibitory effect on PHA-stimulated proliferation using the assay conditions described above (data not shown). The ability of exogenous IL-2 to reverse dexamethazone-mediated inhibition of PHA-induced lymphocyte proliferation has been demonstrated [154]. In fact, this observation lead to the conclusion that the
glucocorticoids mediate their immunosuppressive effects on T-cells by impairing IL-2 production. In summary, this data suggests that although 8-MOP is capable of interfering with IL-2 production or secretion, this may not be the only mechanism by which 8-MOP impairs lectin-induced lymphocyte proliferation.

8-MOP also caused a dose-dependent suppression of IL-2-induced lymphocyte proliferation. The ability of "resting" peripheral blood T-cells to proliferate in response to IL-2 in the absence of other stimuli has been reported [91,155,156]. The mechanism for this effect appears to be related to the possibility that some cells in a resting population already bear IL-2 receptors. These cells may be T-cells that are activated in the course of a current immune response, or memory T cells that are present in every normal individual [156]. It remains to be determined whether the 8-MOP impairment of IL-2-induced proliferation represents a delay in proliferation or an inhibition of proliferation.

Evidence for an effect of 8-MOP on IL-2 receptors

Since an impairment of IL-2 production did not appear to be the only mechanism of action of 8-MOP, experiments were performed to examine 8-MOP's effect on IL-2 receptor expression. 8-MOP caused a delay in the expression of Tac+ cells in cultures which were stimulated with an
optimal concentration of PHA. This effect correlated with a delay of lymphocyte proliferation in a parallel experiment, although the delay in IL-2 receptor expression preceded the delay in proliferation as expected [157] (data not shown). The mean fluorescence of anti-Tac binding (an indication of relative receptor density) for 8-MOP-treated cultures was generally higher than that of control cultures throughout the incubation times examined. Since exogenous IL-2 did not influence the effect of 8-MOP on proliferation induced by an optimal concentration of PHA, it was reasonable to assume that exogenous IL-2 would not influence the effect of 8-MOP on IL-2 receptor expression under these conditions. Indeed, 8-MOP caused a delay in the expression of Tac+ cells regardless of the presence of exogenous IL-2, although this delay occurred later than before.

In contrast, 8-MOP caused a dose-dependent decrease of the expression of Tac+ cells in cultures which were incubated for 96 hours with a suboptimal concentration of PHA. 8-MOP also caused a sustained inhibition, rather than a delay, of the expression of Tac+ cells throughout all the incubation times examined. This effect correlated with a sustained inhibition of lymphocyte proliferation in a parallel experiment. The mean fluorescence of anti-Tac binding for 8-MOP-treated cultures was lower than that of
control cultures until the latest incubation times examined, where there was an equivocal increase in the fluorescence for 8-MOP-treated cultures over that of control cultures. Since exogenous IL-2 allowed lymphocytes to proliferate in the presence of 8-MOP and a suboptimal concentration of PHA, it was reasonable to assume that exogenous IL-2 would allow lymphocytes to express IL-2 receptors under these conditions. Indeed, exogenous IL-2 restored the magnitude of the expression of Tac+ cells in cultures incubated with 8-MOP. However, it also caused a delay in the expression of Tac+ cells, which correlated with its effects on IL-2 receptor expression and lymphocyte proliferation induced by an optimal concentration of PHA. The mean fluorescence of anti-Tac binding for 8-MOP-treated cultures was also increased by exogenous IL-2 and was actually higher than that of control cultures throughout the incubation times examined. The ability of exogenous IL-2 to upregulate its own receptors has been demonstrated [125-128]. In addition, exogenous IL-2 has been shown to overcome the reduced IL-2 receptor expression mediated by anti-Tac [127] and dexamethazone [127,158].

It is interesting that 8-MOP consistently impaired the expression of Tac+ cells, while having variable effects on the relative density of IL-2 receptors. A
possible explanation for this effect is that there are both 8-MOP-sensitive (more abundant) and 8-MOP-insensitive (less abundant) subpopulations of T-cells. Accordingly, the effect of 8-MOP on receptor density would reflect the receptor expression on 8-MOP-insensitive cells, while the effect of 8-MOP on the overall percentage of Tac\(^+\) cells would reflect the receptor expression on 8-MOP-sensitive cells. Interpretation of the data from these experiments, however, must consider an effect of 8-MOP on the expression of low affinity IL-2 receptors. This is particularly important since the anti-Tac monoclonal antibody reacts with both classes of IL-2 receptors [92], and only the less abundant, high affinity receptors are thought to directly participate in the physiological response to IL-2 [91,92]. A more definitive explanation for the effects of 8-MOP on lymphocyte proliferation and IL-2 receptor expression will require analysis of T-cell subpopulations and radiolabeled IL-2 binding experiments.

