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# Modification of Concanavalin A Mitogenesis in Normal and X-Irradiated Murine Splenic Lymphocytes by Tocopherol

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ABSTRACT

Modification of Concanavalin A Mitogenesis in Normal and X-Irradiated Murine Splenic Lymphocytes by Tocopherol

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Mitogenic responses produced by concanavalin A (con A) in C57Bl/6 murine splenic lymphocytes were monitored by tritiated-thymidine uptake. The optimal concentration of con A for cells cultured with 10% fetal calf serum was established at 2 µg/ml. This response was shown to be modifiable by tocopherol in vitro. Specifically, mitogenic responses stimulated by suboptimal (0.5 µg/ml) and optimal levels of con A were significantly enhanced by physiological concentrations (5 µg/ml) of tocopherol. The stimulatory effects of tocopherol on con A mitogenesis were also observed in cultures depleted of adherent accessory cells (macrophages). In contrast, at pharmacological doses (100 µg/ml), tocopherol was inhibitory and seriously curtailed responses to optimal and supraoptimal (5 µg/ml) levels of con A. The stimulation of con A mitogenesis by tocopherol was not mediated by an earlier onset of cell division in lymphocytes. Additional studies are needed

to determine the mechanism of action through which tocopherol exerts its immunomodulatory effects.

Mitogenic responses to optimal con A in C57Bl/6 murine spleen cells decreased in a dose-dependent manner following exposure to 1 - 4 Gy of X-radiation. The biphasic dose-response profiles observed for interphase death and cell proliferation indicated that these cells were heterogeneous with respect to radiosensitivity. The addition of tocopherol (5 µg/ml) to cultures of splenic lymphocytes immediately post-irradiation was radioprotective and partially restored responses to con A in cells exposed to 2 and 4 Gy. Similarly, cell viability, assessed by trypan blue dye-exclusion, was also significantly improved by post-irradiation administration of tocopherol.

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# INTRODUCTION

In recent years, there have been numerous reports and reviews regarding the modification of vertebrate immune responses by vitamin E (Tengerdy, 1980; Panush and Delafuente, 1985; Bendich, 1988).

An important nutritional factor, vitamin E actually comprises eight naturally occurring compounds representing two classes of tocopherols; namely tocols and tocotrienols. Each class contains four members differing in the number and position of methyl groups on the benzene ring of the parent molecule. The tocotrienols are characterized by the presence of an unsaturated side chain whereas the side chain of the tocol family is fully saturated (Kasparek, 1980). D-α-tocopherol (5,7,8-trimethyl tocol) has been reported to be the most potent naturally occurring form of vitamin E. Its synthetic counterpart, DL-α-tocopherol, is slightly less active. These conclusions regarding the relative biological activity of natural and synthetic tocopherols are based on results obtained from the resorption-gestation assay and the erythrocyte hemolysis test (Desai, 1980). The terms tocopherol and DL-α-tocopherol will be used synonymously henceforth in this thesis.

There are several reports from the field of nutritional immunology demonstrating that vitamin E is required for the expression of normal immune responses in certain animal models.

Accordingly, cell-mediated and humoral immune functions, both in vivo and in vitro, have been reported to be significantly depressed as a result of nutritional or experimentally-induced vitamin E deficiency (Marsh et al., 1981; Gebremichael et al., 1984; Saxena et al., 1984; Meeker et al., 1985). A concomitant need for the trace element selenium has also been demonstrated for optimal immune response in certain cases (Peplowski et al., 1980; Meeker et al., 1985). In fact, it appears that the immune system is particularly sensitive to low vitamin E plasma levels. Immunological dysfunction is manifested well before the classical symptoms associated with frank vitamin E deficiency become apparent (Bendich et al., 1986). However, in contrast to results observed in animal models, it is interesting to note that impaired immune function has not yet been described in humans during vitamin E deficiency states (Davey and Dock, 1982).

Enhancement of humoral immunity in vivo has been reported following administration of pharmacological doses of tocopherol (Tengerdy et al., 1973; Tanaka et al., 1979). Barber et al. (1977), for instance, observed that a single high dose (33 IU/kg) of tocopherol administered by intramuscular injection significantly stimulated antibody production in guinea pigs immunized with an attenuated viral vaccine. In other studies, tocopherol was shown to enhance in vitro antibody responses in normal murine spleen cells antigenically stimulated with sheep red blood cells (SRBC). Furthermore, tocopherol also supported in vitro antibody synthesis in

the relative absence of adherent cells (Campbell et al., 1974).

Of particular relevance to this research, are studies in which the effects of tocopherol on mitogen-induced lymphoproliferative responses were investigated. In accordance with published reports for humoral immunity, seriously curtailed blastogenic responses to both B- and T-cell mitogens have been observed in lymphocytes derived from animals maintained on vitamin E-deficient diets (Bendich et al., 1983 and 1984). In dogs, for example, proliferative responses to the T-cell mitogens concanavalin A (con A) and phytohemagglutinin (PHA) were suppressed in animals fed vitamin E-deficient but otherwise nutritionally complete diets. Similarly, mitogenic responses to pokeweed mitogen (PWM) and streptolysin O were also reduced (Sheffy and Schultz, 1979). Responsiveness to the mitogens was restored by supplementing the animal diets with vitamin E. Exogenous tocopherol added directly to lymphocyte cell cultures was also effective. In these experiments, the reversal of immunosuppression by tocopherol was attributed to the ability of the vitamin to overcome the inhibitory effects of a soluble factor present in the serum of vitamin E-deficient dogs (Langweiler et al., 1981 and 1983).

Other studies have also demonstrated that proliferative responses in lymphocytes stimulated with polyclonal mitogens can be potentiated by tocopherol. Bendich et al., (1986) reported a substantial enhancement of T-cell responses produced by optimal

concentrations (1 µg/ml) of con A in male weanling spontaneously hypertensive rats (SHR) fed diets supplemented with increasing amounts of tocopherol acetate. Maximal stimulation of T-cell mitogenesis was reported in lymphocytes from the experimental groups on diets containing 50 and 200 mg/kg of tocopherol. Differences observed between these two groups were not statistically significant. Lymphoproliferative responses produced by suboptimal (0.1 µg/ml) and supraoptimal (5 µg/ml) levels of con A were equivalent in all dietary groups. In the SHR model, lymphocyte responsiveness to con A was shown to be highly correlated with plasma vitamin E concentrations encompassing a range from 0.04 to 18 µg/ml. By comparison, plasma vitamin E levels measured in rats maintained on conventional laboratory diets ranged from 4 to 7 µg/ml. These results indicate that the immune system is extremely sensitive to variations in tocopherol levels. Also, tocopherol concentrations greater than those needed to prevent pathologies associated with vitamin E deficiency are required for the expression of optimal responses to mitogens (Bendich et al., 1986).

There is also some evidence suggesting that tocopherol may, in certain cases, interfere with the expression of normal T-cell immune responses. In support of this view, Prasad (1980) has reported that human peripheral blood lymphocytes obtained from subjects ingesting 300 mg of tocopherol acetate daily for a period of three weeks were remarkably unresponsive to mitogenic stimulation with PHA. The delayed hypersensitivity reaction of the skin to the mitogen was not

affected by megadose vitamin E dietary supplementation. These results are consistent with earlier findings that in vitro addition of tocopherol to mixed lymphocyte cultures (MLC) suppressed the response of human lymphocytes to allogeneic antigen (Mann and Logan, 1970). However, because the experimental designs of these studies did not include a placebo control group, the results need to be interpreted with caution.

T-cell mitogenesis and its modification by dietary tocopherol in murine spleen cells have been extensively studied by Corwin and Shloss (1980a and 1980b) and Corwin et al. (1981). In the referenced studies, dietary tocopherol was reported to enhance primarily mitogenic responses produced by suboptimal (0.6 µg/ml) levels of con A. Responses to con A were increased by factors of three and eight respectively, when commercial diets were supplemented with 50 and 100 mg of tocopherol per 100 grams of diet. In contrast to other reports (Bendich et al., 1986), lymphoproliferative responses generated by optimal (2.5 µg/ml) concentrations of the mitogen were apparently unaffected by tocopherol (Corwin and Shloss, 1980a and 1980b). The reason for this interesting discrepancy is not known. There is some evidence, however, suggesting that in certain instances dietary tocopherol can potentiate responses to optimal con A. The extent of this stimulation is determined by the degree of saturation of the fatty acids present in the diets which experimental animals are fed. A significant enhancement of mitogenic responses produced by optimal

con A has been reported in mice maintained on corn oil-based diets as opposed to other diets containing lard or hydrogenated coconut oil as their primary fat source (Corwin and Shloss, 1980a).

The effects of in vitro tocopherol supplementation in murine spleen cells have also been investigated. In the absence of con A, tocopherol by itself at final concentrations ranging from 1 to 5 μM was found to be slightly mitogenic. The mitogenic responses observed were of comparable magnitude to those produced by 2-mercaptoethanol, and at lower concentrations, tocopherol was a better mitogen (Corwin and Shloss, 1980a).

Murine spleen cell populations depleted of their adherent cell component demonstrate substantially diminished lymphoproliferative responses to mitogens. Corwin et al. (1981) reported that mitogenic responses produced by suboptimal con A (0.6 μg/ml) were reduced by 73% in murine spleen cells depleted of plastic-adherent cells and subsequently treated with antiserum specific for accessory cells. Mitogen responsiveness was restored in these cells through the addition of tocopherol yielding a final concentration of 1 μg/ml.

The precise biochemical mechanism through which tocopherol exerts its immunomodulatory effects is not fully understood at this time and remains to be elucidated. Nonetheless, several theories have been elaborated to explain the mode of action of vitamin E in the stimulation of T-cell mitogenesis. There is considerable evidence

that tocopherol, both in vivo and in vitro, is an important biological antioxidant (Tappel, 1970; Green, 1972; McCay and King, 1980). Furthermore, in the diet, tocopherol is frequently found in association with polyunsaturated fatty acids (PUFA). Increased PUFA intake in animals usually demands a higher tocopherol content in the diet in order to prevent the pathological effects associated with vitamin E deficiency (Corwin and Shloss, 1980b). Therefore, it is believed that the primary metabolic function of tocopherol is that of a free-radical scavenger preventing toxic lipoperoxide formation in cells containing elevated amounts of PUFA (Corwin and Schloss, 1980b). The so-called "antioxidant theory", claims that tocopherol potentiates the response of lymphocytes to suboptimal concentrations of con A by overcoming adverse effects associated with PUFA. That is, with regard to immune function, tocopherol would protect the cell membranes of lymphocytes against the damaging effects of free-radicals and organic peroxides (Diplock and Lucy, 1973; Combs et al., 1975) thereby promoting enhanced blastogenic responses to mitogens. In support of an antioxidant view for the stimulation of T-cell mitogenesis by tocopherol, it has been experimentally verified that other antioxidants also possess mitogenic activity. The synthetic tocopherol analogue, N,N'-diphenyl-p-phenylene diamine (DPPD) and, to a much lesser extent, butylated hydroxytoluene (BHT), were also found to enhance in vitro mitogenic responses to suboptimal con A in spleen cells derived from mice fed diets rich in PUFA (Corwin and Shloss, 1980b). The "antioxidant theory" thus establishes a relationship between experimental evidence and the well-recognized

antioxidant properties of tocopherol in order to account for the stimulation of mitogenic responses by vitamin E. Contrary to this position, it has been demonstrated that compounds such as tocopherol quinone and menadione (vitamin K-3), which unlike tocopherol do not possess any significant antioxidant activity, can also enhance lymphoproliferative responses generated by suboptimal con A. These findings suggest that the stimulation of T-cell mitogenesis produced by tocopherol is perhaps not entirely related to the antioxidant properties of the vitamin E.

A second mechanism proposed to explain the modification of T-cell mitogenesis by tocopherol also originates from the "antioxidant theory" and implicates tocopherol as a possible regulator of prostaglandin biosynthesis (Sheffy and Schultz, 1979; Corwin and Shloss, 1980b). The prostaglandins not unlike tocopherol, are also potent immunomodulators (Henney et al., 1972; Koopman et al., 1973). In particular, the E-type prostaglandins are significant modifiers of cell-mediated immunity (Faulk et al., 1976; Likoff et al., 1978). Elevated levels of this substance are responsible for diminished natural killer cell-mediated cytotoxicity (NKCC) in antioxidant-deficient animals (Brunda et al 1980). Antibody synthesis in vitro and T-cell blast transformation have also been reported to be adversely affected by prostaglandins (Mendelsohn et al., 1973; Mertin and Hughes, 1975). It has been suggested that prostaglandins modify immunological responses indirectly through stimulation of membrane-bound adenyl cyclase activity in lymphocytes with resulting increases

in intracellular cyclic AMP levels (Sheffy and Schultz, 1979). This in turn has been shown to inhibit several immunological responses including lymphocyte blastogenesis (Smith et al., 1971; Strom et al., 1973).

The first step in the biosynthetic prostaglandin cascade involves the peroxidation of arachidonic acid, a polyunsaturated essential fatty acid, by the enzyme fatty acid cyclooxygenase. This oxidation results in the formation of an intermediate endoperoxide which is subsequently acted upon by the enzymes endoperoxide isomerase and reductase to produce prostaglandins PGE and PGF, respectively (Corwin and Shloss, 1980b). It has also been demonstrated that the peroxidation of arachidonic acid can be inhibited by indomethacin, an aspirin-like drug (Vane, 1971), and also in certain systems by tocopherol (Vanderhoek and Lands, 1973). This important observation constitutes the basis for an hypothesis that ascribes a regulatory function to tocopherol for prostaglandin biosynthesis and ultimately, immune modulation.

Vitamin E deficiency is often associated with impaired immune function (Marsh et al., 1981; Gebremichael et al., 1984; Meeker et al., 1985). Sheffy and Schultz, (1978), for example, have reported markedly depressed proliferative responses to mitogens in canine lymphocytes obtained from animals maintained on diets deficient in vitamin E and selenium but containing elevated levels of PUFA. Tocopherol was shown to overcome the immunosuppression in these

experiments thereby restoring mitogen responsiveness to the lymphocytes. Additionally, there is also evidence establishing a correlation between vitamin E deficiency and increased prostaglandin biosynthesis (de Boer et al., 1973). On the basis of these observations, it has been postulated that elevated levels of PUFA may stimulate a rapid conversion of PUFA into prostaglandins by the membrane-bound enzyme prostaglandin synthetase (Sheffy and Schultz, 1979). Tocopherol, by virtue of its antioxidant properties, would presumably modify mitogenic responses by preventing the peroxidation of arachidonic acid thereby restricting the entry of precursors into the prostaglandin cascade. Consistent with this tenable hypothesis, Likoff et al. (1978) have previously reported a significant reduction of mortality in chicks fed aspirin or megatherapeutic quantities of vitamin E following antigenic challenge with E. coli. This response was also accompanied by a corresponding decrease in bursa prostaglandin levels. Vitamin E alone, or in combination with aspirin, did not affect prostaglandin levels in the spleen. In other studies, Corwin and Shloss (1980b) failed to demonstrate an inhibition of prostaglandin biosynthesis in mitogen-stimulated murine spleen cells by dietary supplementation or in vitro administration of tocopherol. Moreover, indomethacin, which is known to antagonize the peroxidation of arachidonic acid, did not potentiate the response of murine spleen cells to suboptimal levels of con A or optimal PHA. Similarly, when tocopherol and indomethacin were administered concurrently in vitro, additional stimulation of T-cell mitogenesis in excess of that produced by

tocopherol alone was not observed. These conflicting results indicate that the modification of T-cell mitogenesis by tocopherol is probably not entirely mediated by the regulation of prostaglandin biosynthesis and hence, further contribute to the uncertainty surrounding the mode of action of tocopherol.

The damaging effects of ionizing radiation on the immune system have been extensively investigated and numerous reviews exist (Dubois et al., 1981; Doria et al., 1982). Briefly, it is known that total-body irradiation causes a selective depletion of lymphocytes from peripheral blood and lymphoid organs resulting in severe lymphopenia (Anderson and Warner, 1976). This response, commonly referred to as acute radiation syndrome, is associated with marked immunosuppression and has been attributed to the unusual radiosensitivity demonstrated by mature lymphocytes (Neta et al., 1986; Manori et al., 1986). In general, B-lymphocytes are more radiosensitive than T-lymphocytes although radioresistant subpopulations have been isolated in both cell lineages (Durum and Gengozian, 1978). For instance, with respect to T-cell function, it has been reported that helper cells are relatively radioresistant compared to their suppressor cell counterparts (Gualde and Goodwin, 1984).

Unlike most non-cycling cells, resting (Go) lymphocytes are extremely radiosensitive and experience rapid interphase death with low doses of radiation (Miller and Raleigh, 1981). Interphase death,

as the name implies, is a process somewhat unique to mitotically inactive lymphocytes and thymocytes in which the cells are killed by low doses of ionizing radiation during interphase of the cell cycle. In BALB/c murine peripheral lymphocytes, X-ray doses as low as 20 rads have been reported to rapidly kill some cells (Lowenthal and Harris, 1985). Resting lymphocytes also differ from other cells in that an inverse dose-rate effect is observed for cell survival. That is, low radiation dose rates appear to be more cytotoxic and are therefore of greater effectiveness in causing interphase death (Konings, 1981; Miller and Raleigh, 1981). Although the mechanism underlying interphase lethality in these cells remains to be determined, it is currently believed that this process reflects damage to cell membranes possibly resulting from peroxidation of polyunsaturated fatty acids.

There is also evidence indicating that the in vitro interphase death response of mitotically inactive lymphocytes is substantially reduced by mitogenic or antigenic activation (Dewey and Brannon, 1976; Dohi et al., 1984). Lowenthal and Harris (1985) have reported that murine T- and B-lymphocytes stimulated with polyclonal mitogens do not exhibit the classical interphase death response typical of non-transformed cells. Instead, activated cells experience a delayed and gradual loss of viability following irradiation. Specifically, it was observed that an X-ray dose of 1000 rads reduced the viability of transformed lymphocytes by only fifty percent when less than one percent of non-stimulated cells survived.

Furthermore, the degree of radioprotection conferred by mitogens is dependent on the temporal relation between activation and irradiation. In murine lymph node cells stimulated with con A, a significant radioprotective effect, as manifested by prevention of rapid cell death, was observed when the mitogen was provided 22 hours prior to irradiation. A smaller percentage of cells were also protected when the mitogenic stimulus was delayed for three hours post-irradiation. These results suggest that the time and duration of exposure to mitogenic lectins are important determinants for the interphase death response of activated lymphocytes.

Immunomodulators have been primarily recognized for their capacity to interact with the complex immunoregulatory network and thus modify specific immune responses (Fauci et al., 1987). There are presently a limited, but increasing number of reports, also ascribing a radioprotective function to some immunomodulators. Of particular interest are studies regarding post-irradiation modification of T-cell effector function (Gerber, 1984) and cell proliferation (Manori et al., 1985 and 1986) by the lymphokine interleukin 2 (IL2). Gerber (1984) demonstrated that supplementation of culture medium with 50% T-cell growth factor (TCGF) post-irradiation, successfully restored the cytotoxic and proliferative responses of irradiated B6 murine splenocytes in mixed lymphocyte cultures. These responses were also enhanced by TCGF in non-irradiated cells. TCGF is essentially a mixture of lymphokines derived from mitogenically-activated lymphocytes and contains significant quantities of

interleukin 2 (Manori et al., 1985).

In similar studies (Manori et al., 1985 and 1986), the effects of TCGF and interleukin-containing preparations on restoration of mitogen-induced lymphoproliferative responses in irradiated cells were investigated. Increasing doses of gamma radiation ranging from 0 to 400 rads were observed to considerably inhibit the blastogenic response of C57Bl/6 murine spleen cells to con A. When addition of the mitogen was deferred for 24 hours post-irradiation, the effects of radiation were more damaging and mitogenic responses to con A were further suppressed. The administration of TCGF concurrently with con A to these cells immediately post-irradiation partially restored blastogenic responses to the mitogen.

The initial experiments of Manori et al. (1985) were later expanded to study the effects of interleukins 1 and 2 on restoration of T-cell mitogenic responses in irradiated thymocytes (Manori et al., 1986). Consistent with their earlier findings reporting the radioprotective effects of TCGF (Manori et al., 1985), it was further demonstrated that thymocytes stimulated with con A or PHA in the presence of interleukin 1 immediately post-irradiation are significantly less radiosensitive than cells treated with mitogens alone. A dose-reducing factor of two was attained with this immunomodulator. The radioprotective effects were abrogated when the addition of interleukin 1 and mitogens was deferred for 24 hours following exposure to radiation. The addition of supernatants containing interleukin 2 to cultures of irradiated thymocytes, at this

time, was radioprotective and partially restored mitogenic responses in these cells.

There is considerable evidence in support of the function of tocopherol as a biological immunomodulator in several experimental models including man (Tengerdy, 1980; Panush and Delafuente, 1985; Bendich et al., 1986). There are also studies demonstrating that in certain cases, post-irradiation tocopherol administration is radioprotective. In particular, it has been reported that animal survival and recovery of hematopoietic stem cells are enhanced by tocopherol following irradiation with doses encompassing bone marrow syndrome (Malick et al., 1978; Roy et al., 1982; Bichay and Roy, 1986).

Experience with modification of radiation-induced immunosuppression by tocopherol is extremely limited. In a previous publication (Roy and Petrella, 1987), the effects of post-irradiation tocopherol administration on the humoral immune response of mice antigenically challenged with sheep red blood cells (SRBC) were reported. A single intraperitoneal injection containing 2.5 mg DL-α-tocopherol given immediately after irradiation and 24 hours prior to inoculation with SRBC was shown to stimulate submaximal IgG antibody responses and was most significant in mice maintained on vitamin E-deficient diets. The effects of post-irradiation tocopherol administration on lymphocyte survival and restoration of mitogenic responses have not been evaluated and require investigation.

The objectives of this research are essentially two-fold. Firstly, the effects of in vitro tocopherol administration on lymphoproliferative responses produced by con A in C57Bl/6 murine spleen cells will be studied in some detail. Increasing concentrations of DL-α-tocopherol encompassing both physiological and pharmacological levels of vitamin E will be utilized in order to identify the doses yielding maximal enhancement of the mitogenic response. It is known that complex vehicles used to dissolve tocopherol may contain toxic components that are immunosuppressive (Davey and Dock, 1982). Consequently, low level mitogenic responses produced by tocopherol or suboptimal concentrations of con A could be potentially abrogated by these inhibitory substances. To circumvent this possibility, the tocopherol used in all experiments will be emulsified in fetal calf serum.

Secondly, the effects of post-irradiation tocopherol administration on interphase death and con A mitogenesis in spleen cells will be evaluated. In these experiments, the concentration of DL-α-tocopherol producing the greatest stimulation of the mitogenic response will be assessed with respect to the modification of radiation-induced suppression of lymphocyte survival and proliferation in cell cultures immediately following irradiation.

# MATERIALS AND METHODS

## Experimental Animals

C57Bl/6 female mice weighing 18 to 22 g were purchased from Charles River Canada Inc. (St. Constant, Quebec). The mice were housed in pairs in lucite cages and held for a minimum of seven days prior to the commencement of experimental procedures. This precautionary measure was taken in order to allow sufficient time for the animals to adapt to the laboratory environment. During this time, the mice were maintained on Agway Prolab Animal Diet (Agway Inc., Country Foods Division, Syracuse, New York). Food and fresh water were present ad libitum at all times. The average temperature of the animal room was 20°C and the relative humidity approximately 50%. Fluorescent tubes were used to illuminate the animal room and a regular 12 hour day : 12 hour night cycle was utilized.

## Tissue Culture Media

For the purposes of this study, mitogen-stimulated murine splenic lymphocytes were cultured in RPMI 1640 basal tissue culture medium and in basal medium supplemented with DL-α-tocopherol. Basal tissue culture medium was prepared by combining RMPI 1640 medium containing L-Glutamine (Gibco Laboratories, Grand Island, New York) with 10% (v/v) fetal calf serum (Whittaker M.A.

Bioproducts, Walkersville, Maryland), 25 mM Hepes Buffer (Flow Laboratories, McLean, Virginia) and 2% (v/v) penicillin-streptomycin solution containing 5000 I.U./ml penicillin and 5000 µg/ml streptomycin (Flow Laboratories, McLean, Virginia). The individual constituents were mixed in sterile 100-ml polystyrene graduated cylinders and the pH of the complete culture medium was adjusted to 7.4 with 1 N sodium hydroxide solution.

Tocopherol-supplemented tissue culture medium was prepared by a modification of the method published by Narayanareddy and Murthy (1982). Between 20 and 40 mg of DL-α-tocopherol (ICN Nutritional Biochemicals, Cleveland, Ohio) was mixed with fetal calf serum in a 100-ml tissue culture medium bottle to produce a 1 mg/ml tocopherol-serum suspension. This mixture was then incubated at 37°C in a water bath for two hours in the absence of light and vigorously agitated at 20 to 30-minute intervals in order to ensure complete dispersion of the tocopherol in the fetal calf serum. Fetal calf serum treated in an identical fashion but without the addition of tocopherol was used for preparing control tissue culture medium. Dilutions were then made from the 1 mg/ml tocopherol-fetal calf serum suspension utilizing control serum as the diluent. Serum containing 10, 50, 100, 250 and 500 µg/ml tocopherol was prepared and subsequently used to make tocopherol-supplemented tissue culture medium. The final concentration of tocopherol in the supplemented culture medium ranged from 1 to 100 µg/ml. The final concentration of fetal calf serum in the tocopherol-supplemented

culture medium, control culture medium and basal tissue culture medium was 10% (v/v) in each case.

Tissue culture medium was filter-sterilized with Nalgene 115-ml Type TC tissue culture sterilization filter units (Nalge Company, Rochester, New York) used in conjunction with the Nalgene pressure filtration adaptor. The positive pressure required to filter the tissue culture medium through the sterilization unit's high efficiency 0.1 μ primary filter was delivered from a 5% $CO_2$ cylinder operated at 10-12 psi. Following filter sterilization, the tissue culture medium was transferred to sterile 100-ml Schott GL 45 tissue culture medium bottles (Bellco Glass Inc., Vineland, New Jersey) and stored at 4°C until required. All operations involved in the preparation of the tissue culture media and media transfers were performed in a Labconco horizontal laminar-flow hood utilizing conventional sterile techniques.

Concanavalin A Solutions

The polyclonal T-cell mitogen, concanavalin A (con A) was purchased from two suppliers (Sigma Chemical Company, St.Louis, Missouri and Difco Laboratories, Detroit, Michigan) in vials containing 5 and 50 mg of sterile lyophilized lectin powder. Commercial batches of con A originating from different production lots were obtained from both suppliers and titrated in a mitogenesis assay based on a standardized protocol before the start of

experiments. This was done in order to determine the concentration of mitogen required to produce optimal cell proliferation with each lot of con A utilized.

