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Ecteinascidia turbinata Extracts Inhibit DNA Synthesis in Lymphocytes After Mitogenic Stimulation by Lectins[1] (39059)

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Previous studies from our laboratory have indicated that aqueous ethanol extracts from the sea squirt (*Ecteinascidia turbinata*) exerted *in vitro* and *in vivo* antitumor cell activity, suppressed antibody production in mice immunized with sheep red blood cells (SRBC) and inhibited graft vs host (GVH) and host vs graft reactions (1). More recently we demonstrated that these extracts are capable of inhibiting blastogenic transformations in human and mouse lymphocytes in response to mitogenic and antigenic stimulation (2). The present account summarizes additional findings which help clarify the mechanism of action of this immunosuppression.

Materials and methods. Normal human peripheral blood lymphocytes were separated by Isopaque-Ficoll density gradient (3), adjusted to 1×10^6 cells/ml and cultured in RPMI No. 1640 medium supplemented with 20% human serum and antibiotics.

Triplicate cultures in volumes of 0.5 ml were treated with mitogens and/or extract from *Ecteinascidia turbinata* (Ete) in concentrations and at time intervals indicated under Results. The cells were incubated for 3 days in 5% CO_2 at 37° and pulsed with 2 μCi/culture of tritiated thymidine 24 hr before harvesting and processing as previously described (4). The results are expressed as counts per minute (cpm) and/or stimulation index (SI) i.e. cpm stimulated cultures/cpm control cultures.

Results. Ete added to human lymphocytes *in vitro* exerted a marked suppressive effect on the uptake of tritiated thymidine in mitogen stimulated cultures.

Table I shows the effect of Ete (100 μg/ml) on lymphocyte responses when added before mitogen, at the same time as mitogen or after mitogen (PHA-P 1:200 final concentration and Con A 5 μg/ml). Continuous incubation with Ete resulted in complete suppression of thymidine uptake. Incubation with Ete for 1 hr followed by washing prior to exposure to mitogen caused a marked decrease in response. The most remarkable finding was the virtual abrogation of DNA synthesis when addition of Ete was delayed 24 hr after exposure to mitogen. Moreover, significant suppression was obtained even when an interval of 48 hr had elapsed between addition of the mitogen and the inhibitor. To rule out cytotoxic effects of Ete, cell viability was ascertained by means of trypan blue exclusion and/or formation of SRBC rosettes. These tests demonstrated that Ete in the concentrations used was not cytotoxic.

In order to obtain additional information on the role of temporal factors in lymphocyte stimulation and inhibition, the various treatments were administered to aliquots of a single preparation of lymphocytes preincubated in initial confrontation with either PHA or PHA and Ete. In one such experiment depicted in Fig. 1, 20×10^6 lymphocytes were aliquoted into two vessels one of which received PHA (1:400 final concentration) and Ete (100 μg/ml final concentration) and the other received PHA in the same concentration and growth medium in place of Ete. After 2 hr of incubation the cells were washed twice, resuspended in growth medium and distributed in tubes. The tubes were randomized, divided into triplicate sets and each set treated as follows: PHA (1:400 final dilution), or Ete (100 μg/ml final concentration), or PHA and Ete (in the respective concentration), or additional growth

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medium. The tubes were incubated for 70 hr; ^{3}H-thymidine was added 24 hr prior to termination.

Several points emerge from this experiment: (a) pretreatment with PHA plus Ete resulted in inhibition of initiation of lymphocyte response (compare S.I. 1.59, bar 8 with S.I. 59.17, bar 4 in Fig. 1). (b) This pretreatment also caused the development of resistance of lymphocytes to new stimulation (compare S.I. 6.44, bar 9 with S.I. 172.67, bar 5). (c) The exposure of lymphocytes to PHA alone for a period of 2 hr sufficed to initiate the blastogenic response in a portion of the cells as evidenced by the increased uptake of thymidine, (S.I. 59.17, bar 4) when the cells were shifted to a PHA-free medium. This may be referred to as an early effect. Additional stimulation reaching a S.I. of 172.67 (bar 5) was obtained by the further addition of PHA suggesting that another part of the cell population was stimulated (late effect). (d) Both effects, early and late, were abrogated by the addition of Ete (S.I. of 0.66, bar 7 and S.I. 1.13, bar 6).

TABLE I. EFFECT OF *Ecteinascidia Turbinata* ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES IN BLASTOGENESIS. [a]

Cultivated in:	PHA[c] Counts per minute	PHA[c] SI	Con A[d] Counts per minute	Con A[d] SI
Growth medium (control)	410	1.00	2564	1.00
Mitogen (Mit.)	123794	302.00	21299	8.30
Mit. + Ete[b]	1002	2.44	2557	0.99
Ete 1 hour, wash, then Mit.	38184	93.00	2362	0.92
Mit. 24 hr later Ete	8904	21.70	3645	1.42
Mit. 48 hr later Ete	56430	137.60	8307	3.23

[a] Comparison of pretreatment with posttreatment; stimulation with PHA and Con A.
[b] Ete = 100 μg/ml final concentration.
[c] PHA = 1:200 final concentration.
[d] Con A = 5 μg/ml.

A similar approach was used in another experiment except for a change in the time factors. The initial incubation was allowed to proceed for 24 hr and the second incubations were of 48 hr, again a total incubation period of 72 hr, with ^{3}H-thymidine having been added 24 hr prior to termination of cultures.