Based on the cumulative data presented here, it is hypothesized that 8-MOP has at least two immunological mechanisms of suppressive activity for lectin-induced lymphocyte proliferation. One mechanism involves a delay in IL-2 receptor expression. This mechanism is independent of IL-2, since it can be observed when either endogenous or exogenous IL-2 is present. Another
mechanism involves an inhibition of IL-2 accumulation, which occurs only when the IL-2-inducing stimulus (lectin) is suboptimal. Addition of exogenous IL-2 under these conditions restores the magnitude of IL-2 receptor expression and lymphocyte proliferation. However, there remains a delay in IL-2 receptor expression.

D. ESTABLISHMENT OF A MODEL SYSTEM FOR STUDIES ON THE BIOCHEMICAL MECHANISM OF ACTION OF 8-MOP

T cell activation and proliferation results from the interaction of several distinct types of cells. It is likely that lectins may have some effect on each of these to directly or indirectly influence the synthesis and secretion of lymphokines and/or the expression of lymphokine receptors. Therefore, an investigation of the biochemical mechanisms involved in lectin-mediated T cell immunoregulation can be an arduous undertaking. Since PBMC consist of various cell types, it is possible that only a small fraction of a lymphocyte population may participate in the biochemical events under investigation. Alternatively, each cell type may participate in the biochemical event at a certain time in culture, and may, therefore, enhance or diminish the overall measured response. To avoid these potential complications, experiments were performed to establish the use of a
lymphoid cell line as a model system for determining the biochemical mechanism of action of 8-MOP.

The choice of a cell model system to study the biochemical effects of 8-MOP

The JURKAT human leukemic T cell line was the first model system tested. The unstimulated JURKAT cell does not express the Tac (IL-2 receptor) antigen and therefore phenotypically resembles a normal resting lymphocyte [130]. Furthermore, the clone of JURKAT cells used in these experiments (obtained from the JURKAT-FHCRC cell line) does not constitutively produce IL-2 when unstimulated [113], although certain JURKAT variants do produce IL-2 constitutively [159]. As in the case of normal T lymphocytes, however, JURKAT cells can be stimulated with the mitogens Con A, PHA and/or phorbol myristate acetate (PMA) to produce IL-2 [113]. Additionally, PMA has been shown to cause a moderate expression of the Tac activation antigen [130].

8-MOP caused a 20-30% reduction of IL-2 accumulation in culture supernatants from PHA-stimulated JURKAT cells. Further studies were not conducted with the JURKAT cells based on their relative insensitivity to 8-MOP. In retrospect, further attempts should have been made to optimize the effect of 8-MOP on these cells. The ability of this homogeneous cell line to be stimulated in an
analogous fashion to normal T lymphocytes has provided a unique system for studying the signal requirements for IL-2 production and lymphokine gene expression [130,160]. It is noteworthy that the immunosuppressant drug cyclosporine inhibits the secretion of IL-2 activity from JURKAT cells when added as long as two hours after activation with PHA and PMA [160].

The alternative cell line which was tested as a model system for biochemical studies was the MOLT-4 T lymphoblastoid cell line. MOLT-4 cells do not produce IL-2 [159] and do not require IL-2 for growth. Additionally, these cells do not express IL-2 receptors and are not reactive with the anti-Tac monoclonal antibody [84]. 8-MOP caused a moderate reduction in the spontaneous proliferation of these cells, which was not the result of reduced cell viability. The ability of 8-MOP to decrease MOLT-4 proliferation established the MOLT-4 cell line as a minimal model system for the study of the biochemical mechanisms by which 8-MOP impairs human lymphocyte proliferation and function.