Mitogen solutions containing 1 and 5 mg/ml con A, respectively, were prepared by rehydrating Sigma type IV-S con A powder with 5 ml of sterile distilled water and similarly, by dissolving the Difco product in twice this amount of sterile water. The reconstituted con A solutions were then centrifuged at 400 X g for five minutes in a clinical centrifuge to precipitate any denatured lectin that may have formed during the rehydration process. Following this purification step, a 1 mg/ml stock solution of the mitogen was prepared by diluting the original Difco con A solution five-fold with serum-free basal tissue culture medium. Dilutions were subsequently made from the 1 mg/ml con A stock solutions (Sigma or Difco) utilizing serum-free culture medium as the diluent. Dilute con A solutions were always stored at 4°C in tightly capped 15-ml centrifuge tubes and used within 24 hours of their preparation. The final concentration of con A in the murine spleen cell cultures prepared for the mitogenesis assays ranged from 0 to 10 µg/ml. Serum-free tissue culture medium without added con A was used for the mitogen control group to determine background cellular proliferation.

Splenic Lymphocyte Cultures

Preparation of Spleen Cell Suspensions.

Mice were sacrificed individually in a polypropylene chamber using carbon dioxide gas inhalation. Immediately thereafter, the mice were completely immersed in a beaker containing 70% ethanol and then carefully positioned with their left side facing up on paper towels soaked with alcohol. This precautionary step was used to minimize the risk of loose hair and dander becoming airborne during subsequent operations. All surgical procedures required initially for the removal of spleens from the mice and later, for the isolation of spleen cells from the excised organ were performed aseptically in a Labconco horizontal laminar-flow hood.

Using a pair of fine-point surgical scissors in conjunction with forceps, an incision approximately 2 cm in length was made in the left side of the mouse in the inguinal region. The skin was carefully retracted and the peritoneal cavity irrigated with 70% alcohol in order to remove loose tissue fragments and other debris. A large U-shaped incision was then cut in the peritoneal wall around the spleen. The spleen was exposed and secured with mouse-tooth forceps as the blood vessels attached to the organ were severed. At this time, any large pieces of adipose tissue present on the spleen were also removed. Following its removal, the spleen was promptly transferred to a Corning 60 x 15 mm tissue culture dish

containing 10 ml of cold (4°C) basal tissue culture medium. At a maximum, six spleens were isolated and retained for further processing during any one experiment. Utilizing fine-point forceps and microscissors, any residual fat tissue attached to the spleen was removed. Extra care was exercised not to rupture the spleen's delicate capsule at this point. The spleens were then washed four times by serial passages through tissue culture dishes containing 10 ml of cold (4°C) tissue culture medium. Immediately thereafter, the spleens were transferred to another tissue culture dish and gently teased in 10 ml of cold tissue culture medium in order to release the lymphocytes into suspension. This was accomplished by repeatedly scraping the surface of the spleens with two scalpels fitted with No. 15 blades. Caution was taken to avoid breaking the spleens into small pieces which would make the isolation of lymphocytes more tedious. Large tissue fragments were discarded and the cell suspension was transferred to a chilled Corning 15-ml sterile polypropylene centrifuge tube. The spleen cell suspension was allowed to stand in a vertical position on crushed ice for 15 minutes allowing small tissue fragments to settle to the bottom of the tube. The supernatant containing the isolated spleen cells was transferred to another sterile centrifuge tube and stored on crushed ice until required.

## Removal of Red Blood Cells from Spleen Cell Suspensions

Red blood cells were removed from the murine spleen cell suspension using the method described by Mishell and Shiigi (1980). The spleen cell suspension was centrifuged for 10 minutes at 200 X g in a clinical centrifuge and the supernatant was discarded. Tris-buffered ammonium chloride solution (pH 7.2) was added to yield 10 ml per ml of packed spleen cells. The cell pellet was thoroughly resuspended in the buffer solution and held at room temperature in a 15-ml sterile centrifuge tube for five minutes. With the aid of a sterile cotton-plugged Pasteur pipette, 2.0 ml of cold (4°C) fetal calf serum were carefully layered below the spleen cell suspension. This was followed by centrifugation at 300 X g for 10 minutes. The supernatant containing lysed red blood cells was discarded and the entire procedure described above was repeated only if a substantial number of red cells were still visible in the cell pellet. Afterwards, the spleen cell pellet was resuspended and washed in 10 ml of cold (4°C) serum-free tissue culture medium. This was achieved by passing the cell suspension through a sterile 10-ml serological pipette several times followed by centrifugation at 200 X g for 10 minutes. The washing procedure was then repeated two more times. Lastly, the washed murine spleen cells were resuspended in 2 to 5 ml of serum-free tissue culture medium, depending on the volume of the resulting cell pellet, and stored in a tissue culture test-tube on crushed ice until needed.

Cell Counting Procedures

Determination of the Nucleated Cell Count

The murine spleen cell suspension was gently vortexed at a low speed setting for a few seconds. A 100-µl aliquot was then aseptically removed and transferred to a 1.5-ml Eppendorf micro test-tube. Nucleated cells were counted using the Unopette 5856 test-system designed for manual determination of white blood cell counts (Becton-Dickinson, Rutherford, New Jersey). The following is a detailed description of the technique employed. Twenty-five microlitres of spleen cell suspension were drawn into a specially designed capillary tube and transferred to a pre-filled plastic reservoir containing 0.475 ml of 3% acetic acid. The spleen cells were thoroughly mixed with the diluent by inverting the capillary tube-reservoir assembly several times in succession. Using this method, the original spleen cell suspension was diluted by a factor of twenty. The spleen cells were allowed to remain in contact with the diluent in the plastic reservoir for 10 minutes before scoring nucleated cells. This ensured the lysis of any residual red blood cells that may have been present in the spleen cell suspension. Thereafter, one-half of a Neubauer hemacytometer was loaded with the diluted cell suspension. Four large grids normally used for counting white blood cells were then scored to obtain the nucleated cell count. The total number of nucleated cells present per ml of spleen cell suspension was calculated with the following formula.

nucleated cells/ml = total number of cells counted X 5 X $10^4$

Similarly, the total number of nucleated cells present in the cell suspension was determined by multiplying the number of nucleated cells per ml of suspension by the volume of the original spleen cell suspension. Sufficient serum-free tissue culture medium was then added to produce a spleen cell suspension containing 1 X $10^7$ cells/ml. This dilute cell suspension was stored in a capped tissue culture test-tube and held on crushed ice until required.

Determination of Cell Viability

Immediately before use, one part of concentrated saline solution (4.25% NaCl w/v) was combined with four parts of 0.2% trypan blue (w/v) in water. Fifty microlitres of trypan blue diluted in saline were then mixed with an equal volume of the original undiluted spleen cell suspension in a 1.5-ml Eppendorf micro test-tube. One drop of this preparation was loaded onto the unused half of the hemacytometer previously used for determining the nucleated cell count. A minimum of 250 nucleated cells were rapidly scored within three minutes from the time the dye was added to the spleen cells as viable cells have been shown to incorporate the trypan blue dye after this time (Mishell and Shiigi, 1980). Viable cells were readily distinguished from dead cells as those which excluded the dye and thus appeared unstained whereas the latter incorporated the trypan

blue and were stained dark blue. The percentage of viable cells present in the spleen cell suspension was calculated. Lymphocyte cultures were established from spleen cell suspensions which contained more than 90% viable cells.

Preparation of Murine Spleen Cell Cultures without Adherent Accessory Cells

Murine spleen cell cultures depleted of adherent accessory cells were prepared by a modification of the method published by Corwin and Shloss (1980a). The technique used is based on the differential affinity for plastic surfaces that exists between adherent and non-adherent spleen cells. A spleen cell suspension containing approximately 1 X $10^8$ nucleated cells was centrifuged at 200 X g for 10 minutes in a clinical centrifuge. The cell pellet was resuspended in 10 ml of 37°C basal tissue culture medium and then transferred to a 25-$cm^2$ polystyrene tissue culture flask (Corning Glass Works, Corning, New York). The tissue culture flask was flushed with 5% $CO_2$ gas (95% air), sealed and incubated in a horizontal position for one hour at 37°C. Following this initial incubation period, the contents of the 25-$cm^2$ flask were transferred to a larger 75-$cm^2$ tissue culture flask (Corning Glass Works, Corning, New York). The inside walls of the smaller flask were vigorously washed two times with 5 ml of 37°C serum-free tissue culture medium to dislodge non-adherent cells and the wash solution was transferred to the larger 75-$cm^2$ tissue culture flask. This flask

was flushed with the 5% $CO_2$ gas mixture and incubated at 37°C for one hour after which its contents were transferred to two 15-ml polystyrene centrifuge tubes and centrifuged at 200 X g for 10 minutes. The non-adherent spleen cell pellets were then individually washed and resuspended in 5 ml cold (4°C) serum-free tissue culture medium. The cell suspensions were pooled and then centrifuged at 200 X g for 10 minutes. The cell pellet was finally resuspended in 5 ml cold (4°C) serum-free tissue culture medium. Cell viability and nucleated cell count were determined as previously described.

Analysis of Concanavalin A Mitogenesis

Optimal Concentration of Concanavalin A

The concentration of con A required to generate maximal lymphoproliferative responses in murine spleen cells was determined using a modification of the procedure described by Spieker-Polet et al. (1979). Lymphocyte cultures were prepared by inoculating 0.2 ml of spleen cell suspension (1 X $10^7$ cells/ml) into 1.8 ml basal tissue culture medium to which 0.1 ml of con A solution or serum-free culture medium had previously been added. Triplicate cell cultures were established in 16 X 125-mm polystyrene tissue culture test-tubes (Corning Glass Works, Corning, New York) for each concentration of con A to be studied. Individual cultures contained 2 X $10^6$ cells in a final cell culture volume of 2 ml (1 X $10^6$ cells/ml). The lymphocyte culture tubes were flushed with 5% $CO_2$,

tightly capped and incubated at 37°C for 48 hours in 45° slant racks. A 50-µl aliquot containing 2.0 µCi of sterile tritiated-thymidine (thymidine, [methyl-$^3$H]) diluted in serum-free tissue culture medium was then dispensed into each of the culture tubes. The tritiated-thymidine utilized in all experiments had a specific activity of 6.7 Ci/mMol and was purchased from New England Nuclear (Boston, Massachusetts) or from ICN Biochemicals Canada Ltd. (Montreal, Quebec).

After the addition of tritiated-thymidine to the lymphocyte cultures, the culture tubes were flushed with 5% $CO_2$, resealed and incubated as before for an additional 18 hours. The total culture time was 66 hours with the final 18 hours in the presence of radioactive thymidine.

Cell Harvesting Procedure

The incorporation of radioactive thymidine by con A-stimulated murine spleen cells was determined after precipitation of macromolecules with trichloroacetic acid (TCA). At the conclusion of the 18-hour feeding period with radiolabelled thymidine, the lymphocyte cultures were removed from the incubator and placed in a refrigerator (4°C) until ready to be processed. Whatman GF/A 2.4 cm glass microfibre filter discs were moistened with cold (4°C) pH 7.4 Dulbecco's modified phosphate buffered saline (Flow Laboratories, McLean, Virginia) and carefully positioned on each of the twelve

filter support screens of a Millipore 1225 sampling manifold (Millipore Corporation, Bedford, Massachusetts). The top plate of the apparatus was secured onto the sampling manifold and a gentle vacuum was applied to the system. Lymphocyte culture tubes were vortexed at a low speed setting for five seconds and the cell suspensions were decanted into the individual sample wells. Twelve lymphocyte cultures could be processed sequentially with the sampling manifold used. The culture tubes were then washed five times in succession as follows. Three ml of cold (4°C) phosphate buffered saline (PBS) were dispensed into each of the lymphocyte culture tubes. The tubes were vortexed and the wash solution was decanted onto the filter discs. Trichloroacetic acid-soluble material was then extracted from the cells by washing the filter discs two times with 10 ml of ice cold 10% (w/v) TCA. The precipitates were subsequently washed three times with 3 ml of cold (4°C) 50% (v/v) ethanol, air dried at room temperature until slightly moist and placed into 20-ml glass liquid scintillation counting vials. Protosol tissue and gel solubilizer (NEN, Boston, Massachussets) was added (0.5 ml) to each vial. The vials were tightly capped and incubated in a heated water bath at 55-60°C for 30 minutes. Afterwards, the vials were permitted to cool to room temperature and 50 µl of glacial acetic acid and 10 ml of Quantaflor liquid scintillation counting fluid (Mallinckrodt, St.Louis, Missouri) were dispensed into each vial. Liquid scintillation counting vials were kept overnight at room temperature and in the absence of light allowing for phosphorescence and chemiluminescence to subside. The tritium

activity present in the TCA precipitates was then measured with an LkB Rack Beta model 1215 liquid scintillation counter. Quench correction using external standard ratio was applied in order to obtain the absolute activity.

## Modification of Concanavalin A Mitogenesis by Tocopherol

### Spleen Cell Cultures Containing Adherent Accessory Cells

Lymphocyte cultures were prepared by inoculating 2 X $10^6$ spleen cells into 1.8 ml of tocopherol-supplemented tissue culture medium to which 0.1 ml of con A solution or serum-free culture medium without mitogen had previously been added. Tissue culture media containing tocopherol at final concentrations of 1, 5, 25, 50 and 100 µg/ml were utilized in this series of experiments. A second set of lymphocyte cultures was similarly established in control tissue culture medium without added tocopherol for each different concentration of con A used. The concentration of con A in the lymphocyte cultures ranged from 0 to 10 µg/ml. Lymphocyte cell cultures were incubated at 37°C for 66 hours with the final 18 hours in the presence of 2 µCi of tritiated-thymidine. The cell cultures were harvested as previously described and the tritium activity present in TCA precipitates was determined by liquid scintillation spectrometry.

Spleen Cell Cultures without Adherent Accessory Cells

Spleen cell cultures containing reduced numbers of macrophages were established in tissue culture medium containing 5 µg/ml DL-α-tocopherol and in control medium without tocopherol. The cell density was 1 X $10^6$ cells/ml. The cells were stimulated with con A at a final concentration of 0.5 and 2 µg/ml. Non-specific cell proliferation was determined using serum-free tissue culture medium without con A as the control for the mitogen. The cells were cultured as previously described and at the conclusion of an 18-hour feeding period with radiolabelled thymidine, the tritium activity present in the cultures was measured by liquid scintillation counting of TCA-precipitated macromolecules.

Time-Course of Tritiated-Thymidine Incorporation

The incorporation of tritiated-thymidine by con A-stimulated murine spleen cells was monitored over a 27-hour period as follows. Lymphocyte cultures were prepared in tocopherol-supplemented tissue culture medium containing 5 µg/ml DL-α-tocopherol and in control culture medium without tocopherol. Con A was added to the cell cultures yielding a final concentration of 2 µg/ml. The lymphocyte cultures were flushed with 5% $CO_2$ and incubated at 37°C in 45° slant racks. At regular 3-hour intervals commencing from the time the cultures were first placed in the incubator, 2.0 µCi of tritiated-thymidine was added to triplicate cell cultures belonging to each of

the two experimental groups. The culture tubes were flushed with 5% $CO_2$, resealed and incubated for three hours with radiolabeled thymidine. At the end of the feeding period, the lymphocyte cultures were removed from the incubator and retained for cell harvesting using the method previously described. Tritiated-thymidine (2.0 μCi) was then added to another set of six lymphocyte cultures at the next scheduled time. The feeding-harvesting cycle was continued until a total of 54 lymphocyte cultures was processed for the nine sampling times studied during the course of this 27-hour experiment. The tritium activity present in the TCA-precipitates was determined by liquid scintillation counting.

Irradiation Procedures

X-radiation for this series of experiments was generated from a Mueller MG-300 X-ray machine operated at 260 kVp and 8 mA with 1.3 mm added aluminum filtration. The exposure dose-rate was measured with a Victoreen model 570 condenser R-meter (Victoreen Instruments Division, Cleveland, Ohio) and the absorbed dose determined by ferrous ammonium sulphate chemical dosimetry (Fricke and Morse, 1927). The average exposure dose-rate was 0.65 Gy/min and the target-to-object distance was 39 cm. Murine spleen cells were irradiated at ambient temperature (20°C).

Interphase Death Response Following Irradiation

Murine spleen cell suspensions were prepared, separated into aliquots and exposed to 0.5, 1, 2, 4 and 8 Gy of X-irradiation at room temperature (20°C). A non-irradiated sample of spleen cell suspension was retained in order to prepare cultures serving as the control group for the irradiation procedure. Parallel spleen cell cultures were established in medium supplemented with DL-α-tocopherol to a final concentration of 5 µg/ml and in medium without added tocopherol. The cell density was 1 X $10^6$ cells/ml. The cultures were incubated at 37°C for 18 hours after which cell viability was determined using the trypan blue dye-exclusion method.

Mitogenic Responses Following Irradiation

Spleen cell suspensions were prepared as described in serum-free tissue culture medium. Cell viability was determined using the trypan blue dye-exclusion method. The cell suspensions were then divided into equal aliquots, transferred to 25-$cm^2$ tissue culture flasks and irradiated. A non-irradiated aliquot of spleen cell suspension was retained and used as a control for the irradiation treatment in these experiments. Spleen cell suspensions exposed to 1, 2, 4 and 8 Gy of X-irradiation were used to prepare lymphocyte cultures containing 5.4 X $10^5$, 6.3 X $10^5$ and 1 X $10^6$ cells/ml in tissue culture medium supplemented with 5 µg/ml DL-α-tocopherol and in control culture medium without tocopherol. All cultures were

established within 60 minutes of the irradiation treatment. Con A was added to the cell cultures yielding a final concentration of 2 µg/ml. The culture tubes were flushed with 5% $CO_2$ and incubated at 37°C for a total of 66 hours with the last 18 hours in the presence of 2.0 µCi of tritiated-thymidine. At the end of the feeding period with radiolabelled thymidine, the lymphocyte cultures were harvested and the tritium activity present in the TCA precipitates was measured by liquid scintillation spectrometry.

# RESULTS

## Optimal Concentration of Concanavalin A

The concentration of concanavalin A (con A) producing the greatest mitogenic response in C57Bl/6 murine spleen cells was determined. The experiment was repeated three times. By convention, this concentration of the mitogen was chosen as the optimal level of con A. Con A concentrations lower or higher than the optimal level are referred to respectively, as suboptimal and supraoptimal concentrations.

The results obtained are presented in tables 1-A, 1-B and 1-C. Henceforth, the three replications of this experiment will be referred to as experiment 1, experiment 2 and experiment 3. The data from these experiments is also graphically illustrated in figure 1.

Lymphoproliferative responses generated by each of the six con A concentrations studied (0, 1, 2, 3, 5 and 10 µg/ml) were indirectly measured as tritiated-thymidine incorporation into TCA-precipitable material. Triplicate spleen cell cultures were used in all cases. In tables 1A, 1B and 1C, mitogenic responses (DPM/2 ml culture) are expressed as the arithmetic mean and standard deviation of the individual observations. The stimulation index (S.I.) was calculated for each con A group. This quantity is defined as the ratio of mitogenic responses observed in con A-stimulated cells to that of

TABLE 1-A

Incorporation of tritiated-thymidine into TCA-precipitable material of C57Bl/6 murine spleen cells mitogenically stimulated with increasing levels of concanavalin A.

Experiment 1

CONCANAVALIN A CONCENTRATION (µg/ml)

| | 0 | 1 | 2 | 3 | 5 | 10 |
|---|---|---|---|---|---|---|
| (1) | 5673 | 713977 | 931699 | 981266 | 497468 | 3629 |
| | 5045 | 543675 | 1191246 | 815595 | 515706 | 3694 |
| | 4030 | 710465 | 1077288 | 856290 | 435403 | 3409 |
| MEAN | 4916 | 656039 | 1066744 | 884384 | 482859 | 3577 |
| S.D. | ±829 | ±97326 | ±130094 | ±86335 | ±42098 | ±149 |
| (2) S.I. (%) | 100 | 13345 | 21699 | 17990 | 9822 | 73 |

(1) Individual observations represent tritiated-thymidine uptake expressed as DPM/2 ml culture.

(2) S.I.: Stimulation index defined as the ratio of mitogenic responses in con A-stimulated cultures to that of the mitogen control group (0 µg/ml con A).

TABLE 1-B

Incorporation of tritiated-thymidine into TCA-precipitable material of C57Bl/6 murine spleen cells mitogenically stimulated with increasing levels of concanavalin A.

Experiment 2

CONCANAVALIN A CONCENTRATION (µg/ml)

| | 0 | 1 | 2 | 3 | 5 | 10 |
|---|---|---|---|---|---|---|
| (1) | 788 | 255880 | 491783 | 225144 | 77685 | 846 |
| | 1314 | 202877 | 642500 | 242422 | 32086 | 1266 |
| | 1284 | 194523 | 616432 | 432932 | 27063 | 785 |
| MEAN | 1129 | 217760 | 583572 | 300166 | 45611 | 966 |
| S.D. | ±295 | ±33276 | ±80553 | ±115303 | ±27890 | ±262 |
| (2) S.I. (%) | 100 | 19288 | 51689 | 26587 | 4040 | 86 |

(1) Individual observations represent tritiated-thymidine uptake expressed as DPM/2 ml culture.

(2) S.I.: Stimulation index defined as the ratio of mitogenic responses in con A-stimulated cultures to that of the mitogen control group ( 0 µg/ml con A).

TABLE 1-C

Incorporation of tritiated-thymidine into TCA-precipitable material of C57Bl/6 murine spleen cells mitogenically stimulated with increasing levels of concanavalin A.

Experiment 3

| | CONCANAVALIN A CONCENTRATION (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 5 | 10 |
| (1) | 6424 | 191343 | 358151 | 310515 | 92759 | 2123 |
| | 4690 | 170267 | 343308 | 402359 | 63632 | 1265 |
| | 5766 | 226016 | 415259 | 528176 | 50277 | 989 |
| MEAN | 5627 | 195875 | 372239 | 413683 | 68889 | 1459 |
| S.D. | ±875 | ±28149 | ±37988 | ±109271 | ±21723 | ±591 |
| (2) S.I. (%) | 100 | 3481 | 6615 | 73.2 | 1224 | 26 |

(1) Individual observations represent tritiated-thymidine uptake expressed as DPM/2 ml culture.

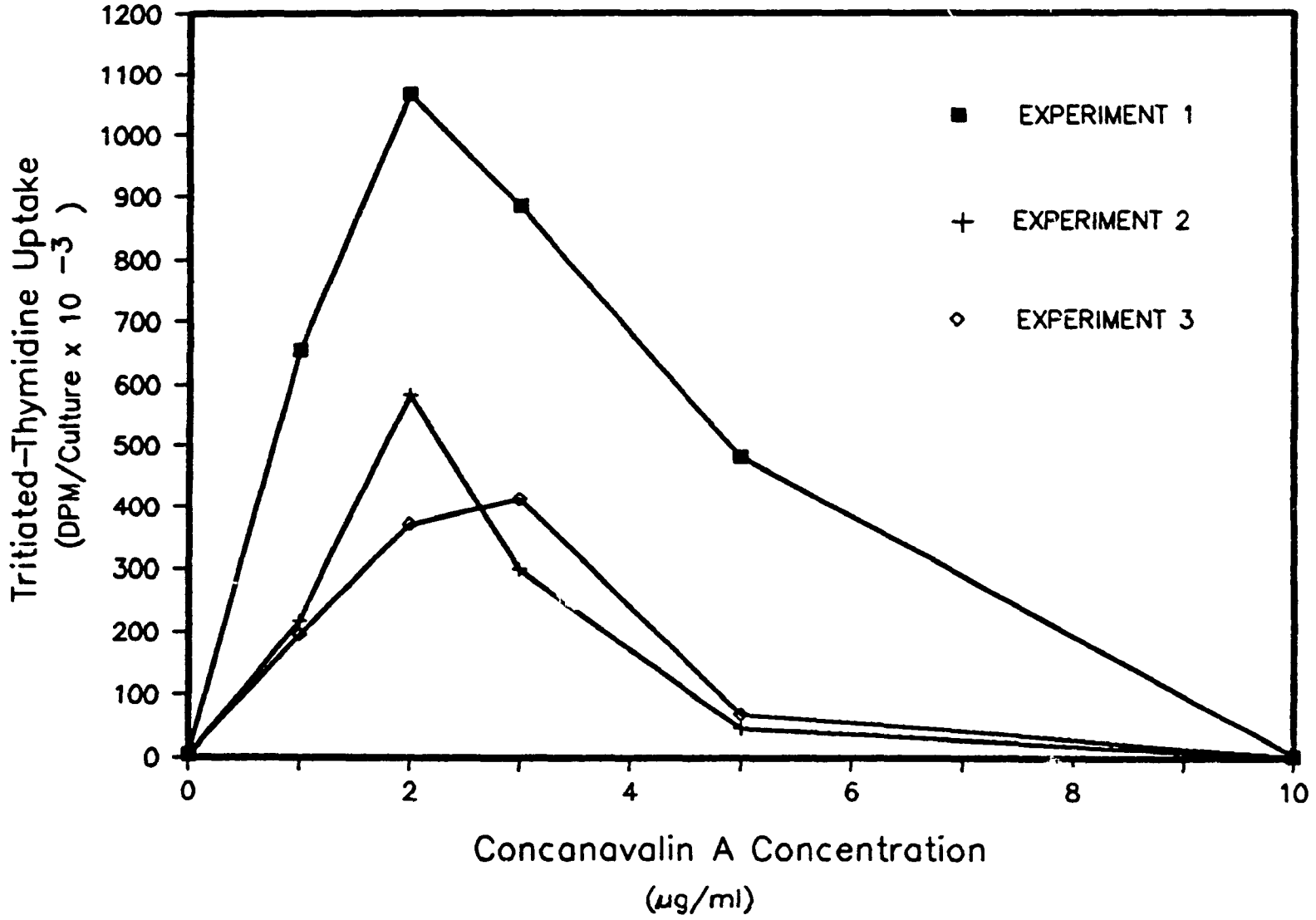
(2) S.I.: Stimulation index defined as the ratio of mitogenic responses in con A-stimulated cultures to that of the mitogen control group (0 µg/ml con A).

the mitogen control group (0 µg/ml con A) and is expressed as a percentage (Mishell and Shiigi, 1980).

Prior to the use of parametric statistical tests, the data from experiments 1, 2 and 3 was transformed. Transformation of the experimental data was required because the basic assumptions for analysis of variance could not be maintained. That is, in order to make valid statistical inferences with parametric tests, the sample data should be normally distributed and homoscedastic (i.e. homogeneous variances) (Sokal and Rohlf, 1981). In addition, when dealing with two-factor designs the assumption of additive treatment effects must also be satisfied (Winer, 1971).

Preliminary examination of the data from the three experiments indicated that the mean of mitogenic responses in the different con A groups was positively correlated with the variance. Furthermore, homogeneous variances were not observed across the six con A treatment groups. These observations clearly indicated that the untransformed experimental data was not normally distributed. Under such circumstances, the logarithmic transformation ($\log_{10}$) has been reported to render variances independent of the mean and thereby normalize data (Sokal and Rohlf, 1981; Woolson, 1987). Heteroscedasticity is also significantly reduced by this monotonic transformation (Winer, 1971). Consequently, the logarithmic transformation was applied to the data from experiments 1, 2 and 3. However, only the original untransformed observations are tabulated.

Figure 1. Lymphoproliferative response of C57Bl/6 murine spleen cells mitogenically stimulated with increasing concentrations of concanavalin A. Data from Tables 1-A, 1-B and 1C.