E. BIOCHEMICAL MECHANISMS OF ACTION OF 8-MOP

Effect of 8-MOP on MOLT-4 intracellular cAMP

Previous work from this laboratory indicated that psoralens in the absence of UV radiation increase the cAMP
content of human fibroblasts, epithelial cells, and PBMC in vitro [17]. Based on this observation and evidence that cAMP can modulate several immune functions, including mitogen-induced lymphocyte proliferation, neutrophil chemotaxis, monocyte bactericidal activity, antibody formation, and tumor cell cytotoxicity [96,105-108], preliminary studies examined the effect of 8-MOP on the cyclic nucleotide concentration of MOLT-4 lymphoblasts. MOLT-4 cells have been used previously in several studies involving cyclic nucleotide metabolism and it is reported that these cells possess both an intact adenylate cyclase complex and a cAMP-dependent protein kinase [161]. 8-MOP caused a dramatic dose-dependent increase of intracellular cAMP in these cells, which was sustained for at least two hours. In contrast, 8-MOP did not have a significant effect on the intracellular cGMP concentration of these cells. If 8-MOP has a similar effect on cyclic nucleotide metabolism in lectin-stimulated normal human lymphocytes then this may be at least one possible biochemical basis for the 8-MOP-mediated suppression of lymphocyte proliferation and function. It is particularly noteworthy that Beckner and Farrar [58] reported opposing effects of IL-2 and PGE$_2$ on the proliferation of an IL-2-dependent cell line. PGE$_2$ which increased cAMP production in these cells caused a decrease in proliferation, while IL-2
which caused a decrease in cAMP production increased proliferation. In addition, IL-2 decreased both basal and PGE₂-stimulated adenylate cyclase activity. Furthermore, Novogrodsky et al. [94] reported that cAMP analogues, PGE₁ and IBMX decreased mouse thymocyte proliferation and IL-2 production.

Effect of 8-MOP on MOLT-4 PDE activity

An early report on psoralens suggested that these agents may be inhibitors of phosphodiesterase (PDE) [131]. Furthermore, previous work from this laboratory demonstrated that antagonists of adenylate cyclase-coupled receptors could not inhibit the ability of 8-MOP to increase the cAMP concentration of fibroblasts [17]. These reports, together with an examination of the time-dependence of the 8-MOP elevation of cAMP in MOLT-4 cells, suggested that 8-MOP was an inhibitor of lymphocyte cAMP PDE. To determine if 8-MOP increased the cAMP concentration of MOLT-4 cells by this mechanism, a crude PDE enzyme was obtained from the 100,000 x g supernatant fraction of sonicated MOLT-4 cells. The PDE enzyme obtained had an apparent \( K_M \) of approximately 0.8 \( \mu M \). This value compares reasonably well to reported \( K_M \) values of 1.1 to 2.5 \( \mu M \) for the high affinity form of soluble PDE obtained from purified human lymphocytes disrupted by sonication [162] and 0.6 to 1.6 \( \mu M \) for the
two high affinity forms of soluble PDE obtained from acute myeloid leukemic blast cells disrupted by sonication [163]. There appears to be much controversy in the literature, however, with regard to the nature of lymphocyte PDE. Discrepancies have been attributed to the method of cell disruption and the extent of cell homogeneity and enzyme purity. Some investigators reported that there is both a high and low affinity form of PDE [162], while others reported that both human lymphoblastoid cell line and normal peripheral blood lymphocyte PDE's have only the high affinity form [164]. Epstein et al. [165] reported that the cAMP PDE's in human lymphoblastoid cell lines are kinetically and physically similar to those in normal human peripheral blood lymphocytes and suggested that these cell lines may prove to be a useful model for the examination of mitogenic effects on cAMP metabolism in isolated human lymphoid cells. It is particularly relevant that mitogens such as PHA and Con A increased PDE activity in normal resting PBMC prior to blastogenesis [166,167] and human PBMC stimulated with PHA showed an increase in cAMP PDE activity which correlated with $^{3}\text{H}-\text{TdR}$ incorporation [165].

8-MOP was found to be a relatively potent inhibitor of MOLT-4 PDE. That this inhibition is independent of an
8-MOP-UV interaction is supported by the finding that 8-MOP appears to have little capacity to photoinactivate enzymes [168]. The apparent $K_i$ of 30 uM for 8-MOP was greater than that observed for papaverine, and less than that observed for IBMX. It is clear, therefore, that 8-MOP can elevate intracellular cAMP via an inhibition of PDE. Although no attempt was made to determine the calmodulin-dependence of MOLT-4 PDE activity, it is interesting to speculate that 8-MOP may be a calmodulin antagonist. In fact, it was recently reported that 8-MOP (20 ug/ml) caused an approximate 20% inhibition of calmodulin-activated heart PDE activity [169]. If this is also true for lymphocyte calmodulin, then 8-MOP's effect on lectin-induced normal lymphocyte proliferation could be related to many of the growth-regulatory properties of calmodulin [170]. In particular, 8-MOP may interfere with the role of calcium in the initiation of lymphocyte activation and proliferation. For example, the known calmodulin antagonists, trifluoperazine and chlorpromazine, were shown to inhibit lymphocyte mitogenesis via an effect on calcium [171]. Interestingly, however, the maximum inhibition observed was only 50%, which correlates with the level of inhibition observed when 8-MOP was added to cultures of PBMC incubated for 3-4 days with an optimal concentration
of lectin. Furthermore, chlorpromazine sulfoxide, which does not antagonize the calmodulin-dependent activation of cAMP PDE [172], did not interfere with lymphocyte mitogenesis.

Although papaverine and IBMX did not interfere with the spontaneous proliferation of MOLT-4 cells, dibutyryl cAMP did cause a dose-dependent decrease of MOLT-4 proliferation. This suggests that the lack of an effect by papaverine and IBMX was due to their inability to sufficiently elevate cAMP in the MOLT-4 cells. However, no attempt was made to verify this. Furthermore, data from experiments with dibutyryl cAMP must be interpreted with caution since the degradation products of cAMP and dibutyryl cAMP (for example, adenosine and butyrate) can exert potent effects on cell growth [173-176]. All three cAMP elevating agents did cause dose-dependent suppression of normal human lymphocyte proliferation induced by a suboptimal concentration of PHA.

The effect of papaverine on suboptimal PHA-induced IL-2 receptor expression was essentially analogous to that seen with 8-MOP, further suggesting the role of PDE inhibition and cAMP elevation in the 8-MOP-mediated impairment of lymphocyte proliferation and function. Addition of exogenous IL-2 to papaverine-treated cultures also resulted in the restored expression of Tac+ cells
and mean fluorescence that it did for 8-MOP-treated cultures. However, there was no apparent delay induced in the expression of Tac⁺ cells, as in the case with 8-MOP. This may implicate yet another mechanism for the effect of 8-MOP on lymphocyte function. In summary, an increase in intracellular cAMP mediated via an inhibition of PDE may be at least one biochemical mechanism by which 8-MOP impairs lymphocyte proliferation and function in vitro. It would be interesting to determine whether 8-MOP can also elevate cAMP via stimulation of adenylate cyclase.

F. SUPPLEMENTAL STUDIES WITH 8-MOP

An experiment was performed to determine if the delay in lymphokine production, receptor expression, and ultimately lymphocyte proliferation was the result of an early elimination or metabolism of 8-MOP in culture. Preliminary attempts to replenish the 8-MOP concentration at a later time of incubation did not appear to result in an additional delay of proliferation. However, it is not clear whether the supplemental concentration of 8-MOP was adequate to induce a greater suppressive effect or an additional delay. Also, the readdition of 8-MOP may not have been timed appropriately. A preliminary experiment performed in collaboration with Dr. Dennis Mays of The Ohio State University, suggested that PHA-stimulated
normal human PBMC do not significantly metabolize
$^3$H-8-MOP after 24 hours of incubation in vitro (data
not shown).

Experiments were designed to determine the in
vitro immunosuppressive effects of 8-MOP in combination
with a known immunosuppressant. 8-MOP had at least an
additive effect with cyclosporine in suppressing the
PHA-induced proliferation of normal human lymphocytes.
The observation that 8-MOP can inactivate cytochrome P-450
[20] suggests that the combined effects of 8-MOP and
cyclosporine in vivo may be significantly greater than
that observed here, since cyclosporine is metabolized in
the liver by P-450 microsomal enzymes [177]. This
suggests that the relatively non-toxic drug, 8-MOP, may be
used in combination with cyclosporine to induce clinical
immunosupression. The potential benefit of this combined
therapy would be that the desired level of
immunosuppression could be maintained while reducing the
toxicity associated with the doses of cyclosporine
currently used.