Tritiated-Thymidine Uptake
(DPM/Culture x 10 -3 )
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
Concanavalin A Concentration
(µg/ml)
EXPERIMENT 1
EXPERIMENT 2
EXPERIMENT 3

Tests of significance were performed with log-transformed variates meeting the assumptions for analysis of variance. Similarly, unless otherwise stated, the parametric statistical tests performed for subsequent experiments were also evaluated with log-transformed data.

Overall differences between mitogenic responses produced by the different con A concentrations utilized were verified for statistical significance ($P \leq 0.050$) with a one-way analysis of variance (Dunn and Clark, 1987). A separate analysis was performed using log-transformed data from each of the three experiments. In all cases, the differences between mitogenic responses were found to be highly significant ($P \leq 0.001$). The analysis of variance tables for experiments 1, 2 and 3 are presented in appendices 1-A, 1-B and 1-C, respectively.

In both experiments 1 and 2, maximal mitogenic stimulation was achieved with 2 µg/ml con A; the corresponding responses (average DPM/2 ml culture) measured with this level of con A were 1066744 ± 130094 DPM/2 ml culture and 583572 ± 80553 DPM/2 ml culture respectively. Incorporation of tritiated-thymidine was greatest in experiment 3 when con A was present at a final concentration of 3 µg/ml (413683 ± 109271 DPM/2 ml culture).

A parametric statistical test, the T-method for unplanned comparisons, was utilized to ascertain if differences observed

between mitogenic responses produced by con A concentrations of 2 and 3 µg/ml were statistically significant (Sokal and Rohlf, 1981). As before, this analysis was performed with log-transformed observations from the three experiments. The results obtained are summarized in appendix 1-D. Statistical significance for this a posteriori comparison was not attained in any of the experiments.

The results obtained in the three experiments indicate that the optimal con A concentration for C57Bl/6 murine spleen cells lies within a relatively narrow concentration range extending from 2 to 3 µg/ml. Although mitogenic responses produced by 2 and 3 µg/ml con A were not significantly different; 2 µg/ml con A yielded the greatest response in two of three experiments. Therefore, in view of these findings, the optimal concentration of con A was established at 2 µg/ml for subsequent experiments.

Suboptimal (1 µg/ml) and supraoptimal (5 and 10 µg/ml) concentrations of con A were noted to generate considerably smaller mitogenic responses than obtained with the optimal level. Differences observed between the responses produced by 2 µg/ml con A and suboptimal and supraoptimal levels of this mitogen were verified by the T-method (appendix 1-D). Statistical significance was demonstrated for these comparisons in both experiments 1 and 2. In experiment 3, mitogenic responses obtained with 1 and 2 µg/ml con A were not significantly different from each other ($P > 0.050$). Furthermore, in the three experiments, the magnitude of

mitogenic responses decreased rapidly as the supraoptimal con A concentration was increased from 5 to 10 µg/ml. Specifically, tritiated-thymidine uptake obtained in the 10 µg/ml con A group was, in all cases, less than that measured in the mitogen control groups (0 µg/ml con A).

Important differences were also observed between the magnitude of mitogenic responses produced by the optimal concentration of con A (2 µg/ml) in the three experiments. For instance, in experiment 2, the response achieved with optimal mitogenic stimulation measured only 54.7% of that obtained with the same level of con A in experiment 1. Similar variability in the magnitude of mitogenic responses obtained with the other levels of con A utilized was also noted between experiments. The exact reason for this discrepancy between mitogenic responses produced by identical concentrations of con A in replicated experiments is not known at this time.

## Modification of Mitogenesis by Tocopherol

### Spleen Cell Cultures Containing Adherent Accessory Cells

The effects of *in vitro* tocopherol supplementation on mitogenic responses produced by suboptimal, optimal and supraoptimal levels of con A in C57Bl/6 murine spleen cells were evaluated in six independent experiments. The results obtained are presented in

tables 2-A through 2-E and in table 3. The data from experiments 2-A, 2-B and experiment 3 are also presented graphically in figures 2-A1 and 2-A2, 2-B1 and 2-B2 and figure 3, respectively. In the following paragraphs, experiments will be referred to by the name of the table containing the data for that particular experiment. As examples, the data tabulated in table 2-A will be referred to as experiment 2-A; that in table 3 as experiment 3 and so forth. Mitogenic responses obtained with each combination of con A and tocopherol are expressed as the arithmetic mean and standard deviation of tritiated-thymidine uptake (DPM /2 ml culture) in triplicate spleen cell cultures. The cell density was 1 X $10^6$ cells/ml in all cases. The relative response (R.R.), expressed as a percentage, was also computed within each con A group. This ratio was obtained by dividing the average mitogenic response of cultures supplemented with tocopherol by that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

The data from experiments 2-A through 2-E were analyzed separately with a two-way analysis of variance allowing for repetition within the experimental groups. As described earlier, the logarithmic transformation ($\log_{10}$) was applied to the data prior to the use of this statistical procedure. Missing observations in a particular group were replaced by the arithmetic mean for that group and the residual and total degrees of freedom adjusted accordingly (Dunn and Clark, 1987). Main effects due to tocopherol and con A, in addition to possible interactions between these two

factors, were tested at the $\alpha = 0.050$ level of statistical significance for a two-tailed test. A one-way analysis of variance was performed on log-transformed data from experiment 3 in order to ascertain significant tocopherol effects in this single-factor experiment (Sokal and Rohlf, 1981). The analysis of variance tables for each experiment described in this section are provided and have been labelled as appendix 2-A through 2-E and appendix 3-1 for ease of reference.

Differences between the mitogenic responses of the different tocopherol groups, at a specified level of con A, were tested for statistical significance ($\alpha = 0.050$) utilizing the T-method for unplanned comparisons as described by Sokal and Ronlf (1981). This parametric statistical test was performed with log-transformed data from each of the six experiments.

In experiment 2-A, C57Bl/6 murine spleen cells were cultured in medium containing 0, 5, 10 and 25 µg/ml DL-α-tocopherol. The suboptimal, optimal and supraoptimal concentrations of con A utilized were respectively 0.1, 2.0 and 10 µg/ml. A control group for the mitogen (0 µg/ml con A) was present. Overall, main effects due to tocopherol ($P \leq 0.001$) and con A ($P \leq 0.001$) were highly significant. The interaction between tocopherol and con A was not statistically significant ($P > 0.050$) in this experiment.

TABLE 2-A

The effects of in vitro tocopherol supplementation (0, 5, 10 and 25 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells

Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.1 | 2.0 | 10.0 |
| 0 | | ---- | 4052 | 280084 | 4150 |
| | | 2248 | 3886 | 350354 | 3192 |
| | | 1937 | 2377 | 270424 | 1984 |
| | MEAN | 2093 | 3438 | 300287 | 3109 |
| | S.D. | ±220 | ±923 | ±43627 | ±1085 |
| | (1) R.R. (%) | 100 | 100 | 100 | 100 |
| 5 | | 2550 | 4862 | 806248 | 5090 |
| | | 2246 | 4734 | 678830 | 6855 |
| | | 4238 | 4125 | 580232 | 6770 |
| | MEAN | 3011 | 4574 | 688437 | 6238 |
| | S.D. | ±1073 | ±394 | ±113314 | ±995 |
| | R.R. (%) | 144 | 133 | 229 | 201 |

TABLE 2-A (cont'd)

The effects of in vitro tocopherol supplementation (0, 5, 10 and 25 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells

Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.1 | 2.0 | 10.0 |
| 10 | | 3672 | 7472 | 625772 | 5121 |
| | | 7646 | 5079 | 660525 | 5081 |
| | | 4526 | 5788 | 534757 | 3929 |
| | MEAN | 5281 | 6113 | 607018 | 1710 |
| | S.D. | ±2092 | ±1229 | ±64948 | ±677 |
| | R.R. (%) | 252 | 178 | 202 | 151 |
| 25 | | 2684 | 3161 | 573416 | 1711 |
| | | 3968 | 3362 | 632488 | 5175 |
| | | 3522 | 4581 | 533339 | 5260 |
| | MEAN | 3391 | 3701 | 579748 | 5050 |
| | S.D. | ±652 | ±768 | ±49877 | ±294 |
| | R.R. (%) | 162 | 108 | 193 | 162 |

TABLE 2-A (cont'd)

The effects of in vitro tocopherol supplementation (0, 5, 10 and 25 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells

Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

Figure 2-A1. Mitogenic effects of *in vitro* tocopherol supplementation in non-stimulated C57Bl/6 murine spleen cells. DL-α-tocopherol present at final concentrations of 0, 5, 10 and 25 µg/ml.

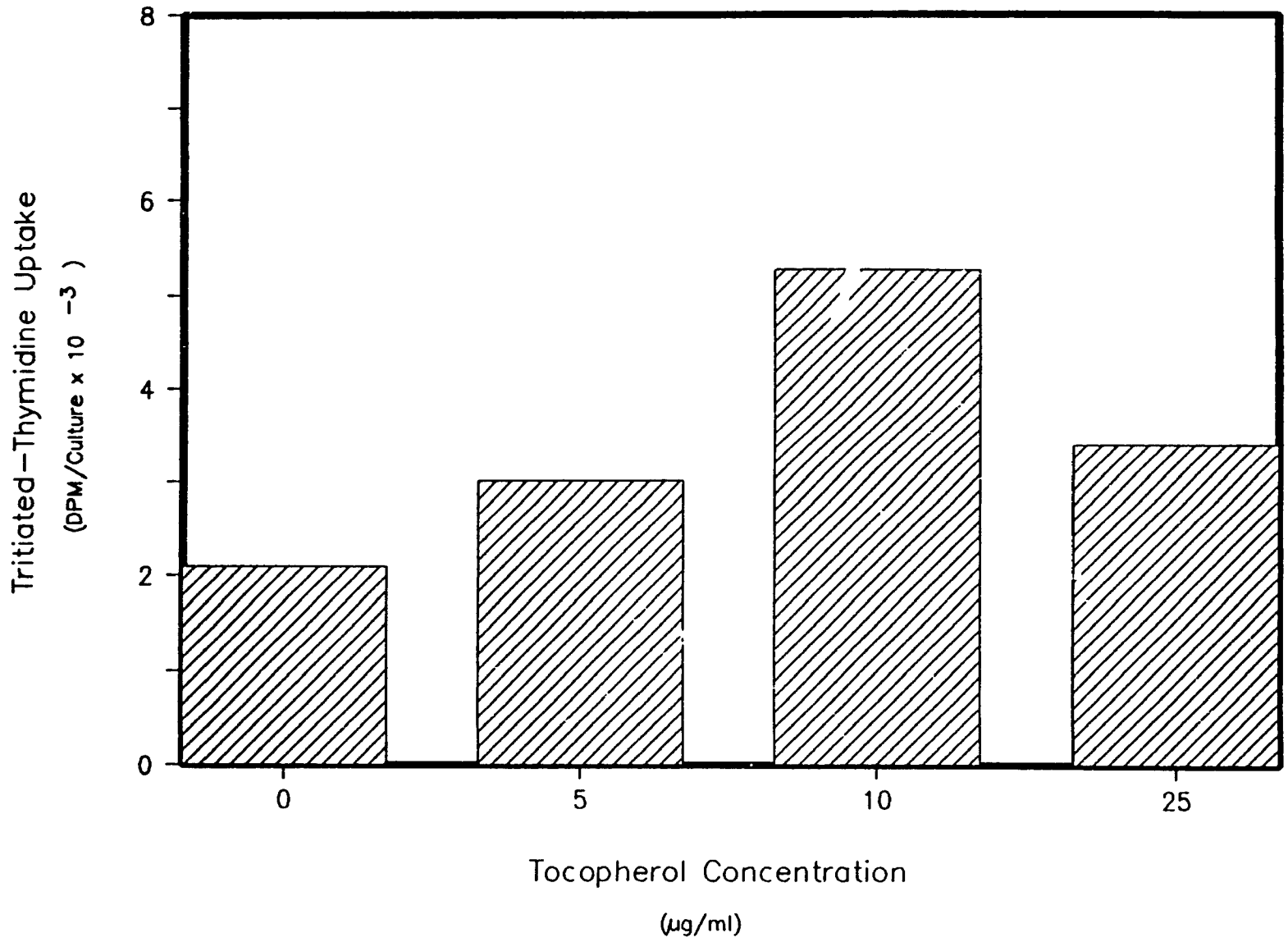
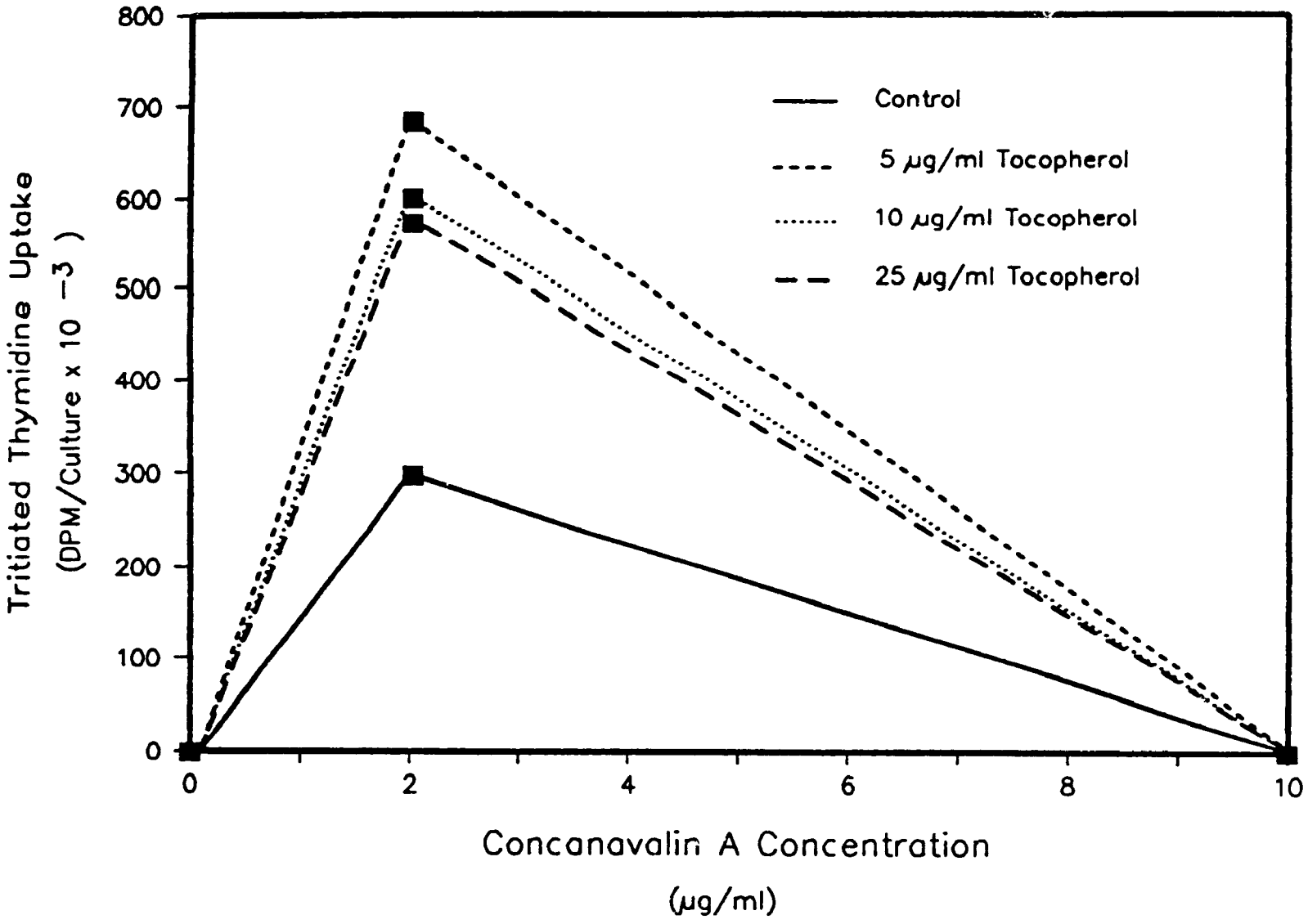

Tritiated-Thymidine Uptake
(DPM/Culture x 10 -3 )
8
6
4
2
0
0
5
10
25
Tocopherol Concentration
(µg/ml)

Figure 2-A2. The effects of *in vitro* tocopherol supplementation on mitogenic responses in concanavalin A-stimulated C57Bl/6 murine spleen cells. DL-α-tocopherol present at final concentrations of 0, 5, 10 and 25 μg/ml.


Tritiated Thymidine Uptake
(DPM/Culture x 10 −3 )
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
Concanavalin A Concentration
(µg/ml)
Control
5 µg/ml Tocopherol
10 µg/ml Tocopherol
25 µg/ml Tocopherol

Although tocopherol appeared to be slightly mitogenic in the absence of con A, no two groups were significantly different at the 0.050 level. At the suboptimal (0.1 µg/ml) level of con A, the mitogenic response of spleen cells cultured with 10 µg/ml DL-α-tocopherol was significantly greater than that measured in the tocopherol control group (0 µg/ml DL-α-tocopherol). Similarly, mitogenic responses to optimal con A (2 µg/ml) were considerably greater ($P \leq 0.050$) in the 5, 10 and 25 µg/ml tocopherol groups. The average stimulation index with tocopherol was 208%. In this experiment, the greatest enhancement of the mitogenic response to optimal con A was achieved with 5 µg/ml DL-α-tocopherol. However, the differences observed between the mitogenic responses of the three tocopherol groups (5, 10 and 25 µg/ml) were not statistically significant. Mitogenic responses to the supraoptimal (10 µg/ml) concentration of con A were also stimulated by tocopherol. Statistical significance was attained with 5 µg/ml DL-α-tocopherol. The extent of this stimulation was similar (R.R. = 201%) to that produced by tocopherol at the optimal level of con A.

Tocopherol concentrations of 5, 50 and 100 µg/ml were evaluated in experiment 2-B with respect to the modification of con A mitogenesis. The suboptimal, optimal and supraoptimal concentrations of con A used in this experiment were 0.5, 2 and 5 µg/ml, respectively. Control groups for both tocopherol and con A were present. As in the previous experiment, the two-way analysis of variance revealed highly significant ($P \leq 0.001$) main effects for

TABLE 2-B

The effects of in vitro tocopherol supplementation (0, 5, 50 and 100 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells

Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.5 | 2.0 | 5.0 |
| 0 | | 3038 | 21496 | 581531 | 81534 |
| | | 2151 | 33722 | 558726 | 87183 |
| | | 1723 | 21443 | 391694 | 61579 |
| | MEAN | 2304 | 25554 | 510650 | 76765 |
| | S.D. | ±671 | ±7074 | ±103648 | ±13452 |
| | (1) R.R. (%) | 100 | 100 | 100 | 100 |
| 5 | | 3698 | 85372 | 1015643 | 77371 |
| | | 3442 | 79171 | 636445 | 99243 |
| | | 5858 | 79575 | 849706 | 110078 |
| | MEAN | 4333 | 81373 | 833931 | 95564 |
| | S.D. | ±1327 | ±3469 | ±190091 | ±16661 |
| | R.R. (%) | 188 | 318 | 163 | 124 |

TABLE 2-B (cont'd)

The effects of in vitro tocopherol supplementation ( 0, 5, 50 and 100 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.5 | 2.0 | 5.0 |
| 50 | | 5053 | 47489 | 427812 | 47497 |
| | | 7508 | 46762 | 464970 | 11363 |
| | | 4839 | 45813 | 452791 | 49123 |
| | MEAN | 5800 | 46688 | 448524 | 45994 |
| | S.D. | ±1483 | ±840 | ±18943 | ±4092 |
| | R.R. (%) | 252 | 183 | 88 | 60 |
| 100 | | 4125 | 32975 | 64054 | 17791 |
| | | 5102 | 34039 | 66008 | 13383 |
| | | 5698 | 30733 | 68196 | 15527 |
| | MEAN | 4975 | 32582 | 66086 | 15567 |
| | S.D. | ±791 | ±1688 | ±2072 | ±2204 |
| | R.R. (%) | 216 | 127 | 13 | 20 |

TABLE 2-B (cont'd)

The effects of in vitro tocopherol supplementation ( 0, 5, 50 and 100 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

Figure 2-B1. Mitogenic effects of *in vitro* tocopherol supplementation in non-stimulated C57Bl/6 murine spleen cells. DL-α-tocopherol present at final concentrations of 0, 5, 50 and 100 µg/ml.

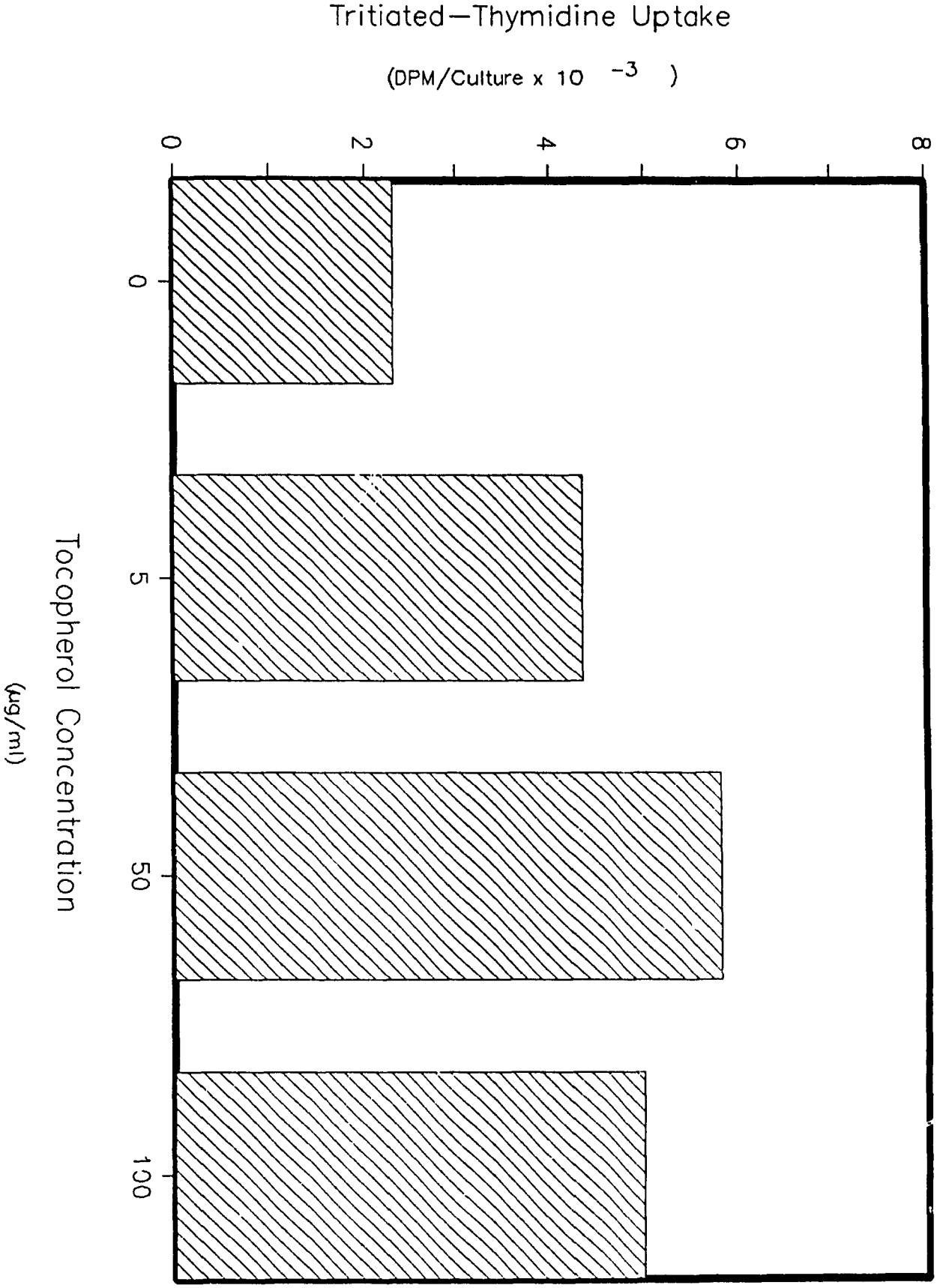
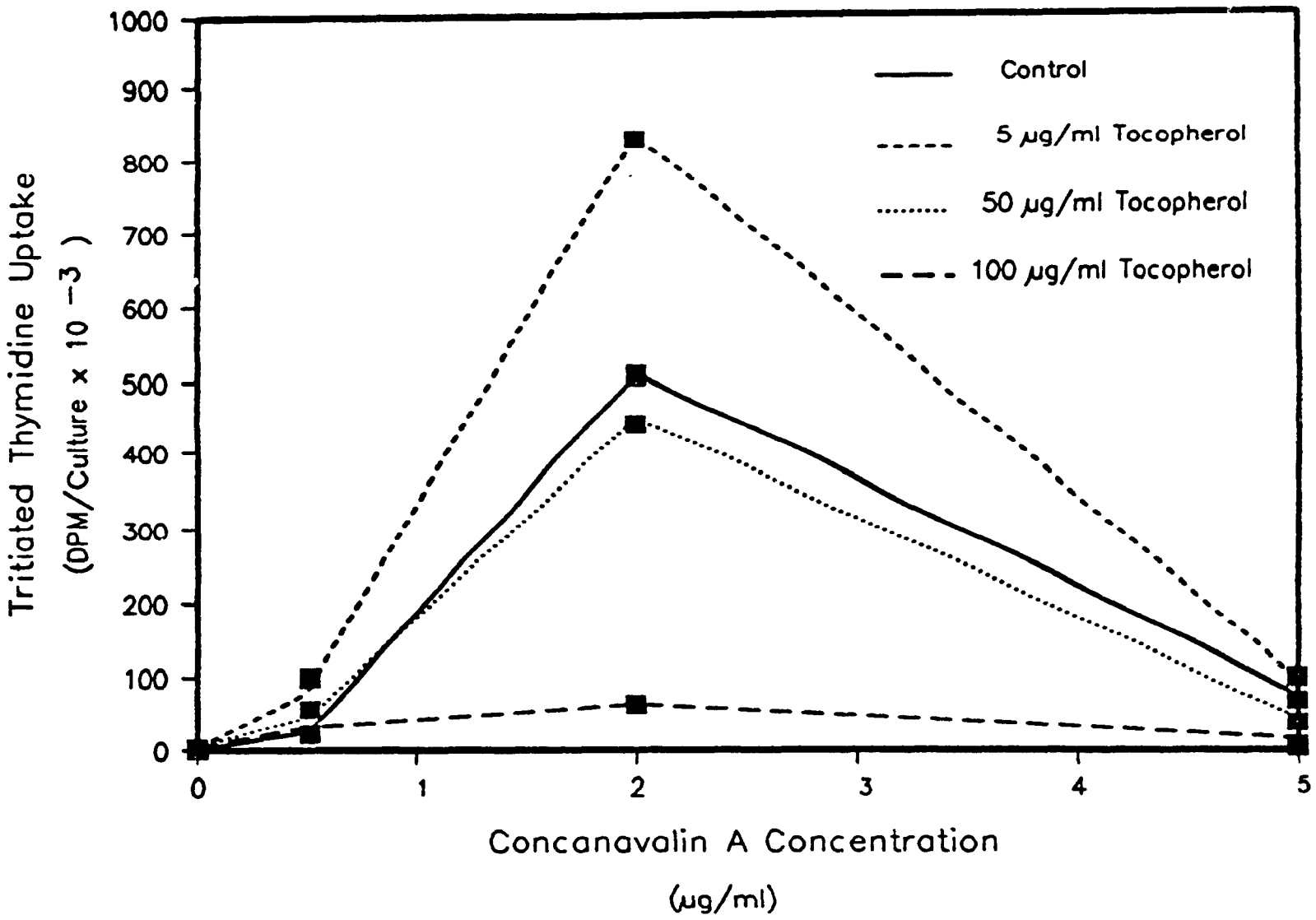

Tritiated—Thymidine Uptake
(DPM/Culture x 10 -3 )
0
2
4
6
8
Tocopherol Concentration
(µg/ml)
0
5
50
100

Figure 2-B2. The effects of in vitro tocopherol supplementation on mitogenic responses in concanavalin A-stimulated C57Bl/6 murine spleen cells. DL-α-tocopherol present at final concentrations of 0, 5, 50 and 100 μg/ml.


Tritiated Thymidine Uptake
(DPM/Culture x 10 −3 )
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Concanavalin A Concentration
(µg/ml)
Control
5 µg/ml Tocopherol
50 µg/ml Tocopherol
100 µg/ml Tocopherol

tocopherol and con A. In addition, the interaction between both factors was also statistically significant ($P \leq 0.001$).

In the absence of con A, statistically significant mitogenic responses were observed in the 50 and 100 µg/ml tocopherol groups. The response at 100 µg/ml DL-α-tocopherol was smaller (4975 ± 794 DPM /2 ml) but not significantly different than that obtained at 50 µg/ml (5800 ± 1483 DPM /2 ml). The response observed with 5 µg/ml DL-α-tocopherol (R.R. = 188) did not differ significantly from that of the control, 50 and 100 µg/ml tocopherol groups. With suboptimal mitogenic stimulation (0.5 µg/ml con A), responses in the 5 (R.R. = 318%) and 50 (R.R. = 183%) µg/ml tocopherol groups were greatly enhanced ($P \leq 0.050$). The response observed with 100 µg/ml DL-α-tocopherol was not significantly different from that of the tocopherol control group. Mitogenic responses to optimal con A (2 µg/ml) were also potentiated in spleen cells cultured with 5 µg/ml DL-α-tocopherol (R.R. = 163%, $P \leq 0.050$). When con A was present at the supraoptimal concentration (5 µg/ml), a slight but not statistically significant enhancement (R.R. = 124%) of the mitogenic response was noted with 5 µg/ml DL-α-tocopherol. Furthermore, there was a tendency for the higher concentrations of tocopherol to progressively inhibit mitogenic responses produced by both optimal and supraoptimal levels of con A. Mitogenic responses to optimal con A were significantly curtailed (R.R. = 13%) in the 100 µg/ml tocopherol group. Inhibitory effects were also observed with supraoptimal con A when tocopherol was present at 50 (R.R. = 60%)

and 100 µg/ml (R.R. = 20%).

In experiment 2-C, DL-α-tocopherol was added to C57Bl/6 murine spleen cell cultures yielding final concentrations of 0, 1 and 5 µg/ml. The concentrations of con A utilized were 0, 0.5, 2 and 5 µg/ml. Overall, the effects due to con A, tocopherol and the interaction between both agents, were highly significant ($P \leq 0.001$).

Within the con A control group, statistically significant mitogenic responses were obtained with both 1 (R.R. = 142%) and 5 µg/ml DL-α-tocopherol (R.R. = 195%). Furthermore, the response to 5 µg/ml tocopherol (R.R. = 195%) was significantly greater than that obtained with the lower (1 µg/ml) concentration of tocopherol (R.R. = 142%). Mitogenic responses produced by suboptimal con A (0.5 µg/ml) were potentiated by both levels of tocopherol in this experiment. The average stimulation index achieved with tocopherol was 190%. However, differences noted between the two tocopherol groups were not significant. In contrast to the findings reported in experiments 2-A and 2-B, tocopherol did not enhance the blastogenic response of murine spleen cells to optimal (2 µg/ml) con A. A significant stimulation (R.R. = 229%) of the response to supraoptimal con A (5 µg/ml) was achieved with 5 µg/ml tocopherol.

The effects of <u>in vitro</u> tocopherol supplementation on mitogenic responses produced by 0, 0.5, 1, 2 and 5 µg/ml con A in C57Bl/6

TABLE 2-C

The effects of in vitro tocopherol supplementation (0, 1 and 5 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.5 | 2.0 | 5.0 |
| 0 | | ---- | 225129 | 2147513 | 770241 |
| | | 4262 | 209090 | 2141032 | 674398 |
| | | 4165 | 194029 | 2128497 | 562829 |
| | MEAN | 4214 | 209416 | 2139014 | 669156 |
| | S.D. | ±68 | ±15552 | ±9667 | ±103805 |
| | (1) R.R. (%) | 100 | 100 | 100 | 100 |
| 1 | | 6786 | 401462 | 2284206 | 888273 |
| | | 4967 | 476168 | 2235742 | 869853 |
| | | 6249 | 410121 | 2143624 | 623112 |
| | MEAN | 6001 | 429250 | 2221191 | 793746 |
| | S.D. | ±934 | ±40862 | ±71412 | ±148069 |
| | R.R. (%) | 142 | 205 | 104 | 119 |

TABLE 2-C (cont'd)

The effects of in vitro tocopherol supplementation (0, 1 and 5 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.5 | 2.0 | 5.0 |
| 5 | | 7632 | 407395 | 2197685 | ------- |
| | | 8424 | 330651 | 2213076 | 1538239 |
| | | 8615 | 364709 | 2248515 | 1534025 |
| | MEAN | 8224 | 367585 | 2219759 | 1536132 |
| | S.D. | ±521 | ±38453 | ±26066 | ±2980 |
| | R.R. (%) | 195 | 175 | 104 | 229 |

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group ( 0 µg/ml DL-α-tocopherol).

murine spleen cells were evaluated in experiment 2-D. The concentrations of tocopherol utilized were 0, 5 and 100 µg/ml. All observations for the 100 µg/ml tocopherol group at 0 µg/ml con A were missing. Therefore, the mitogen control group was excluded from the analysis of variance. Overall, the effects of con A on mitogenic responses were highly significant ($P \leq 0.001$). Similarly, main effects due to tocopherol were also statistically significant ($0.010 < P \leq 0.050$) but to a much lesser degree than for con A. The interaction between tocopherol and con A was not significant ($P > 0.050$).

The effects of tocopherol on the mitogenic responses obtained with the different concentrations of con A utilized were weak and not as delineated as in the previous experiments. Tocopherol at a final concentration of 5 µg/ml, significantly enhanced mitogenic responses to 1 µg/ml con A (R.R.= 185%) and 2 µg/ml con A (R.R. = 195%). At these levels of con A, 100 µg/ml tocopherol yielded somewhat reduced but not significantly different responses than obtained with 5 µg/ml tocopherol.

In experiment 2-E, tocopherol was added to murine spleen cell cultures yielding a final concentration of 5 µg/ml. Mitogenic responses were produced by suboptimal (0.5 µg/ml), optimal (2 µg/ml) and supraoptimal (10 µg/ml) levels of con A. The results of the two-way analysis of variance indicated that the effects of con A on mitogenic responses and the interaction between tocopherol and the

TABLE 2-D

The effects of in vitro tocopherol supplementation (0, 5 and 100 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1.0 | 2.0 | 5.0 |
| 0 | | ---- | 77054 | 258133 | 225974 | 110729 |
| | | 2481 | 50187 | 260727 | 274362 | 91667 |
| | | 1956 | 50736 | 177319 | 235134 | 68156 |
| | MEAN | 2219 | 59426 | 232060 | 245157 | 90184 |
| | S.D. | ±371 | ±15267 | ±47424 | ±25704 | ±21325 |
| | (1) R.R. (%) | 100 | 100 | 100 | 100 | 100 |
| 5 | | 2955 | 50354 | 419823 | 361882 | 83526 |
| | | 1283 | 54194 | 517089 | 568689 | 80297 |
| | | 2022 | 69328 | 355110 | 504311 | 89411 |
| | MEAN | 2087 | 57959 | 430674 | 478294 | 84411 |
| | S.D. | ±838 | ±19031 | ±81533 | ±105830 | ±4621 |
| | R.R. (%) | 94 | 98 | 185 | 195 | 94 |

TABLE 2-D (cont'd)

The effects of in vitro tocopherol supplementation (0, 5 and 100 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1.0 | 2.0 | 5.0 |
| 100 | | ---- | 87235 | 289871 | 284611 | 116634 |
| | | ---- | 97570 | 243023 | 477817 | 89904 |
| | | ---- | 66923 | 300587 | 383368 | 50237 |
| | MEAN | N/A | 83909 | 277827 | 381932 | 85592 |
| | S.D. | N/A | ±15592 | ±30614 | ±96611 | ±33408 |
| | R.R. (%) | N/A | 141 | 120 | 156 | 95 |

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

TABLE 2-E

The effects of in vitro tocopherol supplementation (0 and 5 µg/ml) on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|---|
| | | 0 | 0.5 | 2.0 | 10.0 |
| 0 | | 8942 | 226395 | 1878530 | ------ |
| | | 8285 | 277517 | 1877382 | 270045 |
| | | 7074 | 265227 | 2024117 | 252730 |
| | MEAN | 8100 | 256380 | 1926676 | 261387 |
| | S.D. | ±948 | ±26685 | ±84388 | ±12244 |
| | (1) R.R. (%) | 100 | 100 | 100 | 100 |
| 5 | | ----- | 331702 | 2027412 | ------ |
| | | 10427 | 339376 | 2048738 | 128039 |
| | | 10663 | 346467 | 1960617 | 127480 |
| | MEAN | 10545 | 339182 | 2012256 | 127759 |
| | S.D. | ±167 | ±7384 | ±45974 | ±395 |
| | R.R. (%) | 130 | 132 | 104 | 49 |

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

mitogen were very significant ($P \leq 0.001$). Overall, the main effects due to tocopherol did not attain statistical significance ($P > 0.050$) in this experiment.

As indicated in table 2-E, tocopherol was mitogenic (R.R. = 130%) for murine spleen cells in the absence of con A. Furthermore, the response to suboptimal con A was potentiated by tocopherol (R.R. = 132%). In contrast, at the optimal concentration of con A, mitogenic responses obtained with 5 µg/ml tocopherol were not significantly different than those of the tocopherol control group. Mitogenic responses produced by 10 µg/ml con A were remarkably suppressed (R.R. = 49%) in the presence of tocopherol.

The effects of increasing tocopherol concentrations on the mitogenic response of C57Bl/6 murine spleen cells to optimal (2 µg/ml) con A were evaluated in experiment 3. The concentrations of DL-α-tocopherol used in this experiment were 0, 1, 5 and 100 µg/ml. The overall effect of tocopherol on the blastogenic response of murine spleen cells was tested with a one-way analysis of variance and found to be highly significant ($P \leq 0.001$).

Mitogenic responses were greatly enhanced ($P \leq 0.050$) when tocopherol was present at 1 (R.R. = 202%) and 5 µg/ml (R.R. = 208%).

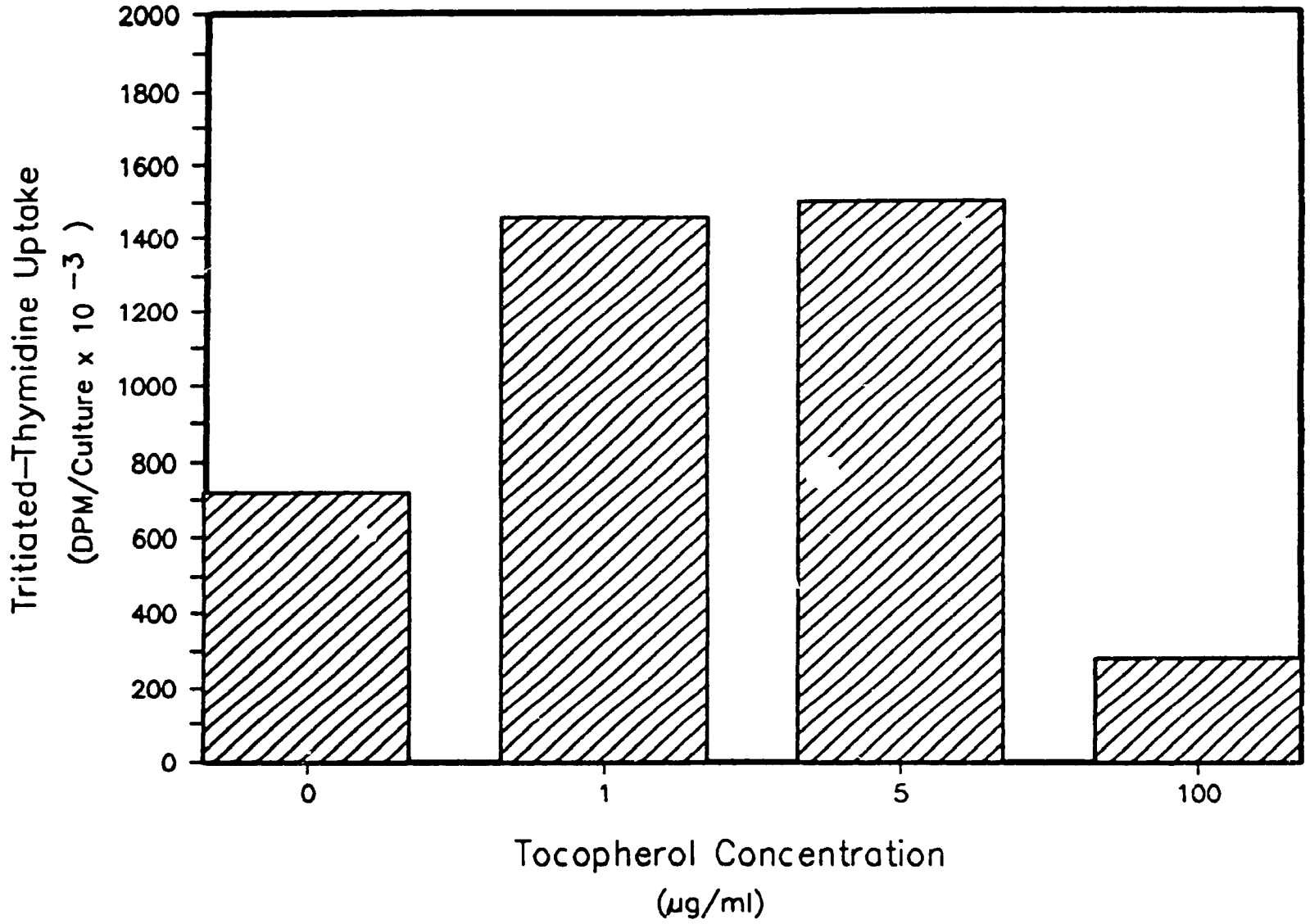
TABLE 3

The effects of increasing tocopherol concentrations on mitogenic responses produced by optimal concanavalin A (2 µg/ml) in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| | DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | | |
|---|---|---|---|---|
| | 0 | 1 | 5 | 100 |
| | 587372 | 1464519 | 1530251 | 336477 |
| | 731924 | 1483862 | 1516778 | 247155 |
| | 841977 | 1415342 | 1446570 | 256813 |
| MEAN | 720424 | 1454574 | 1497866 | 280118 |
| ±S.D. | ±127691 | ±35326 | ±44932 | ±49020 |
| (1) R.R. (%) | 100 | 202 | 208 | 39 |

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

Figure 3. The effects of in vitro tocopherol supplementation on lymphoproliferative responses produced by an optimal concentration of concanavalin A (2 µg/ml) in C57Bl/6 murine spleen cells.


Tritiated-Thymidine Uptake
(DPM/Culture x 10 -3 )
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
5
100
Tocopherol Concentration
(µg/ml)

There was no significant difference between the responses observed at these concentrations of tocopherol. Moreover, when the concentration of DL-α-tocopherol was increased to 100 µg/ml, mitogenic responses to optimal con A were markedly suppressed (R.R. = 39%).

In order to evaluate the overall effects of in vitro tocopherol supplementation on mitogenic responses produced by suboptimal (0.5 µg/ml), optimal (2 µg/ml) and supraoptimal (5 µg/ml) levels of con A in C57Bl/6 murine spleen cells, a non-parametric statistical analysis was performed on combined data derived from experiments 2-A through 2-E and experiment 3. The following is a description of the method employed. Data from groups (specific tocopherol-con A combinations) common to each of the six experiments were combined The individual observations from the three tocopherol groups evaluated (0, 5 and 100 µg/ml) at a particular level of con A were then divided by the average mitogenic response (DPM /2 ml culture) of the tocopherol control group (0 µg/ml DL - tocopherol) for that concentration of mitogen. This ratio-transformed data (total of 127 observations) is tabulated in table 4. Mitogenic responses observed at each concentration of tocopherol for a particular level of con A were compared with the Wilcoxon two-sample test (Sokal and Rohlf, 1981). The statistical analysis is presented in appendix 4. All comparisons were two-tailed at the $\alpha = 0.050$ level of statistical significance.

TABLE 4

The effects of in vitro tocopherol supplementation on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells.

Pooled data from tables 2-A to 2-E and table 3**.

| DL-α-TOCOPHEROL CONCENTRATION (μg/ml) | CONCANAVALIN A CONCENTRATION (μg/ml) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 2.0 | 5.0 |
| 0 | | 0.841 0.883 | 0.993 0.922 | 1.062 |
| | 1.074 1.118 | 1.320 1.082 | 1.167 1.119 | 1.136 |
| | 0.925 0.881 | 0.839 1.035 | 0.901 0.959 | 0.802 |
| | 1.319 1.104 | 1.075 | 1.139 0.975 | 1.151 |
| | 0.934 1.022 | 0.998 | 1.094 0.974 | 1.008 |
| | 0.748 0.873 | 0.927 | 0.767 1.051 | 0.841 |
| | | 1.297 | 1.004 0.815 | 1.228 |
| | 1.011 | 0.850 | 1.001 1.016 | 1.016 |
| | 0.988 | 0.854 | 0.995 1.169 | 0.756 |
| MEAN | 1.000 | 1.000 | 1.000 | 1.000 |
| (1) R.R. (%) | 100 | 100 | 100 | 100 |

TABLE 4 (cont'd)

The effects of in vitro tocopherol supplementation on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells.

Pooled data from tables 2-A to 2-E and table 3**.

| DL-α-TOCOPHEROL CONCENTRATION (μg/ml) | CONCANAVALIN A CONCENTRATION (μg/ml) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 2.0 | 5.0 |
| 5 | 1.218 1.332 | 3.341 1.294 | 2.685 1.476 | 1.008 |
| | 1.073 0.578 | 3.098 1.324 | 2.261 2.320 | 1.293 |
| | 2.025 0.911 | 3.114 1.351 | 1.932 2.057 | 1.434 |
| | 1.605 1.287 | 1.945 | 1.989 1.052 | 2.299 |
| | 1.494 1.316 | 1.579 | 1.246 1.063 | 2.292 |
| | 2.543 | 1.742 | 1.664 1.018 | |
| | 1.811 | 0.847 | 1.068 2.124 | 0.926 |
| | 1.999 | 0.912 | 1.045 2.105 | 0.890 |
| | 2.044 | 1.167 | 1.002 2.008 | 0.991 |
| MEAN | 1.517 | 1.809 | 1.673 | 1.392 |
| R.R. (%) | 152* | 181* | 167* | 139 |

TABLE 4 (cont'd)

The effects of in vitro tocopherol supplementation on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells.

Pooled data from tables 2-A to 2-E and table 3**.

| DL-α-TOCOPHEROL CONCENTRATION (μg/ml) | CONCANAVALIN A CONCENTRATION (μg/ml) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 2.0 | 5.0 |
| 100 | 1.790 | 1.290 | 0.125 | 0.232 |
| | 2.214 | 1.332 | 0.129 | 0.174 |
| | 2.473 | 1.203 | 0.134 | 0.202 |
| | | 1.468 | 1.161 | 1.293 |
| | | 1.642 | 1.949 | 0.997 |
| | | 1.126 | 1.564 | 0.557 |
| | | | 0.467 | |
| | | | 0.343 | |
| | | | 0.356 | |
| MEAN | 2.159 | 1.343 | 0.692 | 0.576 |
| R.R. (%) | 216* | 134* | 69 | 58 |

TABLE 4 (cont'd)

The effects of in vitro tocopherol supplementation on mitogenic responses stimulated by concanavalin A in C57Bl/6 murine spleen cells.

Pooled data from tables 2-A to 2-E and table 3++.

++ Individual observations at each concentration of con A utilized were divided by the average mitogenic response of the tocopherol control group for that con A group. This process was repeated for each experiment from which common data elements were pooled.

(1) R.R.: Relative response defined as the ratio of mitogenic responses in cultures supplemented with tocopherol to that of the tocopherol control group (0 µg/ml DL-α-tocopherol).

* Denotes a response significantly different ($p \leq 0.050$) from that of the tocopherol control group (0 µg/ml DL-α-tocopherol). The exact P-values for the Wilcoxon test-statistic (U) are tabulated in appendix 4.

The results obtained from this statistical analysis clearly indicate that in the absence of con A, tocopherol was mitogenic when present at final concentrations of 5 ($P = 0.004$) and 100 µg/ml ($P = 0.009$). Although the response observed for the 100 µg/ml tocopherol group (mean = 2.159) was greater than that of the 5 µg/ml tocopherol group (mean = 1.517), this difference was not statistically significant ($P = 0.078$). Furthermore, these results need to be interpreted with caution given the relatively small number of observations available ($n = 3$). Perhaps, if a greater number of observations were present for the 100 µg/ml tocopherol group, differences observed between this group and the 5 µg/ml tocopherol group would have attained statistical significance.

When con A was present at the suboptimal concentration (0.5 µg/ml), enhanced mitogenic responses were observed in both the 5 (R.R. = 181%; $P = 0.003$) and 100 µg/ml tocopherol groups (R.R. = 134%, $P = 0.005$). As with the mitogen control group, the differences noted between these responses were not statistically significant ($P = 0.349$).

Mitogenic responses to optimal con A (2 µg/ml) were also greatly enhanced when murine spleen cell cultures were supplemented with tocopherol at a final concentration of 5 µg/ml (R.R. = 167%, $P < 0.001$). When the concentration of tocopherol was increased to 100 µg/ml, mitogenic responses were somewhat suppressed (R.R. = 69%) but were not significantly different ($P = 0.077$) from those of

the tocopherol control group. Mitogenic responses in the 100 µg/ml tocopherol group (mean = 0.692) were significantly smaller (P = 0.033) than those obtained with 5 µg/ml tocopherol (mean = 1.673).

The effects of in vitro tocopherol supplementation on mitogenic responses produced by the supraoptimal level of con A (5 µg/ml) were similar to those observed with optimal (2 µg/ml) con A. Specifically, when tocopherol was present at 5 µg/ml, a slight (R.R. = 139%) but not statistically significant (P = 0.136) stimulation of the blastogenic response was observed. Mitogenic responses obtained with 100 µg/ml DL-α-tocopherol were curtailed and significantly different (R.R. = 58%, P = 0.033) from those noted in the 5 µg/ml tocopherol group (R.R. = 139%).

Spleen Cell Cultures without Adherent Accessory Cells

A single experiment was conducted to evaluate the effects of in vitro tocopherol supplementation on mitogenic responses produced by suboptimal (0.5 µg/ml) and optimal (2.0 µg/ml) concentrations of con A in cultures with and without adherent C57Bl/6 murine spleen cells. Tocopherol was added to cell cultures yielding a final concentration of 5 µg/ml. The results obtained are tabulated in table 5 and are also graphically presented in figure 4. Mitogenic responses observed in each of the experimental groups are expressed as the arithmetic mean (DPM /2 ml culture) and standard deviation of tritiated-thymidine uptake in triplicate spleen cell cultures.

TABLE 5

The effects of in vitro tocopherol supplementation (5 µg/ml) on mitogenic responses produced by suboptimal (0.5 µg/ml) and optimal (2.0 µg/ml) levels of concanavalin A in cultures of C57Bl/6 murine spleen cells containing adherent cells (SC) and in cultures depleted of adherent cells (NASC). Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

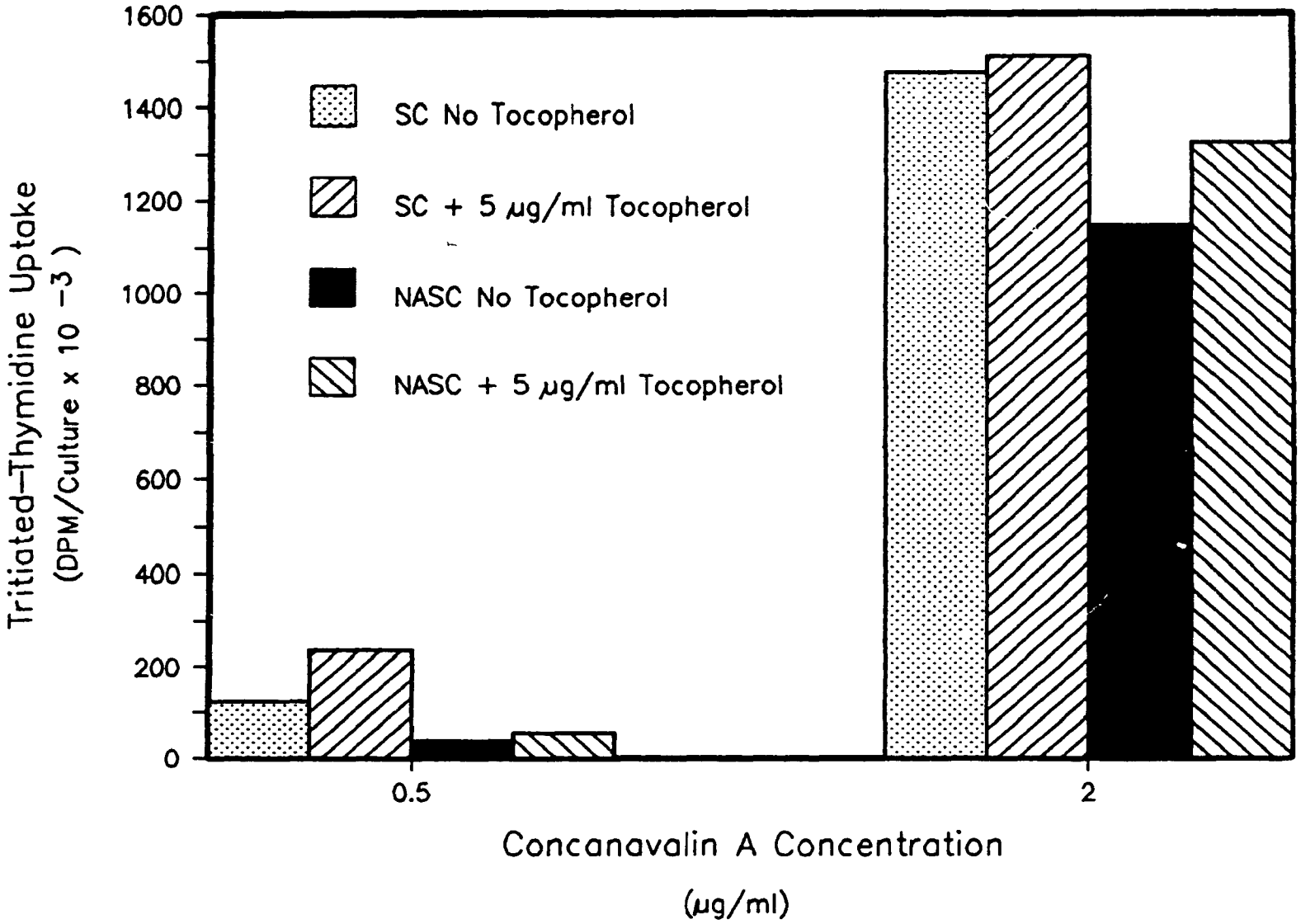
| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | |
|---|---|---|---|
| | | 0.5 | 2.0 |
| 0 | SC | 84652 | 1327504 |
| | | 126391 | 1549217 |
| | | 168654 | 1543812 |
| | MEAN | 126566 | 1473511 |
| | S.D. | ±42001 | ±126475 |
| 0 | NASC | 43729 | 1067211 |
| | | 36154 | 1235507 |
| | | 34012 | 1139017 |
| | MEAN | 37965 | 1147245 |
| | S.D. | ±5105 | ±84449 |

TABLE 5 (cont'd)

The effects of in vitro tocopherol supplementation (5 µg/ml) on mitogenic responses produced by suboptimal (0.5 µg/ml) and optimal (2.0 µg/ml) levels of concanavalin A in cultures of C57Bl/6 murine spleen cells containing adherent cells (SC) and in cultures depleted of adherent cells (NASC). Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| DL-α-TOCOPHEROL CONCENTRATION (µg/ml) | | CONCANAVALIN A CONCENTRATION (µg/ml) | |
|---|---|---|---|
| | | 0.5 | 2.0 |
| 5 | SC | 214399 | 1499835 |
| | | 185165 | 1539355 |
| | | 311325 | 1490606 |
| | MEAN | 236963 | 1509932 |
| | S.D. | ±66037 | ±25895 |
| 5 | NASC | ----- | 1367521 |
| | | 57150 | 1254798 |
| | | 58092 | 1356263 |
| | MEAN | 57621 | 1326194 |
| | S.D. | ±666 | ±62086 |

Figure 4. The effects of *in vitro* tocopherol (5 µg/ml) supplementation on mitogenic responses produced by suboptimal (0.5 µg/ml) and optimal (2.0 µg/ml) levels of concanavalin A in cultures of C57Bl/6 murine spleen cells containing adherent accessory cells (SC) and in cultures depleted of adherent accessory cells (NASC).