6. CONCLUSIONS

The data presented in this dissertation indicates
that 8-MOP impairs the lectin-induced proliferation of
normal human lymphocytes in the absence of UV irradiation.
This impairment is characterized as either a delay in
proliferation (optimal lectin stimulation) or a sustained inhibition of proliferation (suboptimal lectin stimulation). One immunological mechanism for these effects is a delay of IL-2 receptor expression (both optimal and suboptimal lectin stimulation), which is independent of endogenous or exogenous IL-2. Another mechanism for these effects is an inhibition of IL-2 production (supoptimal lectin stimulation only). The 8-MOP-mediated impairment may be specific for a particular subpopulation of lymphocytes. At least one biochemical mechanism for these effects may be an increase of the intracellular cAMP concentration of lymphocytes, which is mediated, at least in part, by an inhibition of PDE.

H. FUTURE EXPERIMENTS

The following areas of investigation should further characterize the effects of 8-MOP on immune cell function in the absence of UV irradiation:

1) structure-activity studies with psoralen analogues
2) elucidation of 8-MOP binding sites on lymphocyte membrane
3) in vivo studies of cell-mediated immunity, for example, delayed-type hypersensitivity and allograft survival
4) studies on IL-2 and IL-2 receptor gene transcription and protein translation
5) radiolabeled IL-2 binding assays to distinguish effects on high- versus low-affinity IL-2 receptors
6) studies of specific subpopulations of lymphocytes
7) studies with calmodulin, PDE and/or calcium
8) studies with adenylate cyclase

I. SIGNIFICANCE

The ability of 8-MOP, in combination with UV irradiation (PUVA therapy), to impair DNA replication has been postulated as the primary mechanism of action of 8-MOP in the treatment of psoriasis. This mechanism has also been proposed to explain the various effects of 8-MOP on immune cell function. However, there is accumulating evidence that 8-MOP has functional and biochemical effects on cells which are independent of UV irradiation. This document represents the first comprehensive study of 8-MOP's effect on normal human lymphocyte proliferation in the absence of UV irradiation. The results presented here demonstrate that 8-MOP alone impairs lectin-, alloantigen-, and IL-2-induced lymphocyte proliferation in vitro. New immunological and biochemical mechanisms have been elucidated to explain this impairment. 8-MOP specifically impairs the generation of
IL-2 and its receptors. It also alters the cyclic nucleotide metabolism of human lymphoblastoid cells, and presumably that of normal human lymphocytes.

It is difficult to determine whether 8-MOP alone has similar effects in vivo or during the course of PUVA therapy. Therefore, no claim can be made as to the clinical significance of these results for the treatment of psoriasis. However, the immunological effects of 8-MOP that have been observed may have importance in light of reports that psoriasis may arise as a result of immunological abnormalities [109]. In particular, it has recently been suggested that psoriasis is a disease of abnormal keratinocyte proliferation which is induced by T lymphocytes [110]. Supporting this hypothesis are reports that cyclosporine, which does not directly affect epidermal cell kinetics [178], is effective in treating psoriasis [179,180].

8-MOP's ability to inhibit PDE and elevate the concentration of cAMP may also be relevant considering that an alteration in cyclic nucleotide metabolism has been reported in psoriasis [181]. Additionally, elevated calmodulin levels have been reported in psoriasis [182,183], and phosphodiesterase inhibitors and calmodulin antagonists have been shown to improve psoriatic lesions [169,184-185].
Evidence for new effects and mechanisms of action of 8-MOP are significant in light of recent reports which suggest that 8-MOP's clinical utility may no longer be restricted to the field of dermatology. For example, Edelson et al. [35] have successfully used 8-MOP in a novel new therapy for treating systemically disseminated cutaneous T cell lymphoma, and Oesterwitz et al. [186,187] have successfully used PUVA in combination therapy with azathioprine and prednisolone to maintain rat renal allograft survival.

Pharmacologic agents and techniques have aided in the study of lymphocyte activation and proliferation. The development of new pharmacologic approaches which could be used to manipulate discrete events of both early and late activation may offer further insight into the mechanisms of lymphocyte immunoregulation. The experiments presented in this document indicate that 8-MOP has effects on IL-2 production, IL-2 receptor expression, and lymphocyte proliferation that are determined by lectin concentration. The ability of 8-MOP and lectin to differentially modulate these systems, together with 8-MOP's diverse molecular and biochemical effects, suggests that this compound might be a useful and unique tool for dissecting the different stages of lectin-induced lymphocyte activation.