1600
1400
1200
1000
800
600
400
200
0
Tritiated-Thymidine Uptake
(DPM/Culture x 10 -3 )
SC No Tocopherol
SC + 5 µg/ml Tocopherol
NASC No Tocopherol
NASC + 5 µg/ml Tocopherol
0.5
2
Concanavalin A Concentration
(µg/ml)

Although performed with preliminary data from a single experiment, the overall effects of tocopherol, con A and cell culture-type (either with or without adherent cells) on the resulting mitogenic responses were assessed with a three-way analysis of variance (Dunn and Clark, 1987). Planned (a priori) comparisons between the responses of selected groups were evaluated with Student's t-test for independent observations (Sokal and Rohlf, 1981). All statistical tests were two-tailed at the $\alpha = 0.050$ level of significance and performed with log-transformed data. The results of the three-way analysis of variance and planned comparisons are presented in appendices 5-1 and 5-2, respectively.

The analysis of variance revealed highly significant main effects ($P \leq 0.001$) for tocopherol, con A and cell culture-type. This information suggests that each of these factors affected mitogenic responses to some extent in this experiment. Furthermore, statistically significant interactions were noted between tocopherol and con A ($P \leq 0.050$) and between con A and cell culture-type ($P \leq 0.001$).

Mitogenic responses produced by suboptimal con A (0.5 µg/ml) were enhanced by tocopherol in vitro in both cultures with (R.P. = 187%) and without adherent murine spleen cells (R.R. = 152%). This stimulation was statistically significant for cultures depleted of adherent accessory cells ($P = 0.023$) but not for cultures containing adherent cells ($P = 0.064$). However, when the comparison was

repeated using a one-tailed test to determine if mitogenic responses in tocopherol-supplemented cultures with adherent cells were greater than in cultures containing adherent cells and not supplemented with 5 µg/ml DL-α-tocopherol; differences between these groups attained statistical significance (P = 0.032).

When murine spleen cell suspensions were depleted of macrophages (adherent accessory cells), mitogenic responses observed in cultures without adherent cells were significantly suppressed (R.R. = 30%; P = 0.005) compared to the corresponding responses measured in cultures containing adherent cells. The addition of tocopherol in vitro to cultures without adherent cells was stimulatory and partially restored (R.R. = 46%, P = 0.023) mitogen responsiveness to suboptimal con A. There was no significant difference (P = 0.063) between mitogenic responses observed in cultures without adherent cells supplemented with 5 µg/ml DL-α-tocopherol and cultures containing adherent cells without tocopherol supplementation.

At the optimal level of con A (2 µg/ml), mitogenic responses in cultures without adherent spleen cells were significantly enhanced (R.R. = 116%; P = 0.044) in the presence of tocopherol. In contrast to this finding, tocopherol in vitro (5 µg/ml) did not stimulate mitogenic responses to optimal con A in cultures containing adherent cells (R.R. = 102%; P = 0.632). As before, significantly reduced mitogenic responses (R.R. = 78%; P = 0.020) were noted in cultures depleted of adherent accessory cells (macrophages). When

tocopherol was added to spleen cell cultures without adherent cells, mitogenic responses to optimal con A were partially restored (R.R. = 90%) but were not significantly different (P = 0.148) from responses obtained in cultures containing adherent cells and without tocopherol supplementation.

Time-Course of Tritiated-Thymidine Incorporation

The time-course of tritiated-thymidine uptake in C57Bl/6 murine spleen cells mitogenically stimulated with 2 µg/ml con A and cultured with and without 5 µg/ml DL-α-tocopherol was monitored over a 27-hour period. Table 6 summarizes the results of this experiment. The responses obtained during each of the 3-hour feeding intervals with radiolabelled thymidine are expressed as the arithmetic mean and standard deviation of tritiated-thymidine uptake (DPM /2 ml culture) in triplicate spleen cell cultures. The individual observations for each sampling time are tabulated in appendix 6. The responses observed for the control and 5 µg/ml tocopherol groups at each sampling time (3, 6, 9, 12, 15, 18, 21, 24 and 27 hours) are also presented graphically in figure 5.

As indicated in table 6, the tritiated-thymidine uptake profiles of both groups were very similar. Some mitotic activity was observed in the control (15412 ± 1213 DPM /2 ml culture) and tocopherol (15987 ± 898 DPM /2 ml culture) groups three hours following the start of spleen cell cultures with con A. Thereafter,

TABLE 6

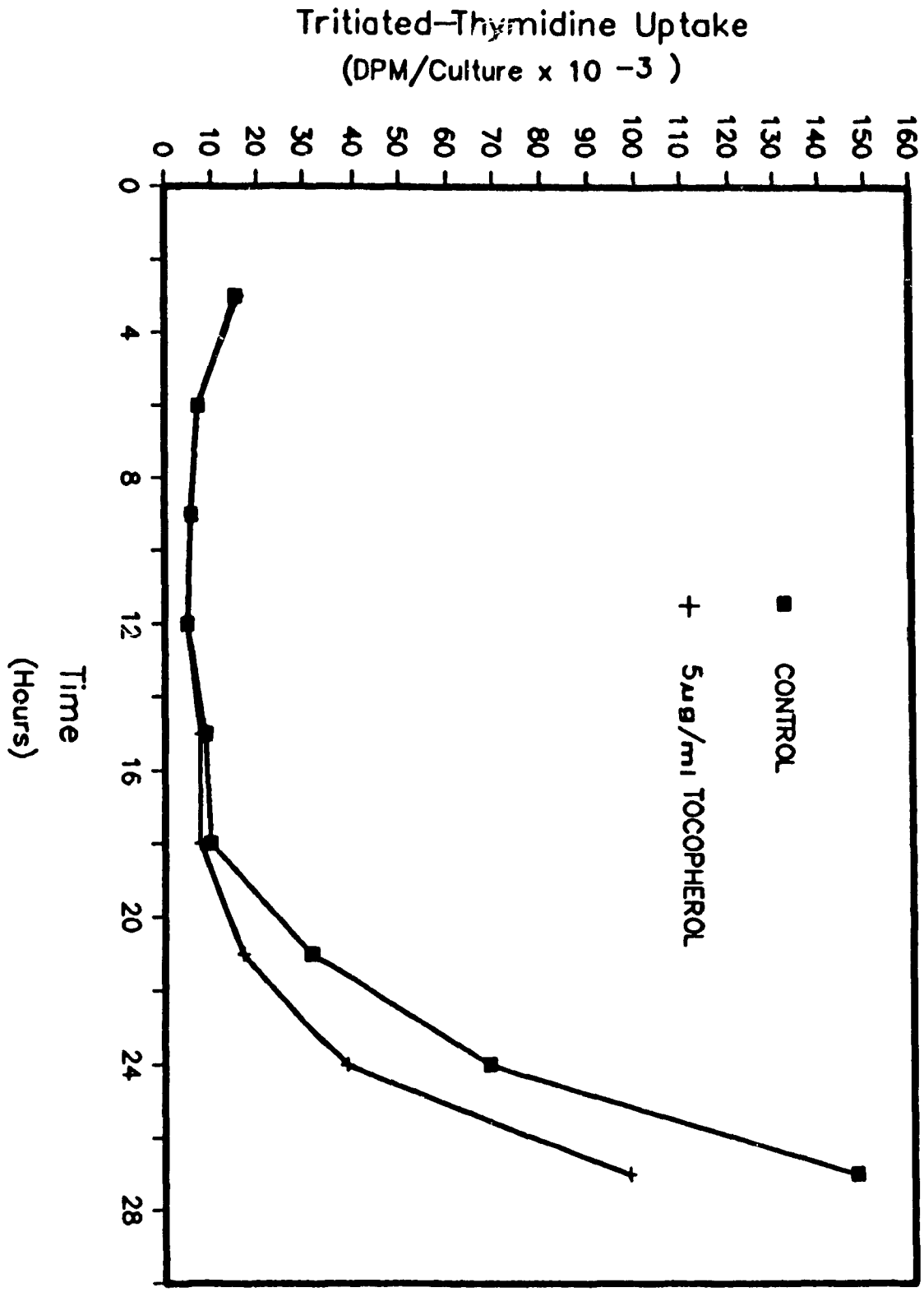
The time-course of tritiated-thymidine uptake in concanavalin A-stimulated C57Bl/6 murine spleen cells cultured with and without tocopherol.

| TIME (hours) | TRITIATED-THYMIDINE UPTAKE++ (DPM/2 ml culture) DL-α-TOCOPHEROL (0 µg/ml) | DL-α-TOCOPHEROL (5 µg/ml) |
|---|---|---|
| 3 | 15412 ± 1213 | 15987 ± 898 |
| 6 | 7391 ± 845 | 7205 ± 421 |
| 9 | 5836 ± 1131 | 5667 ± 880 |
| 12 | 4959 ± 260 | 4846 ± 402 |
| 15 | 8913 ± 551 | 7782 ± 327 |
| 18 | 10104 ± 1300 | 7837 ± 755 |
| 21 | 31728 ± 1455 | 17039 ± 1010 |
| 24 | 68850 ± 3533 | 38534 ± 8704 |
| 27 | 147490 ± 8418 | 98309 ± 5838 |

++ The response (DPM/2 ml) reported at each sampling time represents the arithmetic mean and standard deviation of tritiated-thymidine uptake in triplicate spleen cell cultures.

Note: The individual observations for each sampling time are tabulated in Appendix 6.

Figure 5. Time-course of tritiated-thymidine uptake in concanavalin A-stimulated C57Bl/6 murine spleen cells cultured with and without 5 μg/ml DL-α-tocopherol.


Tritiated-Thymidine Uptake
(DPM/Culture x 10 -3 )
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
0
4
8
12
16
20
24
28
Time
(Hours)
CONTROL
5 µg/ml TOCOPHEROL

mitotic activity gradually subsided during the following 15 hours. Tritiated-thymidine uptake, in both groups, started during the 18-21 hour interval and continued to increase exponentially afterwards. Student's t-test for paired observations (Woolson, 1987) was used to compare responses obtained at 18 and 21 hours. The differences noted in tritiated-thymidine uptake at both sampling times were highly significant for the control ($P = 0.003$, two-tailed test) and 5 µg/ml tocopherol ($P = 0.007$, two-tailed test) groups.

It is also interesting to note that mitogenic responses to optimal con A (2 µg/ml) were notably smaller in the tocopherol group at 21, 24 and 27 hours. The reason for this difference in tritiated-thymidine uptake between groups is not known.

Interphase Death Response Following Irradiation

The interphase death response of C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol was determined 18 hours following exposure to 0, 0.5, 1, 2, 4 and 8 Gy of X-radiation. Cell viability was assessed with the trypan blue dye-exclusion method. In table 7, the percent survival at each dose of radiation was calculated for the control and tocopherol groups. The percent change in cell survival of the tocopherol group with respect to that of the control group was similarly computed. The interphase death dose-response profiles for this experiment are also graphically illustrated in figure 6.

TABLE 7

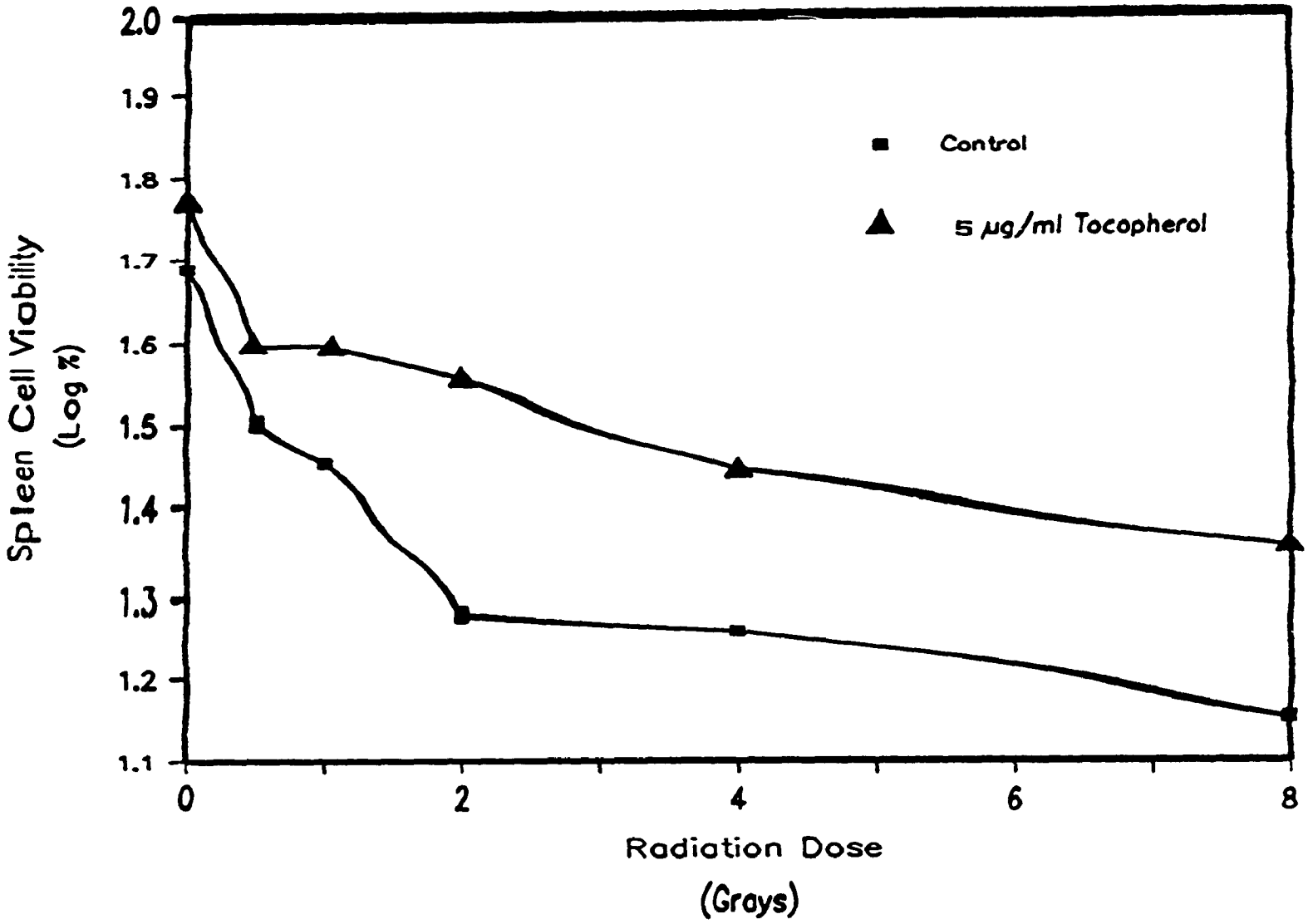
Interphase death response of C57Bl/6 murine spleen cells cultured with and without tocopherol. Cell viability determined 18 hours post-irradiation.

| RADIATION DOSE (Gy) | % SURVIVAL (VIABLE CELLS) 0 µg/ml DL-α-TOCOPHEROL | 5 µg/ml DL-α-TOCOPHEROL | % CHANGE+ |
|---|---|---|---|
| 0 | 35.8 | 48.1 | 34.4* |
| 0.5 | 23.3 | 36.0 | 54.5* |
| 1.0 | 20.9 | 39.9 | 90.9* |
| 2.0 | 16.5 | 35.7 | 116.4* |
| 4.0 | 12.1 | 24.7 | 104.1* |
| 8.0 | 8.6 | 19.8 | 130.2* |

\+ $\%\ \text{CHANGE} = \frac{(\text{TOCOPHEROL} - \text{CONTROL})}{\text{CONTROL}} \times 100\%$

* Denotes a statistically significant difference ($P \leq 0.050$). The exact P-values for the $X^2$ test are tabulated in appendix 7.

Figure 6. Interphase death response of C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol and expressed as percent viable cells present 18 hours post-irradiation.


2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
Spleen Cell Viability
(Log %)
Control
5 µg/ml Tocopherol
0
2
4
6
8
Radiation Dose
(Grays)

Differences observed between the interphase death response of the control and 5 µg/ml tocopherol group were verified by contingency table analysis (Woolson, 1987). A separate chi-square ($X^2$) statistic was computed for each dose of radiation utilized. All tests were two-tailed. The results of this statistical analysis are summarized in appendix 7.

In the tocopherol control group, cell survival decreased rapidly with increasing doses of X-radiation. A considerable number of cells (12.5%) were killed at the lowest dose of radiation utilized (0.5 Gy). By comparison, a "shouldered" dose-response curve was observed for the 5 µg/ml tocopherol group. Cell survival was significantly ($P < 0.050$) enhanced by tocopherol at all doses of radiation. The greatest improvement of cell survival was noted at 8 Gy (130.2%; $P = 0.001$).

Mitogenic Responses Following Irradiation

The suppressive effects of X-radiation on mitogenic responses produced by optimal con A (2 µg/ml) in C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol were studied in two independent experiments (tables 8-A and 8-B). A third experiment (appendix 8-C) was also conducted in order to evaluate the effects of increasing X-ray doses on mitogenic responses (2 µg/ml con A) in spleen cells cultured without tocopherol.

Responses obtained at each dose of radiation are expressed as the arithmetic mean and standard deviation of tritiated-thymidine uptake (DPM /2 ml culture) in triplicate spleen cell cultures. The percent change in the mitogenic response of the tocopherol group with respect to the control group was also calculated. In addition, the relative response (R.R.) of the irradiated groups with respect to the control group (0 Gy) was also computed. This ratio was obtained by dividing the average profilerative response of irradiated cell cultures by that of the control group and is expressed as a percentage. The dose-reducing factor (DRF) was calculated for experiments 8-A and 8-B as follows. The radiation dose which reduced mitogenic responses in the control and 5 µg/ml tocopherol groups by 50% was determined by linear regression (Dunn and Clark, 1987). The dose obtained for the tocopherol group (5 µg/ml) was then divided by that for the control group in order to obtain the DRF (Manori et al., 1986). The DRF obtained in experiments 8-A and 8-B were used to calculate the average DRF achieved with tocopherol. A graphical presentation of the data for experiments 8-A and 8-B is provided in figures 7-A and 7-B, respectively.

A two-way analysis of variance allowing for repetition within the experimental groups (Dunn and Clark, 1987) was utilized to evaluate the significance of main effects due to tocopherol and the irradiation treatment. The statistical analysis was performed with log-transformed data. The analysis of variance tables for experiments 8-A and 8-B are presented in appendices 8-A1 and 8-B1,

respectively. Missing observations in a particular experimental group were replaced by the arithmetic mean for that group. The residual and total degrees of freedom (df) were also adjusted accordingly in order to compensate for missing observations (Dunn and Clark, 1987). All tests of significance were two-tailed at the $\alpha = 0.050$ level of statistical significance. Planned comparisons between the response of the control and 5 µg/ml tocopherol groups, at each dose of radiation, were performed with Student's t-test for independent observations (Sokal and Rohlf, 1981). The logarithmic transformation ($\log_{10}$) was applied to the data from experiments 8-A and 8-B prior to the use of this statistical procedure. All comparisons were evaluated with a two-tailed test. Differences between groups were considered statistically significant if the P-value obtained for the t-statistic was less than or equal to 0.050. The exact P-values determined for each of the t-tests are tabulated in appendices 8-A2 (experiment 8-A) and 8-B2 (experiment 8-B).

In experiment 8-A, murine spleen cell cultures containing 5.4 X $10^5$ cells/ml were exposed to 0, 1, 2 and 4 Gy of X-radiation. Overall, mitogenic responses to optimal con A (2 µg/ml), were significantly affected by tocopherol ($P \leq 0.001$) and radiation ($P \leq 0.001$). The interaction between tocopherol and radiation was also statistically significant ($0.001 < P \leq 0.01$) in this experiment.

Mitogenic responses to con A in the control and tocopherol groups were suppressed following irradiation, especially at the higher

TABLE 8-A

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2 and 4 Gy) C57Bl/6 murine spleen cells.

| RADIATION DOSE (Gy) | | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) 0 µg/ml DL-α-TOCOPHEROL | 5 µg/ml DL-α-TOCOPHEROL | % CHANGE* |
|---|---|---|---|---|
| 0 | | ------- | 1115907 | |
| | | 1474462 | 1525316 | |
| | | 1217030 | 1355575 | |
| | MEAN | 1345746 | 1332266 | -1.0 |
| | S.D. | ±182032 | ±205697 | |
| | (1) R.R. (%) | 100 | 100 | |
| 1 | | ------- | ------- | |
| | | ------- | 1309987 | |
| | | 1264150 | 1322000 | |
| | MEAN | 1264150 | 1315994 | 4.1 |
| | S.D. | N/A | ±8494 | |
| | R.R. (%) | 94 | 99 | |

TABLE 8-A (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2 and 4 Gy) C57Bl/6 murine spleen cells.

| RADIATION DOSE (Gy) | | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) 0 µg/ml DL-α-TOCOPHEROL | 5 µg/ml DL-α-TOCOPHEROL | % CHANGE* |
|---|---|---|---|---|
| 2 | | 766660 | ------- | |
| | | 335994 | 1288504 | |
| | | 599593 | 1239750 | |
| | MEAN | 567416 | 1264127 | 122.8 |
| | S.D. | ±217129 | ±34474 | |
| | R.R. (%) | 42 | 95 | |
| 4 | | 231880 | 613391 | |
| | | 232308 | 673636 | |
| | | 225020 | 387879 | |
| | MEAN | 229736 | 558302 | 143.0* |
| | S.D. | ±4090 | ±150633 | |
| | R.R. (%) | 17 | 42 | |

TABLE 8-A (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2 and 4 Gy) C57Bl/6 murine spleen cells.

\+ $\%\ \text{CHANGE} = \left( \frac{\text{TOCOPHEROL} - \text{CONTROL}}{\text{CONTROL}} \right) \times 100\%$

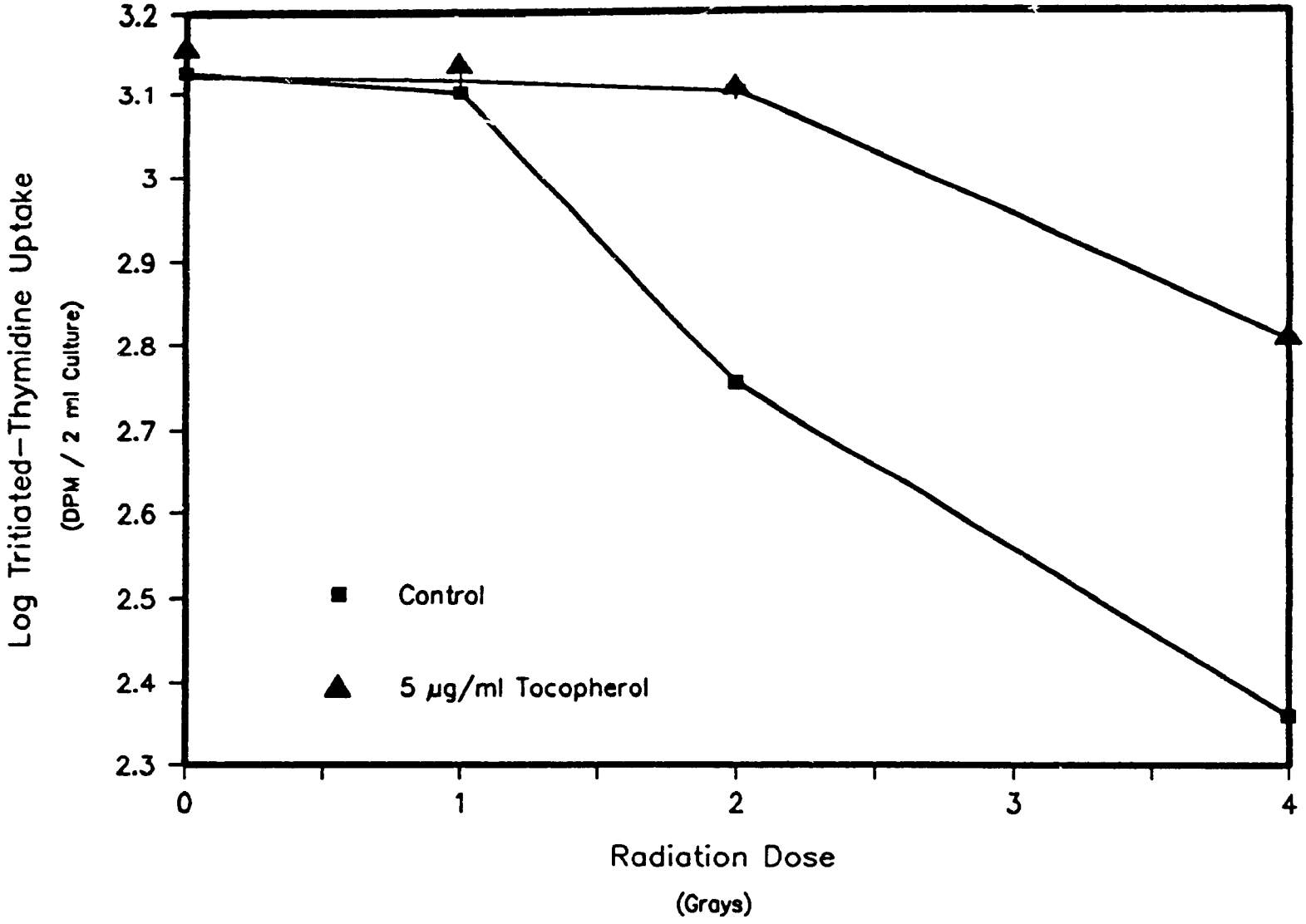
(1) R.R.: Relative response defined as the relative ratio of mitogenic responses in irradiated cell cultures to that of the non-iradiated control group (0 Gy).

A dose-reducing factor (DRF) of 1.99 was computed for this experiment.

* Denotes a statistically significant difference ($P \leq 0.050$). The exact P-values for the t-test are tabulated in appendix 8-A2.

NOTE: The cell density was $5.4 \times 10^5$ cells/ml.

Figure 7-A. Modification of concanavalin A-stimulated lymphoproliferative responses in X-irradiated (0, 1, 2 and 4 Gy) C57Bl/6 murine spleen cells by tocopherol.


Log Tritiated-Thymidine Uptake
(DPM / 2 ml Culture)
3.2
3.1
3
2.9
2.8
2.7
2.6
2.5
2.4
2.3
Control
5 µg/ml Tocopherol
0
1
2
3
4
Radiation Dose
(Grays)

doses (2 and 4 Gy). The addition of tocopherol to spleen cell cultures immediately post-irradiation was observed to partially restore mitogenic responses at these doses of radiation. Differences noted between the control and 5 µg/ml tocopherol groups approached statistical significance at 2 Gy (122.8%; P = 0.073) and were highly significant at 8 Gy (143.0%; P = 0.007). The DRF computed for this experiment was 1.99. A radioprotective effect was not observed in murine spleen cells exposed to 1 Gy of X-radiation. Moreover, tocopherol did not stimulate mitogenic responses in non-irradiated cells (P = 0.927).

In experiment 8-B, C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol were irradiated with 1, 2, 4 and 8 Gy of X-radiation. A control group for the irradiation treatment (0 Gy) was present. The cell density was 1 X $10^6$ cells/ml. Responses obtained at each dose of radiation (DPM /2 ml cultured) were corrected by multiplying individual observations by 0.54. This was done in order to facilitate comparisons with the data obtained in experiment 8-A (5.4 X $10^5$ cells/ml). Both the original and corrected observations are tabulated in table 8-A. Corrected data was used to prepare figure 7-B.

The results of the analysis of variance revealed highly significant ($P \leq 0.001$) main effects for both tocopherol and the irradiation treatment. Similarly, the interaction between these factors was also statistically significant ($0.001 < P \leq 0.010$).

TABLE 8-B

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2, 4 and 8 Gy) C57Bl/6 murine spleen cells.

| RADIATION DOSE (Gy) | | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) 0 µg/ml DL-α-TOCOPHEROL | | 5 µg/ml DL-α-TOCOPHEROL | | % CHANGE+ |
|---|---|---|---|---|---|---|
| | | OBS. | (1) CORR. | OBS. | (1) CORR. | |
| 0 | | 1940737 | 1042176 | 2063189 | 1107932 | |
| | | 1936914 | 1040123 | 2293882 | 1231815 | |
| | | 2030597 | 1090430 | 2344986 | 1259257 | |
| | MEAN | 1969416 | 1057576 | 2234019 | 1199668 | 13.4* |
| | S.D. | ±53019 | ±28471 | ±150134 | ±80622 | |
| | (2) R.R. (%) | | 100 | | 100 | |
| | | OBS. | CORR. | OBS. | CORR. | |
| 1 | | 1168754 | 627621 | 1550603 | 832674 | |
| | | 1729649 | 928822 | 1699240 | 912492 | |
| | | 1657835 | 890257 | 1577442 | 847086 | |
| | MEAN | 1518746 | 815567 | 1609095 | 864084 | 5.9 |
| | S.D. | ±305221 | ±163904 | ±79212 | ±42537 | |
| | R.R (%) | | 77 | | 72 | |

TABLE 8-B (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2, 4 and 8 Gy) C57Bl/6 murine spleen cells.

| RADIATION DOSE (Gy) | | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) 0 µg/ml DL-α-TOCOPHEROL | | 5 µg/ml DL-α-TOCOPHEROL | | % CHANGE+ |
|---|---|---|---|---|---|---|
| | | OBS. | CORR. | OBS. | CORR. | |
| 2 | | 1426560 | 766063 | 1756863 | 943435 | |
| | | 1315793 | 706581 | 1532095 | 822735 | |
| | MEAN | 1371177 | 736322 | 1644479 | 883085 | 19.9 |
| | S.D. | ±78324 | ±42060 | ±158935 | ±85348 | |
| | R.R. (%) | | 70 | | 74 | |
| | | OBS. | CORR. | OBS. | CORR. | |
| 4 | | 437966 | 235188 | 603890 | 324289 | |
| | | 339187 | 182143 | 835167 | 448485 | |
| | | 214059 | 114950 | 566380 | 304146 | |
| | MEAN | 330404 | 177427 | 668479 | 358973 | 102.3* |
| | S.D. | ±112212 | ±60258 | ±145569 | ±78171 | |
| | R.R (%) | | 17 | | 30 | |

TABLE 8-B (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2, 4 and 8 Gy) C57Bl/6 murine spleen cells.

| RADIATION DOSE (Gy) | | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) 0 µg/ml DL-α-TOCOPHEROL | | 5 µg/ml DL-α-TOCOPHEROL | | % CHANGE* |
|---|---|---|---|---|---|---|
| | | OBS. | CORR. | OBS. | CORR. | |
| 8 | | 101694 | 54610 | ------ | ----- | |
| | | 93249 | 50075 | 124526 | 66870 | |
| | | 91193 | 48971 | 131511 | 70621 | |
| | MEAN | 95379 | 51219 | 128019 | 68746 | 34.2* |
| | S.D. | ±5565 | ±2988 | ±4939 | ±2652 | |
| | R.R (%) | | 5 | | 6 | |

TABLE 8-B (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated (0, 1, 2, 4 and 8 Gy) C57Bl/6 murine spleen cells.

\+ $\%\ \text{CHANGE} = \left( \frac{\text{TOCOPHEROL} - \text{CONTROL}}{\text{CONTROL}} \right) \times 100\%$

(1) The individual observations for this experiment ($1 \times 10^6$ cells/ml) were corrected for cell density by multiplying by 0.54 in order to allow comparisons with the data presented in table 8-A ($5.4 \times 10^5$ cells/ml).

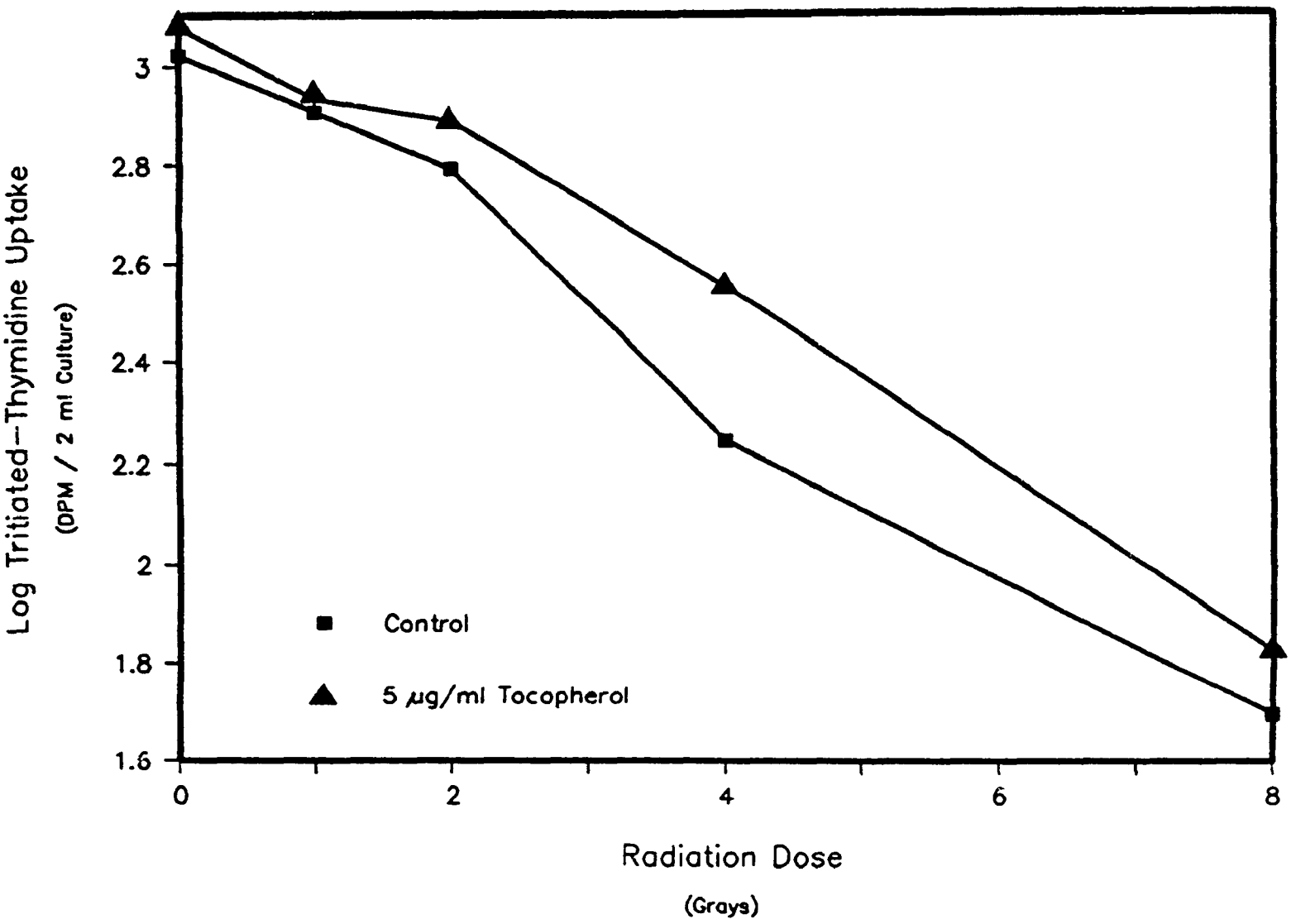
(2) R.R.: Relative response defined as the relative ratio of mitogenic responses in irradiated cell cultures to that of the non-irradiated control group (0 Gy).

A dose-reducing factor (DRF) of 1.14 was computed for this experiment.

* Denotes a statistically significant difference ($P \leq 0.050$). The exact p-values for the t-test are tabulated in appendix 8-B2.

NOTE: The cell density was $1 \times 10^6$ cells/ml.

Figure 7-B. Modification of concanavalin A-stimulated lymphoproliferative responses in X-irradiated (0, 1, 2, 4 and 8 Gy) C57Bl/6 murine spleen cells by tocopherol.


Log Tritiated-Thymidine Uptake
(DPM / 2 ml Culture)
3
2.8
2.6
2.4
2.2
2
1.8
1.6
0
2
4
6
8
Control
5 µg/ml Tocopherol
Radiation Dose
(Grays)

Consistent with the findings from experiment 8-A, mitogenic responses in irradiated cells were suppressed at all doses of radiation. The extent of suppression of mitogen-induced proliferation was found to be positively correlated with the dose of radiation. As before, tocopherol demonstrated radioprotective effects and partially restored mitogenic responses to optimal con A (2 µg/ml) in spleen cells exposed to 4 (102.3 percent change; $P = 0.039$) and 8 Gy of X-radiation (34.2 percent change; $P = 0.008$). A DRF of 1.14 was achieved with tocopherol in this experiment. Differences noted between the blastogenic response of cells cultured with and without tocopherol at 1 and 2 Gy did not attain statistical significance ($P > 0.050$). However, in non-irradiated cells (0 Gy), mitogenic responses were stimulated ($P = 0.042$) by tocopherol.

A non-parametric statistical analysis was performed in order to evaluate the overall effects of X-irradiation on mitogenic responses produced by optimal con A (2 µg/ml) in C57Bl/6 murine spleen cells cultured with and without tocopherol. The following is a description of the statistical methodology utilized. Individual observations obtained at 0, 1, 2 and 4 Gy, in both the control and 5 µg/ml tocopherol groups for each of the three experiments (tables 8-A, 8-B and appendix 8-C) were used. The observations belonging to a particular experiment were then divided by the average mitogenic response obtained for the radiation control group (i.e., 0 Gy) for that experiment. This process was repeated for each experiment. The ratio-transformed observations utilized for the overall statistical

TABLE 9

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated C57Bl/6 murine spleen cells. Pooled data from tables 8-A, 8-B and appendix 8-C**.

| RADIATION DOSE (Gy) | DL-α-TOCOPHEROL CONCENTRATION (μg/ml) 0 | 5 |
|---|---|---|
| 0 | 1.096 | 0.829 |
| | 0.904 | 1.133 |
| | 0.985 | 1.007 |
| | 0.983 | 1.048 |
| | 1.031 | 1.165 |
| | 1.000 | 1.191 |
| | 0.951 | |
| | 1.049 | |
| MEAN | 1.000 | 1.062 |
| (1)% CHANGE | | 6.2 |
| 1 | 1.000 | 1.036 |
| | 0.769 | 1.046 |
| | 1.139 | 1.021 |
| | 1.092 | 1.119 |
| | 1.062 | 1.039 |
| | 0.938 | |
| MEAN | 1.000 | 1.052 |
| % CHANGE | | 5.2 |

TABLE 9 (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated C57Bl/6 murine spleen cells. Pooled data from tables 8-A, 8-B and appendix 8-C**.

| RADIATION DOSE (Gy) | DL-α-TOCOPHEROL CONCENTRATION (μg/ml) 0 | | 5 |
|---|---|---|---|
| 2 | 1.351 | | 2.271 |
| | 0.592 | | 2.185 |
| | 1.057 | | 1.281 |
| | 1.040 | | 1.117 |
| | 0.960 | | |
| | 0.928 | | |
| | 1.128 | | |
| | 0.944 | | |
| MEAN | 1.000 | | 1.714 |
| % CHANGE | | | 71.4* |
| 4 | 1.009 | | 2.670 |
| | 1.011 | | 2.932 |
| | 0.979 | | 1.688 |
| | 1.326 | 0.911 | 1.828 |
| | 1.027 | 0.936 | 2.528 |
| | 0.648 | 1.153 | 1.714 |
| MEAN | 1.000 | | 2.227 |
| (1)% CHANGE | | | 122.7* |

TABLE 9 (cont'd)

The effects of in vitro tocopherol supplementation on concanavalin A-stimulated mitogenic responses in X-irradiated C57Bl/6 murine spleen cells.

Pooled data from tables 8-A, 8-B and appendix 8-C++.

++ Individual observations for each dose of X-radiation utilized (0 to 4 Gy) were divided by the average mitogenic response of the tocopherol control group for that particular dose of radiation. This process was repeated for data belonging to the tocopherol control and experimental groups for each experiment.

* Denotes a statistically significant difference ($P \leq 0.050$). The exact P-values for the Wilcoxon test-statistic (U) are tabulated in appendix 9.

(1) $$\% \text{ CHANGE} = \frac{(\text{TOCOPHEROL} - \text{CONTROL})}{\text{TOCOPHEROL}} \times 100\%$$

analysis are tabulated in table 9.

The Wilcoxon two-sample test (Sokal and Rohlf, 1981) was used to compare responses observed in the control and 5 µg/ml tocopherol groups at 0, 1, 2 and 4 Gy. All comparisons were two-tailed. Differences noted between groups were considered statistically significant if a P-value less than or equal to 0.050 was obtained for the Wilcoxon test-statistic (U). The results of this statistical analysis are summarized in appendix 9.

Overall, mitogenic responses to optimal con A were significantly improved by tocopherol in cells exposed to 2 (P = 0.027) and 4 Gy (P = 0.001) of X-radiation. There was no significant difference (P = 0.715) between both groups at 1 Gy. Furthermore, it was interesting to note that in non-irradiated cells (0 Gy), mitogenic responses to optimal con A (2 µg/ml) were not enhanced by tocopherol (P = 0.155).

# DISCUSSION AND CONCLUSION

## Optimal Concentration of Concanavalin A

The polyclonal mitogen concanavalin A (con A), is a plant-derived lectin with specific binding affinity for cell surface glycoprotein receptors containing glucose and mannose (Goldstein et al., 1973). Exposure of murine spleen cells to mitogenic levels of con A has been shown to trigger cell division in these cells followed by clonal expansion into effector cells required for the expression of both humoral and cell-mediated immune responses (Persson et al., 1978; Larsson and Coutinho, 1979). The spleen is a peripheral lymphoid organ which contains a spectrum of immunocompetent cells including T- and B-lymphocytes as well as adherent accessory cells (Andersson et al., 1972; Möller, 1975). It has also been reported that both T and B-cells contain an equal number of cell surface receptors capable of binding con A (Andersson et al., 1972). Findings from additional studies have demonstrated that solubilized con A preparations can induce lymphoproliferative responses in B-cells in addition to their T-cell counterparts when both cell types are present in the cell culture system (Coutinho et al., 1973; Andersson et al., 1972). In support of these findings, B-cell responses, measured by the appearance of plaque forming cells (PFC), have been demonstrated in spleen cell cultures stimulated with con A. Antibody forming cells are first noticed in mixed cell cultures 50 to 60 hours after exposure to con A. Following this lag

period, the B-cell response peaks approximately 120 hours after stimulation with the mitogen (Andersson and Melchers, 1976). Tritiated-thymidine uptake in cultures containing both T- and B-cells is reported to increase significantly 16 to 18 hours after introduction of the mitogenic stimulus. This response attains maximal activity 50 hours following the start of cell cultures with con A.

When preparations of spleen cells are depleted of their T-cell component by treatment with anti-theta (anti-θ) serum and complement, the mitogenic response of the remaining cells to con A is significantly curtailed (Andersson et al., 1972). Similarly, spleen cells from thymectomized or congenitally athymic (nude) mice do not respond to con A (Andersson et al., 1972; Andersson and Melchers, 1976). Conversely, preparations of cortisone-treated murine thymus cells, which do not contain B-lymphocytes, are stimulated to proliferation by con A and exhibit a narrow dose-response profile to the mitogen. These results have been interpreted to demonstrate that con A is specifically a T-cell mitogen. Therefore, it is believed that the mitogenic activation of B-cells by con A in murine spleen cells is secondary to the T-cell response. A prevalent explanation to account for con A-stimulated B-cell responses suggests that the T-cell mitogen initially promotes the proliferation and differentiation of T-cells into helper cells which in turn trigger and mediate the resulting antibody response (Andersson and Melchers, 1976; Katz, 1977). In support of this view, a host of T-cell-elaborated factors

including B-cell growth and differentiation factors and a low molecular weight (11 kilodalton) factor have been shown to play important roles in the proliferative response of B-cells (Kehrl et al., 1984; Bowden et al., 1986).

The findings reviewed thus far regarding the mitogenic activation of B-cells indirectly by con A, in cultures of murine spleen cells are of particular relevance to the present research. Specifically, the results obtained in the three con A dose-response experiments (experiments 1, 2 and 3), represent proliferative responses measured in an essentially heterogeneous population of mitogenically stimulated lymphoid cells. In addition, although con A is clearly recognized as a T-cell mitogen, the extent to which a secondary B-cell response contributed to the overall mitogenic response generated by con A is undetermined. The importance of the B-cell response in relation to the overall mitogenic response will be discussed in greater detail in a later section.

The activation of lymphocytes to cell division by polyclonal mitogens is commonly referred to as blast transformation. In the following paragraphs, the results obtained in experiments 1, 2 and 3 will be discussed with reference to information currently available in the literature on the study of con A mitogenesis in murine spleen cells. An in-depth review of T-cell mitogenesis is also provided.

The mitogenic response of T-cells to con A is a complex biological process resulting from the interaction between the activating lectin, T-cells and non-T accessory cells (Larsson and Coutinho, 1979; Corwin et al., 1981). The sequence of events triggered by con A and leading to the expression of a lymphoproliferative response can basically be described as a "two-signal" process. The mitogenic lectin, in this case con A, is considered to be the first "signal" required for the blastogenic transformation of lymphocytes. Initially, T-cells respond to con A by synthesizing receptor sites for a glycoprotein growth factor known as lymphocyte activating factor (LAF). The LAF-specific receptors are subsequently expressed on the surface of the con A-reactive cells thereby rendering these cells competent to respond to LAF. Larsson and Coutinho (1979) demonstrated that exposure of murine spleen cells to mitogenic doses of con A for periods of time as short as 3 hours effectively induced the expression of cell surface receptor sites for LAF. The synthesis of LAF receptors by T-cells evidently does not require the participation of accessory cells. More importantly, it was also demonstrated that the simple binding of con A to purified preparations of T-cells cannot, in the absence of LAF, initiate a lymphoproliferative response. Both the activating lectin and growth factors synthesized by accessory cells are required to initiate and sustain a mitogenic response in T-cells.

The accessory cells implicated in the mitogenic activation of lymphocytes have been identified as non-T and non-B cells bearing

Ia antigens on their suface (Ahmann et al., 1978; Habu et al., 1979). These cells have been further characterized as adherent by virtue of their high affinity for glass or plastic surfaces and are observed to be radioresistant. It is believed that these accessory cells which play an important supportive role in the proliferative response of lymphocytes are in fact macrophages or blood monocytes (Johnston, 1988; Fauci et al., 1987).

The macrophage-monocyte lineage of accessory cells has been shown to provide two important functions during the mitogenic activation of lymphocytes. First, macrophages are required for processing and presentation of the mitogen to T-cells (Cantor et al., 1984; Johnston, 1988). This step results in the expression of receptor sites specific for LAF on the surface of the T-cells. Furthermore, macrophages respond to con A by actively synthesizing and secreting in situ the growth factor LAF. Accessory cells are the source of LAF which in turn is required during subsequent steps of the mitogenic process (Shaw et al., 1980).

The macrophage-derived product LAF, also known as interleukin 1 (IL1), induces sensitized T-cells, expressing receptor sites for this growth factor, to produce the lymphokine interleukin 2 (IL2, co-stimulator) (Shaw et al., 1980). Interleukin 2 is the second biological "signal" required for the mitogenic transformation of lymphocytes and serves an autocrine function for T-cells (Fauci et al., 1987). In addition, IL2 is an important immunomodulator and

promotes the proliferation and differentiation of various subclasses of T-cells into the effector cells of the cell-mediated immune response (Haynes and Fauci, 1986). It is noteworthy to mention, that in the absence of con A, the interleukins (IL1 and IL2) are incapable of initiating a mitogenic response. The rate limiting step during the activation of lymphocytes by mitogenic lectins has been shown to be the synthesis of growth factors by accessory cells (Larsson and Coutinho, 1979).

The proliferative response of lymphocytes to mitogenic lectins is commonly measured indirectly as the incorporation of tritiated-thymidine into TCA-precipitable material (Andersson and Melchers, 1976; Mishell and Shiigi, 1980). It has been demonstrated that murine spleen cells cultured with 5 to 10% fetal calf serum (FCS) exhibit a broad dose-response profile to con A. Andersson et al. (1972) for instance, reported significant mitogenic responses with con A concentrations ranging from 1.2 to 10 µg/ml. Maximal stimulation of C57Bl murine spleen cells was achieved with 5 µg/ml con A. In other experiments, CBA (Corwin and Shloss, 1980a) and C57Bl (Spieker-Polet et al., 1979) spleen cells responded well to 0.5 to 6 µg/ml con A and gave optimal responses when the mitogen was present at 2.5 and 2 µg/ml, respectively. The results obtained in experiments 1, 2 and 3 are in good agreement with these findings. Specifically, it was observed that con A at concentrations ranging from 1 to 5 µg/ml, produced significant mitogenic responses in C57Bl/6 murine spleen cells cultured with 10% FCS. The optimal

mitogenic dose was established at 2 µg/ml. In accordance with the reports of Andersson et al. (1972) and Spieker-Polet et al. (1979), the supraoptimal levels of con A (5 and 10 µg/ml) were inhibitory and thus generated responses considerably smaller than those obtained with the optimal concentration of the mitogen. The highest concentration of con A utilized (10 µg/ml) suppressed mitogenic activity to levels measured in the mitogen control groups (tables 1-A, 1-B and 1-C).

The dose-response profile to con A observed in experiments 1, 2 and 3 (figure 1) was fact similar to that reported by Spieker-Polet et al. (1979). This observation contrasts results from other experiments performed with purified murine T-cells, which unlike spleen cells, demonstrate relatively narrow dose-response profiles to con A (Andersson et al., 1972). It is believed that the broad dose-response profile observed for murine spleen cells reflects the heterogeneous nature of the lymphoid cells stimulated to cell division by con A. Consequently, the presence of mitotically active B-cells, indirectly triggered to proliferation by T-cell-derived factors, may partially account for the type of dose-response profile observed with spleen cells. In support of this conclusion, Andersson et al. (1972) have reported that secondary B-cell responses are an important determinant in the overall mitogenic response of spleen cells to con A. Additionally, it has also been demonstrated that distinct subclasses of T-cells respond differently to low and high concentrations of con A (Persson et al., 1978). This information

together with the finding that B-cells are indirectly activated to proliferation by con A, strongly suggests that the dose-response profile observed for murine spleen cells is not as delineated as that for pure T-cells, because it encompasses the individual mitogenic responses of different classes of immunocompetent cells present in the spleen.

Modification of Mitogenesis by Tocopherol

The modification of concanavalin A mitogenesis in murine spleen cells by tocopherol in vitro has been previously reported (Corwin and Shloss, 1980a and 1980b; Corwin et al., 1981). In these studies, tocopherol was found to be stimulatory but somewhat selective in its action. Specifically, physiological concentrations (1 µg/ml) of tocopherol in vitro significantly enhanced mitogenic responses produced by suboptimal (0.6 µg/ml) levels of con A. However, when con A was present at optimal levels (2.5 µg/ml), proliferative responses to the mitogen were not stimulated by tocopherol. It was also demonstrated that in the absence of Con A, tocopherol by itself was mitogenic for murine spleen cells when added to cell cultures at final concentrations of 1, 2 and 5 µM. The lymphoproliferative responses achieved with these levels of tocopherol in vitro were of comparable magnitude to those produced by the antioxidant 2-mercaptoethanol (5, 10, 50 and 200 µM). Also, at low concentrations (5 µM), tocopherol was found to be a more potent mitogen than 2-mercaptoethanol (Corwin and Shloss, 1980a).

The initial studies reported by Corwin and Shloss (1980a and 1980b) and Corwin et al. (1981) have been expanded for the purposes of the current study in order to evaluate the effects of physiological and pharmacological concentrations of tocopherol in vitro in murine splenic lymphocytes stimulated with different levels of con A. The results obtained are in general consistent with previously reported findings although some important differences were also noted. In the following paragraphs, these results will be reviewed and discussed in relation to published information regarding the modification of con A mitogenesis by tocopherol.

As indicated in table 4, tocopherol in vitro was slightly mitogenic for C57Bl/6 murine spleen cells in the absence of con A. Statistically significant mitogenic responses were obtained with both physiological (5 µg/ml) and pharmacological (100 µg/ml) concentrations of tocopherol. Furthermore, it was noted that the mitogenic responses produced by tocopherol were considerably smaller than those generated by optimal con A (2 µg/ml) alone (tables 1-A, 1-B and 1-C) or in combination with physiological concentrations of tocopherol (table 4).

In accordance with the findings reported by Corwin and Shloss (1980a and 1980b) and Corwin et al. (1981), mitogenic responses produced by suboptimal (0.5 µg/ml) levels of con A were significantly enhanced by tocopherol in vitro. Both physiological and

pharmacological concentrations of tocopherol were stimulatory. Mitogenic responses in spleen cells cultured with 100 µg/ml DL-α-tocopherol were also somewhat smaller (R.R. = 134%) than those obtained with the physiological (5 µg/ml) concentration of tocopherol (R.R. = 181%). This difference, however, was not statistically significant (P = 0.349).

Interestingly, the stimulation of con A mitogenesis by tocopherol in vitro was not limited to suboptimal levels of the mitogen. The results of the non-parametric statistical analysis (Table 4, appendix 4), performed with data from several replicated experiments, clearly demonstrated a highly significant enhancement (R.R. = 167%; P < 0.001) of proliferative responses by tocopherol (5 µg/ml) in murine spleen cells stimulated with optimal levels (2 µg/ml) of con A. The extent to which physiological concentrations of tocopherol stimulated mitogenic responses was similar whether suboptimal (R.R. = 181%) or optimal (R.R. = 167%) levels of con A were utilized. Moreover, mitogenic responses generated by supraoptimal (5 µg/ml) levels of con A were not enhanced (R.R. = 139%, P = 0.229) in spleen cell cultures containing 5 µg/ml DL-α-tocopherol. These findings represent a significant departure from existing reports in the literature indicating that the stimulatory effects of tocopherol on con A mitogenesis are selective and limited to suboptimal levels of con A.

A significant enhancement of mitogenic responses to optimal con A was also observed in spleen cell cultures supplemented with considerably lower levels of tocopherol. In experiment 3 (table 3), for example, proliferative responses in C57Bl/6 murine splenic lymphocytes stimulated with 2 µg/ml con A were increased 102 percent (R.R. = 202%) when tocopherol was added to cell cultures yielding a final concentration of 1 µg/ml. Differences noted between the blastogenic response of the 1 (R.R. = 202%) and 5 µg/ml (R.R. = 208%) tocopherol groups were minimal and did not attain statistical significance. Similar results demonstrating a substantial stimulation of T-cell mitogenesis in cultures of CBA/J murine spleen cells containing 1 µg/ml tocopherol have also been reported by Corwin et al. (1981).

The observation that a significant stimulation of mitogenic responses to optimal con A can be achieved with tocopherol concentrations in vitro as low as 1 µg/ml is somewhat remarkable. It has been reported that plasma tocopherol levels in mice maintained on commercial diets containing adequate amounts of vitamin E range from 5 to 12 µg/ml (Corwin and Shloss, 1980a). Furthermore, although the tocopherol content of the fetal calf serum (FCS) used in this study was not measured, it has been shown that FCS generally contains less than 0.75 µg/ml tocopherol (Corwin and Shloss, 1980a). Therefore, the final concentration of tocopherol in murine spleen cell cultures containing 10% FCS and 1 µg/ml exogenous tocopherol is estimated to be approximately 1.08 µg/ml.

It is interesting that a significant stimulation of con A mitogenesis also occurred in cell cultures containing at least 5 times less tocopherol (i.e. 1 µg/ml) than that utilized in most of the other experiments. These findings also contrast with reports published by Bendich et al. (1986) demonstrating that tocopherol levels greater than those required to prevent symptoms associated with classical vitamin E deficiency in rats (4 to 7 µg/ml) are needed for optimal response to mitogens. The reason for these conflicting reports between in vivo and in vitro experimental models is not known.

It is also noteworthy that in two separate experiments (experiments 2-C and 2-E), lymphoproliferative responses in C57Bl/6 murine spleen cells stimulated by optimal con A (2 µg/ml) were not enhanced by tocopherol in vitro. The reason for this discrepancy between these findings and the results of the overall statistical analysis (table 4 and appendix 4) is not entirely clear. In both experiments, the magnitude of the blastogenic responses achieved with optimal con A alone was approximately five to ten times greater than observed in experiments 2-A, 2-B, 2-D and experiment 3. Consequently, it is possible that at these excessively high levels of cell proliferation, the rapid depletion of nutrients from the cell culture system prevented further stimulation of the mitogenic response by physiological (5 µg/ml) and lower (1 µg/ml) concentrations of tocopherol.

Several interesting hypotheses have been elaborated in order to explain the mechanism of action of tocopherol with respect to the stimulation of con A mitogenesis. Many of these are based on the vitamin's well-recognized antioxidant properties in biological systems (Green, 1972; Harman et al., 1977). For instance, a protective function has been ascribed to tocopherol by virtue of its ability to act as a free-radical scavenger and thereby prevent the formation of damaging lipoperoxides in cells of the immune system (Horwitt, 1965; Tappel, 1970; Corwin and Shloss, 1980b). It has also been suggested that tocopherol may play an important role in stabilizing cell membranes containing elevated levels of polyunsaturated fatty acids (Diplock and Lucy, 1973). Other mechanisms proposed have also implicated tocopherol as a possible regulator of prostaglandin biosynthesis (Goodwin et al., 1977; Goodwin and Webb, 1980; Corwin and Shloss, 1980b; Takenaga et al., 1981). More recently, Bellas and Corwin (1982), have proposed that tocopherol, like insulin, may stimulate mitogenic responses to con A by maintaining interleukin 2 (IL2) receptors on the surface of lymphocytes thereby increasing the number of cells entering S-phase.

In spite of the number and originality of the theories advocating tocopherol's mode of action in the stimulation of mitogenesis, none at this time have been accepted as definitive by the scientific community. The answer appears to be complex and it is possible that tocopherol exerts its immunomodulatory effects by more than one mechanism. Alternatively, it is also possible that the

underlying mode of action of vitamin E remains to be determined. In this thesis, several explanations for the stimulatory effects of tocopherol on mitogenesis will be explored and discussed with reference to the experimental findings of the current study.

The stimulation of mitogenesis by tocopherol may be explained on the basis of a biological interaction between tocopherol and T-cells. Corwin and Shloss (1980a), for example, have proposed that tocopherol in vitro enhances proliferative responses in murine spleen cells stimulated with suboptimal levels of con A by increasing the sensitivity of T-lymphocytes to con A. Presumably, such an effect would result in a shift in the dose-response profile to con A. This hypothesis, however, is not supported by experimental evidence. The results of this study, in addition to those reported by Corwin (1980a), have shown that the con A dose-response curve is not shifted by tocopherol (figure 2-B2). That is, the optimal concentration for con A remained unchanged at 2 µg/ml even though a significant stimulation of mitogenic responses to suboptimal (0.5 µg/ml) con A was achieved with physiological concentrations (5 µg/ml) of tocopherol in vitro (table 4).

It is also possible that the stimulation of proliferative responses to con A obtained with tocopherol in vitro reflects an earlier onset of cell division in T-cells. This hypothesis was tested experimentally in this study but was subsequently rejected on the basis of the results obtained. Briefly, the data reported in table 6

indicate that cell division in C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol began at the same time following exposure to con A. It has also been previously demonstrated that the rate-limiting step in the mitogenic activation of lymphocytes is in fact the synthesis of growth factors (LAF or IL1) by accessory cells (Larsson and Coutinho, 1979). In view of these observations, it appears unlikely that tocopherol promotes an earlier expression of growth factors by macrophages thereby stimulating con A mitogenesis.

There is considerable evidence demonstrating that the expression of proliferative responses in murine splenic lymphocytes stimulated with mitogenic levels of con A requires the participation of metabolically active accessory cells (Ahmann et al., 1978; Persson et al., 1978; Habu et al., 1979). In agreement with these studies, it was found that the depletion of macrophages from cultures of C57Bl/6 murine spleen cells significantly reduced the magnitude of mitogenic responses to suboptimal (R.R. = 30%) and optimal (R.R. = 78%) concentrations of con A (table 5). However, the stimulatory effects of tocopherol on con A mitogenesis in cultures containing reduced numbers of adherent cells were not eliminated even though blastogenic responses to the mitogen were seriously curtailed. It was also noted that in these cultures, tocopherol in vitro (5 µg/ml) enhanced mitogenic responses produced by suboptimal con A (R.R. = 152%) to a greater extent than those obtained with optimal levels of the mitogen (R.R. = 116%). Similar results have been reported by Corwin et al. (1981). Accordingly, it was demonstrated that following a substantial reduction of mitogenic responses to suboptimal (0.6 µg/ml) con A by anti-Ia serum, proliferative responses to the mitogen in spleen cells and cultures of spleen cells depleted of accessory cells were enhanced by tocopherol in vitro (1 µg/ml) two-fold and four-fold, respectively. By comparison, tocopherol did not stimulate mitogenic responses to optimal con A (2.5 µg/ml) in cultures of CBA/J murine spleen cells containing anherent cells with or without antiserum treatment. On the basis of these results, it was concluded that the stimulation of con A

mitogenesis by tocopherol does not require the presence of Ia-positive accessory cells. Consequently, it is possible that tocopherol exerts its effects through an Ia-negative helper T-cell thus bypassing the requirement for macrophages and macrophage-derived growth factors. In this respect, it should be noted that tocopherol in vitro shares a functional resemblance with 2-mercaptoethanol (Koren and Hodes, 1977) and phorbol myristic acetate (Farrar et al., 1980) in its ability to replace lymphocyte activating factor (LAF, IL1). These substances, including tocopherol, promote the synthesis of interleukin 2 (IL2, co-stimulator) by helper T-cells and thus effectively support lymphoproliferative responses to con A in the relative absence of accessory cells.

There are also a limited number of studies reporting inhibition of mitogenesis in murine spleen cells by tocopherol. Yasunaga et al. (1982), for instance, demonstrated that mitogenic responses produced by con A (33 µg/ml), PHA (13 µg/ml) and lipopolysaccharide (333 µg/ml) were significantly suppressed in male C3H/He mice injected intraperitoneally with 80 IU/kg all-rac-α-tocopherol daily for fourteen days. The serum tocopherol levels at the steady state in these mice measured 21.91 µg/ml and were considerably greater than the 5-12 µg/ml reported for mice maintained on conventional diets. In other studies (Corwin and Shloss, 1980a), a slight but nonetheless statistically significant inhibition of mitogenic responses to optimal (2.5 µg/ml) and higher levels of con A was reported with physiological (2 µM) concentrations of tocopherol in vitro.

The results presented in table 4 indicate that the addition of tocopherol in vitro to cultures of murine splenic lymphocytes yielding pharmacological concentrations (100 µg/ml) suppressed blastogenic responses to optimal (2 µg/ml) and supraoptimal (5 µg/ml) levels of con A. By comparison, physiological concentrations of tocopherol (5 µg/ml) were not inhibitory, but instead, enhanced the proliferative responses of murine spleen cells stimulated to suboptimal (0.5 µg/ml), optimal (2 µg/ml) and supraoptimal (5 µg/ml) levels of con A.

At least two explanations can be proposed to account for the suppression of con A mitogenesis produced by elevated levels of tocopherol in vitro. Firstly, it is possible that synergistic effects between pharmacological concentrations of tocopherol (100 µg/ml) and con A (2 and 5 µg/ml) may have potentiated the cytotoxic effects of con A thereby diminishing the magnitude of blastogenic responses to the mitogen. In support of this position, it has been demonstrated that con A by itself can be toxic for murine spleen cells especially at the higher (10 µg/ml) supraoptimal levels (tables 1-A, 1-B and 1-C). The existence of interactions between tocopherol and con A has similarly been confirmed by statistical methods. Furthermore, although physiological concentrations of tocopherol (5 µg/ml) in vitro were clearly stimulatory, it was noted that the extent of this stimulation decreased progressively with increasing levels of con A (table 4). These effects were greatly exaggerated when pharmacological concentrations (100 µg/ml) of

tocopherol were utilized. Taken together, these findings suggest that the cytotoxicity inherent to con A was greatly augmented in murine spleen cell cultures containing elevated levels of tocopherol and therefore interfered with the expression of normal mitogenic responses to the mitogen.

Secondly, it is also possible that tocopherol by itself is cytotoxic for murine spleen cells when it is present in vitro at pharmacological concentrations. In this respect, tocopherol would inhibit mitogenic responses independently of con A. Consistent with this view, Narayanareddy and Murthy (1982) have demonstrated that pharmacological concentrations of tocopherol (50 and 500 µg/ml) were in fact cytotoxic for human peripheral blood lymphocytes. Cell survival as assessed by the trypan blue dye exclusion method during a 96-hour period was found to decrease progressively with time and increasing concentrations of tocopherol. The rate of cell death was maximal 48 to 78 hours following the start of cell cultures with tocopherol. It was also reported that at physiological concentrations (5 µg/ml), tocopherol in vitro was essentially non-toxic for human lymphocytes. In other studies, Corwin and Shloss (1980a) demonstrated that the addition of tocopherol in vitro (2 µM) to cultures of CBA/J murine spleen cells did not adversely affect cell survival. It was also noted that cell survival at 48 hours was improved 49% with tocopherol. In summary, it is believed that the suppression of proliferative responses to con A produced by tocopherol in vitro may be due to the increased cytotoxicity

manifested by pharmacological concentrations of the vitamin, potentiation of con A toxicity, or alternatively, a combination of both effects.

## Post-Irradiation Modification of Spleen Cell Survival and Proliferation by Tocopherol

The effectiveness of tocopherol as a radioprotective agent has been demonstrated in several studies assessing the lethality of whole animals and single cells following administration of tocopherol before (Huber and Schroder, 1962; Sakamoto and Sakka, 1973; Hoffer and Roy, 1975; Prasad and Rama, 1984) and after (Malick et al., 1978; Roy et al., 1982; Bichay and Roy, 1986) irradiation. The precise mechanism through which tocopherol modifies radiation responses is not known at this time. Evidence from studies in which protective effects were obtained when tocopherol was present prior to or at the time of irradiation, suggests that it may be related to the well-recognized antioxidant properties of vitamin E (Tappel, 1972; Diplock and Lucy, 1973; McCay and King, 1980). However, in other studies, radioprotection was not observed with tocopherol (Haley et al., 1954; Ershoff and Steers, 1960; Rostock et al., 1980). A few studies have also shown that tocopherol can potentiate the lethal effects of radiation in tumor cells both in vivo (Kagerud et al., 1978; Kagerud and Peterson, 1981) and in vitro (Prasad et al., 1979; Sarria and Prasad, 1984). The reason for conflicting results regarding radioprotection by tocopherol in normal and neoplastic cells has not

been determined. Different forms of the vitamin, dose, mode and route of administration and the time relative to irradiation are all factors which may account for some of these differences. The cell-type utilized in different experiments is also undoubtedly an important factor. In addition, there is some indication that the differential radiation response modification produced by tocopherol in normal and neoplastic cells may also be partly mediated by antioxidant mechanisms (Sarria and Prasad, 1984).

Considering the extensive literature that exists regarding the suppressive effects of ionizing radiation (reviewed in Dubois et al., 1981) and reports on the modification of both humoral and cell-mediated immune functions by tocopherol (Panush and Delafuente, 1985; Bendich, 1988), it is somewhat surprising that studies to assess the radioprotective effects of tocopherol on lymphocyte proliferation following mitogenic activation have not previously been reported. In this study, the effects of post-irradiation tocopherol administration on lymphocyte survival and con A mitogenesis were investigated. The results obtained demonstrating the ameliorating effects of tocopherol in vitro are reviewed and will be discussed with reference to studies reporting similar radioprotective effects with other immunomodulators.

The interphase death response of C57Bl/6 murine spleen cells cultured with and without 5 µg/ml DL-α-tocopherol was determined 18 hours post-irradiation using the trypan blue dye-exclusion method.

The results presented in table 7 indicate that cell viability in the tocopherol control group decreased in a dose-dependent manner over a range extending from 0.5 to 2.0 Gy. It was noted that a significant number of cells were killed (12.5%) at 0.5 Gy. Thereafter, cell survival following exposure to 4.0 to 8.0 Gy of X-radiation remained approximately constant at 8.6 to 12.1%.

It is important to note that the shape of the dose-effect curve (figure 6) observed for the interphase death response of murine spleen cells is typical of a heterogeneous population of cells (Song and Levitt, 1978; Kwan and Norman, 1977). In addition, the biphasic dose-response profile suggests that murine spleen cells are heterogeneous with respect to radiosensitivity. Biphasic dose-responses with apparent radioresistance at higher doses have similarly been reported for other immune responses including cell-mediated cytotoxicity (Gerber, 1984).

The results of this study indicate that post-irradiation tocopherol administration (5 µg/ml) significantly enhanced cell survival in C57Bl/6 murine spleen cells exposed to 0.5 to 8.0 Gy of X-radiation. The greatest ameliorating effect of tocopherol in vitro was observed at 8.0 Gy (130.2%; P = 0.001). It was also noted that the viability of non-irradiated cells at 18 hours was improved by 34.4% (P = 0.21) in the presence of tocopherol. This finding is in agreement with previous reports that cell viability at 48 hours was enhanced by physiological concentrations of tocopherol in vitro (2

μM) in CBA/J murine spleen cells (Corwin and Shloss, 1980a).

As expected, a biphasic dose-effect curve, similar to that observed for the tocopherol control group, was noted for murine spleen cells cultured with 5 μg/ml tocopherol. However, this curve could also be readily distinguished from that of the control group by the presence of a shouldered region extending from 0.5 to 2.0 Gy. The presence of this shoulder indicates that the spleen cells treated with tocopherol could withstand more radiation damage than cells cultured without tocopherol. The shouldered dose-response profile may also reflect a greater capacity for DNA repair in spleen cells cultured with tocopherol immediately after irradiation.

There are a number of reports that certain treatments, including mitogenic and antigenic activation, can modify the radiation response of non-cycling ($G_0$) lymphocytes thereby rendering these cells less susceptible to rapid cell death during interphase (Schrek and Stefani, 1964; Schrek, 1968; Sato, 1970; Sprent et al., 1974; Dickinson, 1981). More recently, Lowenthal and Harris (1985) demonstrated that the extent of radioprotection conferred by mitogenic lectins is strongly dependent on the temporal relation between irradiation and exposure to the mitogen. It was shown that for murine lymph node cells, the greatest radioprotective effect was achieved when the cells were stimulated with con A (5 μg/ml) 22 hours before exposure to 1000 rads of X-radiation. The number of cells surviving irradiation was considerably less when con A was

present 48 or 72 hours before irradiation. It was also observed that a small proportion of cells (10 to 15%) could be protected when the mitogenic stimulus was delayed 3 hours post-irradiation. However, when lymph node cells were treated with con A 5 hours following exposure to X-radiation, the radioprotective effects in blast cells were abrogated and the rate of cell death increased to levels observed in non-stimulated cells.

The mechanism responsible for prevention of rapid cell death by ionizing radiation in cells stimulated with mitogens is essentially unknown. The results of several studies have shown that untransformed lymphocytes are extremely radiosensitive and experience rapid interphase death with low doses of radiation. By comparison, activated cells demonstrate a delayed response and possibly experience a reproductive death after one or two cell divisions (Lowenthal and Harris, 1985; Webb and Sheldon, 1984). There is also evidence indicating that mitogen-induced radioprotection in activated cells is mediated by DNA repair processes (Castellani et al., 1980). Consistent with this view, the presence of a shouldered dose-response profile in murine spleen cells cultured with tocopherol indicates that the radioprotective effects of vitamin E on lymphocyte survival may in fact be due to a stimulation of DNA repair processes in these cells. However, the magnitude of the radioprotective effects produced by tocopherol in vitro is remarkable considering that tocopherol was found to be only slightly mitogenic by itself (table 4) yielding proliferative responses

much smaller than achieved, for instance, with 5 µg/ml con A (tables 1-A, 1-B and 1-C). It is also conceivable, that tocopherol exerts its radioprotective effects in murine spleen cells through the induction of some other immunomodulator (Neta et al., 1986). Previous studies have demonstrated that radiation effects are cell- cycle dependent and that cells in late S-phase are generally more radioresistant (Sinclair and Morton, 1966). In addition, although tocopherol was shown not to induce an earlier onset of cell division in murine spleen cells (table 6), there are some reports that tocopherol may maintain interleukin 2 (IL2) receptors on the surface of lymphocytes thereby increasing the number of cells entering S-phase (Bellas and Corwin, 1982). Taken together, these findings suggest that tocopherol could delay or prevent interphase death in lymphocytes by promoting the entry of a greater proportion of lymphoid progenitor cells into S-phase of the cell cycle. A similar hypothesis has been proposed by Neta et al. (1986) in order to explain the radioprotective effects of interleukin 1 (IL1) with respect to increased survival in mice exposed to lethal doses of gamma radiation. Additional studies are needed to test these hypotheses in order to acquire a better understanding of the mechanism through which mitogens, interleukins and tocopherol reduce interphase death in immune cells.

In continuation of this study, the effects of post-irradiation tocopherol administration on con A mitogenesis were evaluated. The results obtained (table 9) indicate a significant enhancement of

mitogenic responses to optimal (2 µg/ml) con A in murine spleen cells exposed to 2 and 4 Gy of X-radiation by physiological concentrations (5 µg/ml) of tocopherol in vitro. However, stimulatory effects were not observed in control and 1 Gy-irradiated cells. These findings, particularly in non-irradiated cells, were unexpected considering the overall results of the mitogenesis assays (table 4) which demonstrated a significant enhancement of proliferative responses to optimal con A (2 µg/ml) by tocopherol. The absence of stimulatory effects with tocopherol at optimal concentrations of mitogen was also observed in two other experiments (experiments 2-C and 2-E).

It is recognized that immune responses requiring cell proliferation are generally more radiosensitive than those mediated by terminally differentiated cells (Song and Levitt, 1978). For instance, the production of migration inhibition factors (MIF) by mature T-cells (Salvin and Nishio, 1972) and the synthesis of immunoglobulins by plasma cells (Vann and Makinodan, 1969) have been shown to be relatively radioresistant responses. In contrast, it has been reported that cytotoxic responses by T-lymphocytes are highly radiosensitive (Song and Levitt, 1978; Gerber, 1984). Similarly, proliferative responses stimulated by polyclonal mitogens have been shown to be seriously curtailed even at low doses of radiation (Gualde and Goodwin, 1984; Webb and Sheldon, 1984). The results of the current study are in agreement with these findings regarding the marked radiosensitivity of dividing lymphocytes.

Accordingly, mitogenic responses to optimal con A (2 µg/ml) decreased in a dose-dependant manner following exposure of murine spleen cells to graded doses of X-radiation. It was also noted that the radiation response of these cells was variable especially at lower doses (1 and 2 Gy) of radiation. Specifically, a dose of 1 Gy suppressed blast transformation by only 6% in experiment 8-A whereas in experiment 8-B this response was reduced by 23%. These results concur with previous studies demonstrating some variability in the radiation response of murine spleen cells stimulated with con A (Manori et al., 1985) and similarly, murine thymocytes mitogenically activated with con A and phytohemagglutinin (Manori et al., 1986). This variability reflects the presence of lymphocytes in the spleen that are heterogeneous with respect to radiosensitivity and therefore, explains the biphasic shape of the dose-response curves observed for experiment 8-A and, to a lesser extent, experiment 8-B. Similar responses have also been previously reported for the incorporation of tritiated-thymidine by human lymphocytes (Hedges and Hornsay, 1981).

The results of this study have shown that C57Bl/6 murine spleen cells stimulated in the presence of con A and tocopherol immediately post-irradiation were less radiosensitive than cells treated with con A alone. An average dose-reducing factor of 1.6 was achieved with 5 µg/ml tocopherol. In light of these findings, it is believed that the tocopherol effects observed at 2 and 4 Gy (table 3) are, in part, the result of radioprotection effects in cells that

survived interphase death at the time of irradiation. Radioprotective effects in immune cells stimulated to proliferation with mitogenic lectins have also been reported with the interleukins. Manori et al. (1985), for instance, demonstrated that the addition of T-cell growth factor, which contains interleukin 2, to cultures of murine spleen cells immediately following irradiation partially restored mitogenic responses to con A. In other studies, radioprotection was demonstrated with cell culture supernatants containing the monokine interleukin 1 (Manori et al., 1986). Briefly, it was found that mitogenic responses to con A and PHA in irradiated murine thymocytes were enhanced when the cells were stimulated in the presence of interleukin 1 immediately after irradiation. However, when the addition of mitogens and interleukin 1 was delayed for 24 hours, the radioprotective effects were abrogated. Interestingly, it was also demonstrated that under identical conditions, interleukin 2 was partially radioprotective and thus supported proliferative responses to the mitogens. These observations indirectly confirmed the radiosensitivity of interleukin 2-producing cells. On the basis of these results and current knowledge regarding the mechanism of T-cell activation by mitogens, it was concluded that interleukin 1 acts as radioprotective agent through the induction of interleukin 2 synthesis. This conclusion was also supported by previous work demonstrating the radioprotective effects of T-cell growth factor in irradiated murine spleen cells (Manori et al., 1985). There is also some evidence indicating that tocopherol can replace the requirement for interleukin 1 in several immunological responses. The results of

the current study (table 5), in addition to published reports (Corwin and Shloss, 1980a; Corwin et al., 1981) that tocopherol in vitro can support proliferative responses stimulated by mitogens in the relative absence of accessory cells, argue in favour of the functional resemblance between interleukin 1 and tocopherol. Consequently, it is possible that tocopherol, like interleukin 1, also mediates radioprotection by inducing the production of interleukin 2 by helper T-cells.

The mechanism of action governing radioprotection in proliferating lymphocytes by the interleukins and more recently, tocopherol, remains to be determined. Nonetheless, it has been proposed that the radioprotective effects associated with these agents may, in fact, result from an increase in DNA repair processes in irradiated cells. In support of this hypothesis, Manori et al. (1985), reported that con A blasts cultured with T-cell growth factor immediately post-irradiation contained fewer DNA strand-breaks than cells cultured without T-cell growth factor. Alternatively, it is also possible that post-irradiation administration of interleukins or tocopherol, in concert with mitogens, reduces radiation-induced division delay in blast cells thereby resulting in an earlier onset of cell division and increased levels of tritiated-thymidine incorporation.

Another interesting mechanism has also been proposed by Gerber (1984) in order to explain radioprotection by T-cell growth factor with respect to immune T-cell cytotoxic function. With this

experimental model, it was demonstrated that the administration of T-cell growth factor to cultures of immune splenic lymphocytes immediately post-irradiation enhanced the proliferative response and lytic activity of irradiated cells. It was also found that the stimulatory effects of T-cell growth factor were greater at low cell densities. On the basis of these results, Gerber postulated that TCGF simply compensated for the irradiation effects by facilitating the proliferation of lymphocytes surviving interphase death. Accordingly, it has also been suggested that the restorative effects of TCGF should be more evident at higher doses of radiation because an excess of the lymphokine is shared by a reduced number of cells. The results of Gerber's study have argued in favour of this hypothesis. Similarly, in the current study, it was also noted that there was a trend for greater tocopherol effects at higher doses of X-radiation. Unfortunately, at this time, it is not known if tocopherol acts as a radioprotective agent by compensating radiation effects by biological response modification, as described by Gerber (1984), or through some other mechanism possibly involving modulation of cellular DNA repair processes. Additional studies are needed to resolve these issues.

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## APPENDIX 1-A

Analysis of variance table for $LOG_{10}$-transformed data presented in table 1-A++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to con A | 20.4191 | 5 | 4.0838 | 1464.929*** | 3.89 |
| Residual | 0.0334 | 12 | 0.0028 | | |
| Total | 20.4525 | 17 | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 1-B

Analysis of variance table for $LOG_{10}$-transformed data presented in table 1-B++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to con A | 23.0110 | 5 | 4.6022 | 228.519*** | 3.89 |
| Residual | 0.2417 | 12 | 0.0201 | | |
| Total | 23.2527 | 17 | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 1-C

Analysis of variance table for $LOG_{10}$-transformed data presented in table 1-C++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to con A | 15.8338 | 5 | 3.1667 | 269.478*** | 3.89 |
| Residual | 0.1410 | 12 | 0.0118 | | |
| Total | 15.9748 | 17 | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 1-D

A posteriori comparisons between mitogenic responses produced by suboptimal, optimal and supraoptimal concentrations of con A. T-method for equal sample sizes applied to $LOG_{10}$-transformed data presented in tables 1-A, 1-B and 1-C. Comparisons made at the $\alpha = 0.050$ level of statistical significance**.

TABLE 1-A

(1) $S_Y = 0.0305$

(2) $MSD = S_Y \times 4.751 = 0.1448$

| | | | |
|---|---|---|---|
| (3) | $\lvert Y_2 - Y_1 \rvert = 0.2123 > MSD$ | significant | $P \leq 0.050$ |
| (4) | $\lvert Y_2 - Y_3 \rvert = 0.0806 < MSD$ | not significant | $P > 0.050$ |
| (5) | $\lvert Y_2 - Y_5 \rvert = 0.3432 > MSD$ | significant | $P \leq 0.050$ |
| (6) | $\lvert Y_2 - Y_{10} \rvert = 2.4726 > MSD$ | significant | $P \leq 0.050$ |

TABLE 1-B

(1) $S_Y = 0.0819$

(2) $MSD = S_Y \times 4.751 = 0.3893$

| | | | |
|---|---|---|---|
| (3) | $\lvert Y_2 - Y_1 \rvert = 0.4284 > MSD$ | significant | $P \leq 0.050$ |
| (4) | $\lvert Y_2 - Y_3 \rvert = 0.3054 < MSD$ | not significant | $P > 0.050$ |
| (5) | $\lvert Y_2 - Y_5 \rvert = 1.1535 > MSD$ | significant | $P \leq 0.050$ |
| (6) | $\lvert Y_2 - Y_{10} \rvert = 3.1615 > MSD$ | significant | $P \leq 0.050$ |

## APPENDIX 1-D (cont'd)

A posteriori comparisons between mitogenic responses produced by suboptimal, optimal and supraoptimal concentrations of con A. T-method for equal sample sizes applied to $LOG_{10}$-transformed data presented in tables 1-A, 1-B and 1-C. Comparisons made at the $\alpha = 0.050$ level of statistical significance**.

TABLE 1-C

(1) $S_Y = 0.0626$

(2) $MSD = S_Y \times 4.751 = 0.2974$

| | | |
|---|---|---|
| (3) $\lvert Y_2 - Y_1 \rvert = 0.2803 < MSD$ | not significant | $P > 0.050$ |
| (4) $\lvert Y_2 - Y_3 \rvert = 0.0371 < MSD$ | not significant | $P > 0.050$ |
| (5) $\lvert Y_2 - Y_5 \rvert = 0.7452 > MSD$ | significant | $P \leq 0.050$ |
| (6) $\lvert Y_2 - Y_{10} \rvert = 2.4279 > MSD$ | significant | $P \leq 0.050$ |

## APPENDIX 1-D (cont'd)

A posteriori comparisons between mitogenic responses produced by suboptimal, optimal and supraoptimal concentrations of con A. T-method for equal sample sizes applied to $LOG_{10}$-transformed data presented in tables 1-A, 1-B and 1-C. Comparisons made at the $\alpha = 0.050$ level of statistical significance++.

(1) Standard error for the T-test defined as:

$$S_Y = \sqrt{\frac{MS\ (RESIDUAL)}{n}}$$

n = 3 observations per group.

(2) MSD = minimum significant difference defined as:

$$S_Y \times Q_\alpha [\ K, V\ ]$$

where; Q = critical value of the studentized range (Sokal and Rohlf, 1981)
$\alpha = 0.050$
K = number of groups = 6
V = degrees of freedom for the residual mean square (MS) = 12

(3) Comparison of mitogenic responses produced by con A concentrations of 2 and 1 µg/ml.

(4) Comparison of mitogenic responses produced by con A concentrations of 2 and 3 µg/ml.

(5) Comparison of mitogenic responses produced by con A concentrations of 2 and 5 µg/ml.

(6) Comparison of mitogenic responses produced by con A concentrations of 2 and 10 µg/ml.

Note: Differences between groups are considered statistically significant ($p \leq 0.050$) if and only if the absolute difference of the means for the two groups is greater than or equal to the MSD (Sokal and Rohlf, 1981).

++ Sokal and Rohlf, 1981.

APPENDIX 2-A

Analysis of variance table for LOG10-transformed data presented in table 2-A++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 0.5639 | 3 | 0.1880 | 21.668*** | 3.59 |
| Due to con A | 40.4457 | 3 | 13.4819 | 1554.149*** | 3.59 |
| Tocopherol X con A interaction | 0.1766 | 9 | 0.0196 | 2.262 | 2.57 |
| Residual | 0.2776 | 31+ | 0.0087 | | |
| Total | 41.4638 | 46+ | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by one (1) in order to compensate for a single missing observation in table 2-A. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987).

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 2-B

Analysis of variance table for $LOG_{10}$-transformed data presented in table 2-B††.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 1.9256 | 3 | 0.6419 | 103.819*** | 3.59 |
| Due to con A | 22.1331 | 3 | 7.3777 | 1193.287*** | 3.59 |
| Tocopherol X con A interaction | 1.9944 | 9 | 0.2216 | 35.843*** | 2.57 |
| Residual | 0.1978 | 32 | 0.0062 | | |
| Total | 26.2510 | 47 | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

†† Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 2-C

Analysis of variance table for $LOG_{10}$-transformed data presented in table 2-C++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 0.3175 | 2 | 0.1587 | 83.415*** | 4.38 |
| Due to con A | 34.8435 | 3 | 11.6145 | 6102.690*** | 3.78 |
| Tocopherol X con A interaction | 0.1933 | 6 | 0.0322 | 16.926*** | 3.05 |
| Residual | 0.0457 | 22+ | 0.0019 | | |
| Total | 35.3999 | 33+ | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by two (2) in order to compensate for two missing observations in table 2-C. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987).

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

## APPENDIX 2-D

Analysis of variance table for $LOG_{10}$-transformed data presented in table 2-D*.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 0.1112 | 2 | 0.0556 | 5.870** | 4.32 |
| Due to con A | 3.7633 | 3 | 1.2544 | 132.395*** | 3.72 |
| Tocopherol X con A interaction | 0.1797 | 6 | 0.0299 | 3.160* | 2.99 |
| Residual | 0.2274 | 24 | 0.0095 | | |
| Total | 4.2816 | 35 | | | |

APPENDIX 2-D (cont'd)

Analysis of variance table for $LOG_{10}$-transformed data presented in table 2-D++.

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

Note: An analysis of variance based on three rows and four columns (3 X 4) was utilized in order to exclude the 0 µg/ml con A group from the analysis. All observations for this group at 100 µg/ml DL-α-tocopherol are missing.

++ Dunn and Clark, 1987.

* $0.020 < P \leq 0.050$

** $0.010 < P \leq 0.050$

*** $P \leq 0.001$

## APPENDIX 2-E

Analysis of variance table for $LOG_{10}$-transformed data presented in table 2-E++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 0.0010 | 1 | 0.0010 | 1.442 | 6.41 |
| Due to con A | 16.7397 | 3 | 5.5799 | 7938.175*** | 4.35 |
| Tocopherol X con A interaction | 0.1875 | 3 | 0.0625 | 88.905*** | 4.35 |
| Residual | 0.0112 | 13+ | 0.0007 | | |
| Total | 16.9394 | 20+ | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by three (3) in order to compensate for three missing observations in table 2-E. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987).

++ Dunn and Clark, 1987.

*** $P \leq 0.001$

# APPENDIX 3-1

Analysis of variance table for $LOG_{10}$-transformed data presented in table 3++.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to tocopherol | 1.0662 | 3 | 0.3554 | 120.142*** | 5.42 |
| Residual | 0.0237 | 8 | 0.0030 | | |
| Total | 1.0899 | 11 | | | |

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

++ Sokal and Rohlf, 1981.

*** $P \leq 0.001$

## APPENDIX 3-2

A posteriori comparisons between mitogenic responses produced by optimal concanavalin A (2 µg/ml) in C57Bl/6 murine spleen cells cultured with increasing levels of DL-α-tocopherol. T-method for equal sample sizes applied to LOG10-transformed data presented in table 3. Comparisons made at the α = 0.050 level of statistical significance††.

(1) $S_Y$ = 0.0316

(2) MSD = $S_Y$ X 4.529 = 0.1432

| | | | |
|---|---|---|---|
| (3) $\lvert Y_0 - Y_1 \rvert$ | = 0.3098 > MSD | significant | $P \leq 0.050$ |
| $\lvert Y_0 - Y_5 \rvert$ | = 0.3224 > MSD | significant | $P \leq 0.050$ |
| $\lvert Y_0 - Y_{100} \rvert$ | = 0.4097 > MSD | significant | $P \leq 0.050$ |
| $\lvert Y_1 - Y_5 \rvert$ | = 0.0127 < MSD | not significant | $P > 0.050$ |
| $\lvert Y_1 - Y_{100} \rvert$ | = 0.7195 > MSD | significant | $P \leq 0.050$ |
| $\lvert Y_5 - Y_{100} \rvert$ | = 0.7322 > MSD | significant | $P \leq 0.050$ |

APPENDIX 3-2 (cont'd)

A posteriori comparisons between mitogenic responses produced by optimal concanavalin A (2 µg/ml) in C57Bl/6 murine spleen cells cultured with increasing levels of DL-α-tocopherol. T-method for equal sample sizes applied to $LOG_{10}$-transformed data presented in table 3. Comparisons made at the $\alpha = 0.050$ level of statistical significance".

(1) Standard error for the T-test defined as:

$$S_Y = \sqrt{\frac{MS\ (RESIDUAL)}{n}}$$

n = 3 observations per group.

(2) MSD = minimum significant difference defined as:

$$S_Y \times Q_\alpha\ [\ K,\ V\ ]$$

where; Q = critical value of the studentized range (Sokal and Rohlf, 1981)
α = 0.050
K = number of groups = 4
V = degrees of freedom for the residual mean square (MS) = 8

## APPENDIX 3-2 (cont'd)

A posteriori comparisons between mitogenic responses produced by optimal concanavalin A (2 µg/ml) in C57Bl/6 murine spleen cells cultured with increasing levels of DL-α-tocopherol. T-method for equal sample sizes applied to $LOG_{10}$-transformed data presented in table 3. Comparisons made at the $\alpha = 0.050$ level of statistical significance++.

(3) Comparison of mitogenic responses produced by optimal con A at different levels of tocopherol. The subscripts denote the concentrations of tocopherol being compared as follows:

$Y_0$ = 0 µg/ml DL-α-tocopherol group.
$Y_1$ = 1 µg/ml DL-α-tocopherol group.
$Y_5$ = 5 µg/ml DL-α-tocopherol group.
$Y_{100}$ = 100 µg/ml DL-α-tocopherol group.

Note: Differences between groups are considered to be statistically significant ($p \leq 0.050$) if and only if the absolute difference of the mean for the two groups is greater than or equal to the MSD (Sokal and Rohlf, 1981).

++ Sokal and Rohlf, 1981.

## APPENDIX 4

Non-parametric statistical analysis of the data presented in table 4 utilizing the Wilcoxon two-sample test for ranked observations[44].

| CONCANAVALIN A CONCENTRATION (µg/ml) | DL-α-TOCOPHEROL CONCENTRATIONS COMPARED (µg/ml) | $n_1$ (1) | $n_2$ (2) | Computed U (3) | Tabled U (4) | P-value |
|---|---|---|---|---|---|---|
| 0 | 0 - 5 | 14 | 12 | 140 | 123 | 0.004 |
| | 0 - 100 | 12 | 3 | 36 | 32 | 0.009 |
| | 5 - 100 | 14 | 3 | 35 | 37 | 0.078 |
| 0.5 | 0 - 5 | 12 | 12 | 123 | 107 | 0.003 |
| | 0 - 100 | 12 | 6 | 65 | 58 | 0.005 |
| | 5 - 100 | 12 | 6 | 46 | 58 | 0.349 |

## APPENDIX 4 (cont'd)

Non-parametric statistical analysis of the data presented in table 4 utilizing the Wilcoxon two-sample test for ranked observations++.

| CONCANAVALIN A CONCENTRATION (µg/ml) | DL-α-TOCOPHEROL CONCENTRATIONS COMPARED (µg/ml) | $n_1$ (1) | $n_2$ (2) | Computed U (3) | Tabled U (4) | P-value |
|---|---|---|---|---|---|---|
| 2 | 0 - 5 | 18 | 18 | 289 | 225 | < 0.001 |
| | 0 - 100 | 18 | 9 | 110 | 120 | 0.136 |
| | 5 - 100 | 18 | 9 | 138 | 120 | 0.003 |
| 5 | 0 - 5 | 9 | 8 | 48.5 | 57 | 0.229 |
| | 0 - 100 | 9 | 6 | 42 | 44 | 0.077 |
| | 5 - 100 | 8 | 6 | 40.5 | 40 | 0.033 |

(1) $n_1$ = number of observations in the larger sample.

(2) $n_2$ = number of observations in the smaller sample.

(3) Wilcoxon test-statistic (U).

(4) Tabled U-value for $\alpha = 0.050$, two-tailed test.

++ Sokal and Rohlf, 1981.

## APPENDIX 5-1

Analysis of variance table for $LOG_{10}$-transformed data presented in table 5[1].

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Main Effects | | | | | |
| Tocopherol | 0.118 | 1 | 0.118 | 20.897*** | 6.20 |
| Con A | 7.899 | 1 | 7.899 | 1398.852*** | 6.20 |
| Cell culture-type | 0.549 | 1 | 0.549 | 97.176*** | 6.20 |
| Two-Way Interactions | | | | | |
| Tocopherol X Con A | 0.055 | 1 | 0.055 | 9.826** | 6.20 |
| Tocopherol X Cult-type | 0.000 | 1 | 0.000 | 0.075 | 6.20 |
| Con A X Cult-type | 0.312 | 1 | 0.312 | 55.230*** | 6.20 |
| Three-Way Interactions | | | | | |
| Tocopherol X Con A X Cell culture-type | 0.008 | 1 | 0.008 | 1.343 | 6.20 |
| Residual | 0.085 | 15[1] | 0.006 | | |
| Total | 9.000 | 22[1] | | | |

APPENDIX 5-1 (cont'd)

Analysis of variance table for LOG10-transformed data

presented in table 5++.

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by one (1) in order to compensate for a single missing observation in table 5. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987).

++ Dunn and Clark, 1987.

** $0.010 < P \leq 0.050$

*** $P \leq 0.001$

## APPENDIX 5-2

Planned comparisons between LOG10-transformed mitogenic responses presented in table 5. Comparisons made with Student's t-test for independent observations[1].

| Con A Concentration (µg/ml) | DL-α-Tocopherol Concentrations Compared (µg/ml) | Cell culture-types compared (1) | Computed T (2) | d.f. (3) | P-value (4) |
|---|---|---|---|---|---|
| 0.5 | 0<br>5 | SC<br>SC | 2.538 | 4 | 0.064 |
| 0.5 | 0<br>0 | SC<br>NASC | 5.480 | 4 | 0.005 |
| 0.5 | 0<br>5 | SC<br>NASC | 2.900 | 3 | 0.063 |
| 0.5 | 0<br>5 | NASC<br>NASC | 4.322 | 3 | 0.023 |

APPENDIX 5-2 (cont'd)

Planned comparisons between LOG10-transformed mitogenic responses presented in table 5. Comparisons made with Student's t-test for independent observations++ .

| Con A Concentration (µg/ml) | DL-α-Tocopherol Concentrations Compared (µg/ml) | Cell-types Compared (1) | Computed T (2) | d.f. (3) | P-value (4) |
|---|---|---|---|---|---|
| 2.0 | 0<br>5 | SC<br>SC | 0.518 | 4 | 0.632 |
| 2.0 | 0<br>0 | SC<br>NASC | 3.768 | 4 | 0.020 |
| 2.0 | 0<br>5 | SC<br>NASC | 1.791 | 4 | 0.148 |
| 2.0 | 0<br>5 | NASC<br>NASC | 2.894 | 4 | 0.044 |

(1) Cell culture-types:
- SC: cultures of C57Bl/6 murine spleen cells containing adherent accessory cells.
- NASC: cultures of C57Bl/6 murine spleen cells depleted of adherent accessory cells.

(2) Computed T-statistic for Student's t-test (unpaired observations).

(3) Degrees of freedom.

(4) P-value for a two-tailed test.

++ Sokal and Rohlf, 1981.

## APPENDIX 6

The time-course of tritiated-thymidine uptake in concanavalin A-stimulated C57Bl/6 murine spleen cells cultured with and without tocopherol.

| TIME (hours) | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) DL-α-TOCOPHEROL (0 µg/ml) | DL-α-TOCOPHEROL (5 µg/ml) |
|---|---|---|
| 3 | 16306 | 16226 |
| | 15899 | 14994 |
| | 14031 | 16742 |
| 6 | 8337 | 7086 |
| | 7126 | 6857 |
| | 6711 | 7673 |
| 9 | 7123 | 5486 |
| | 5389 | 4892 |
| | 4997 | 6623 |
| 12 | 5238 | 4844 |
| | 4724 | 4445 |
| | 4915 | 5251 |
| 15 | 9378 | 8151 |
| | 9058 | 7669 |
| | 8304 | 7526 |

APPENDIX 6 (cont'd)

The time-course of tritiated-thymidine uptake in concanavalin A-stimulated C57Bl/6 murine spleen cells cultured with and without tocopherol.

| TIME (hours) | TRITIATED-THYMIDINE UPTAKE (DPM/2 ml culture) DL-α-TOCOPHEROL (0 µg/ml) | DL-α-TOCOPHEROL (5 µg/ml) |
|---|---|---|
| 18 | 11582 | 7767 |
| | 9594 | 7120 |
| | 9137 | 8625 |
| 21 | 31070 | 15903 |
| | 33396 | 17836 |
| | 30719 | 17379 |
| 24 | 65506 | 28846 |
| | 68500 | 45693 |
| | 72545 | 41064 |
| 27 | 156422 | 95374 |
| | 146346 | 94521 |
| | 139702 | 105032 |

## APPENDIX 7

Contingency table (2 X 2) analysis of the data presented in table 7**.

| RADIATION DOSE (Gy) | NO. DEAD CELLS (-T) (1) | (+T) (2) | NO. VIABLE CELLS (-T) | (+T) | Computed $X^2$ (3) | P-value (4) |
|---|---|---|---|---|---|---|
| 0 | 113 | 84 | 63 | 78 | 5.294 | 0.021 |
| 0.5 | 165 | 146 | 50 | 82 | 8.544 | 0.003 |
| 1.0 | 242 | 122 | 64 | 81 | 21.595 | < 0.001 |
| 2.0 | 212 | 146 | 42 | 81 | 23.091 | < 0.001 |
| 4.0 | 153 | 207 | 21 | 68 | 10.745 | 0.001 |
| 8.0 | 288 | 215 | 27 | 53 | 15.355 | 0.001 |

(1) -T: without tocopherol (0 µg/ml DL-α-tocopherol).

(2) +T: with tocopherol (5 µg/ml DL-α-tocopherol).

(3) $X^2$-value for one (1) degree of freedom.

(4) P-value for a two-tailed test.

++ Woolson, 1987.

# APPENDIX 8-A1

Analysis of variance table for LOG10-transformed data

presented in table 8-A††.

| Source of variation | S.S. (1) | d.f. (2) | M.S. (3) | Computed F | Tabled F (4) |
|---|---|---|---|---|---|
| Due to radiation | 1.2979 | 3 | 0.4326 | 60.867*** | 4.63 |
| Due to tocopherol | 0.2148 | 1 | 0.2148 | 30.214*** | 6.72 |
| Radiation X tocopherol interaction | 0.2030 | 3 | 0.0677 | 9.522** | 4.63 |
| Residual | 0.1137 | 11+ | 0.0071 | | |
| Total | 1.8294 | 18+ | | | |

(1) Sums of squares.
(2) Degrees of freedom.
(3) Mean square.
(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by five (5) in order to compensate for five missing observations in table 8-A. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987).

†† Dunn and Clark, 1987.

** $0.002 < P \leq 0.010$

*** $p \leq 0.001$

## APPENDIX 8-A2

Planned comparisons between LOG10-transformed mitogenic responses observed for the control and 5 µg/ml tocopherol groups at various doses of X-radiation (Experiment 8-A). Comparisons made with Student's t-test for independent observations++.

| RADIATION DOSE (Gy) | % CHANGE (1) | Computed t (2) | d.f. (3) | P-value (4) |
|---|---|---|---|---|
| 0 | -1.0 | 0.099 | 3 | 0.927 |
| 1 | 4.1 | N/A | N/A | N/A |
| 2 | 122.8 | 2.711 | 3 | 0.073 |
| 4 | 143.0 | 5.037 | 4 | 0.007 |

(1) % CHANGE = $\frac{(\text{TOCOPHEROL} - \text{CONTROL})}{\text{CONTROL}} \times 100\%$

(2) Computed t-statistic for Students' t-test (unpaired observations).

(3) Degrees of freedom.

(4) P-value for a two-tailed test.

++ Sokal and Rohlf, 1981.

# APPENDIX 8-B1

Analysis of variance table for $LOG_{10}$-transformed data presented in table 8-B††.

| Source of variation (1) | S.S. (2) | d.f. (3) | M.S. (4) | Computed F | Tabled F |
|---|---|---|---|---|---|
| Due to radiation | 6.7675 | 4 | 1.6919 | 374.720*** | 3.66 |
| Due to tocopherol | 0.1116 | 1 | 0.1116 | 24.711*** | 6.04 |
| Radiation X tocopherol interaction | 0.0797 | 4 | 0.0199 | 4.412** | 3.66 |
| Residual | 0.0903 | 17† | 0.0045 | | |
| Total | 7.0491 | 26† | | | |

## APPENDIX 8-B1 (cont'd)

Analysis of variance table for $LOG_{10}$-transformed data presented in table 8-B++.

(1) Sums of squares.

(2) Degrees of freedom.

(3) Mean square.

(4) Tabled F-value is for $\alpha = 0.050$, two-tailed test.

\+ The residual and total degrees of freedom (d.f.) were reduced by three (3) in order to compensate for three missing observations in table 8-B. For computational purposes, a missing observation in a specific treatment group was replaced with the arithmetic mean for that treatment group (Dunn and Clark, 1987). Note, that the corrected observations were used to perform the two-factor analysis of variance.

++ Dunn and Clark, 1987.

* $0.020 < P \leq 0.050$

*** $P \leq 0.001$

# APPENDIX 8-B2

Planned comparisons between $LOG_{10}$-transformed mitogenic responses observed for the control and 5 µg/ml tocopherol groups at various doses of X-radiation (Experiment 8-B). Comparisons made with Student's t-test for independent observations++.

| RADIATION DOSE (Gy) | % CHANGE (1) | Computed t (2) | d.f. (3) | P-value (4) |
|---|---|---|---|---|
| 0 | 13.4 | 2.941 | 4 | 0.042 |
| 1 | 5.9 | 0.564 | 4 | 0.603 |
| 2 | 19.9 | 2.268 | 2 | 0.151 |
| 4 | 102.3 | 3.030 | 4 | 0.039 |
| 8 | 34.2 | 6.212 | 3 | 0.008 |

(1) % CHANGE = $\frac{(\text{TOCOPHEROL} - \text{CONTROL})}{\text{CONTROL}} \times 100\%$

(2) Computed t-statistic for Students' t-test (unpaired observations)

(3) Degrees of freedom.

(4) P-value for a two-tailed test.

++ Sokal and Rohlf, 1981.

## APPENDIX 8-C

The effects of X-irradiation on concanavalin A-stimulated mitogenic responses in C57Bl/6 murine spleen cells. Mitogenic responses are expressed as tritiated-thymidine incorporation into TCA-precipitable material (DPM/2 ml culture).

| | RADIATION DOSE (Gy) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| | 1407971 | ------- | 1235993 | 469971 | 121154 |
| | 1339703 | 1705660 | 1502286 | 482933 | 177651 |
| | 1477314 | 1505772 | 1257394 | 594567 | 219308 |
| MEAN | 1408329 | 1605716 | 1331891 | 515824 | 172704 |
| S.D. | ±68806 | ±141342 | ±147954 | ±68501 | ±49264 |
| (1) R.R. (%) | 100 | 114 | 95 | 37 | 12 |

(1) R.R.: Relative response defined as the relative ratio of mitogenic responses in irradiated cell cultures to that of the non-irradiated control group (0 Gy).

Note: The cell density was 6.3 X $10^5$ cells/ml.

## APPENDIX 9

Non-parametric statistical analysis of the data presented in table 9 utilizing the Wilcoxon two-sample test for ranked observations++.

| RADIATION DOSE (Gy) | $n_1$ (1) | $n_2$ (2) | Computed U (3) | Tabled U (4) | P-value |
|---|---|---|---|---|---|
| 0 | 8 | 6 | 35 | 40 | 0.155 |
| 1 | 6 | 5 | 17 | 27 | 0.715 |
| 2 | 8 | 4 | 29 | 28 | 0.027 |
| 4 | 9 | 6 | 54 | 44 | 0.001 |

(1) $n_1$ = number of observations in the larger sample.

(2) $n_2$ = number of observations in the smaller sample.

(3) Wilcoxon test-statistic (U).

(4) Tabled U-value for $\alpha = 0.050$, two-tailed test.

++ Sokal and Rohlf, 1981.