As shown in Fig. 2 analogous results were

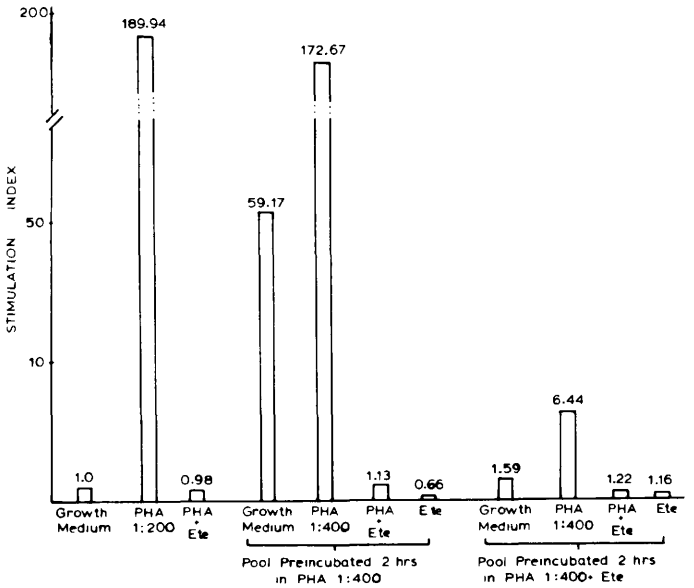

FIG. 1. Inhibitory effects of Ete (100 μg/ml final concentration) on blastogenic responses of lymphocytes in relation to time of inhibitor presentation. Two hour preincubation with mitogen or with mitogen plus inhibitor followed by 70 hr incubation after secondary treatment.

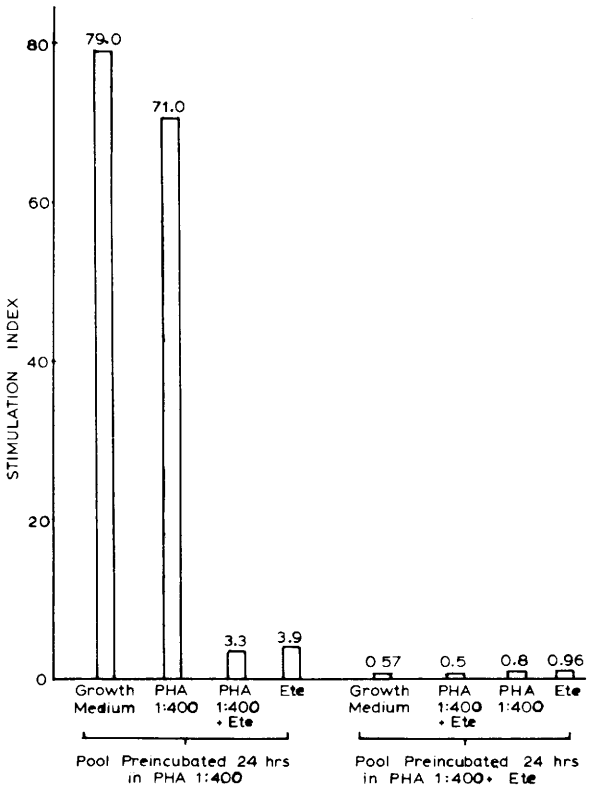

FIG. 2. Inhibitory effects of Ete (100 μg/ml final concentration) on blastogenic responses of lymphocytes in relation to time of inhibitor presentation. Twenty-four hours preincubation period with mitogen or with mitogen plus inhibitor followed by 48 hr incubation after secondary treatment.

obtained. Two findings are noteworthy: (a) the initial exposure of cells to PHA for 24 hr resulted in maximal stimulation as evidenced by the same level of response in cells transferred to fresh growth medium with or without additional PHA and, (b) Ete was capable of neutralizing this maximal effect. These results are interpreted to mean that Ete was able to stop thymidine incorporation into the TCA insoluble fraction, even when given after the maximum commitment to mitogenic stimulation had taken place.

Discussion. Extracts of *Ecteinascidia turbinata* inhibited the mitogenic effects of PHA and Con A in cultures of human lymphocytes. It was previously shown that these preparations were also inhibitory to mouse spleen cells confronted with the same mitogens or a tumor antigen to which they were sensitive (2, 5). On the assumption that these mitogens and antigens bind to different receptors on the lymphocyte membrane, the results imply that Ete did not exert this effect by virtue of competition for a specific binding site. While ruling out competition for site, these experiments did not exclude the possibility that Ete acted by combining with the mitogens and reducing their availability to the cells. This point was tested (unpublished observations) by experiments in which increasing amounts of mitogen were presented to cells treated with Ete. There was no indication that mitogen was being consumed by coupling with the extract. However, the most convincing evidence against the action of Ete being at the level of receptors comes from the findings with inhibition of stimulation demonstrable in full at 24 hr of contact with the mitogen at a time when maximal commitment to mitogenic response had already taken place. In fact, significant inhibition was still

demonstrable at 48 hr. These findings distinguish the activity of Ete from the activities of sugars which are known to inhibit the effect of plant lectins. α-methylmannoside inhibits the effect of Con A and N-acetylgalactosamine inhibits the action of PHA but only within the first 20 hr of exposure (6). These substances apparently act at the receptor level. Our findings suggest that Ete is capable of canceling or reversing a signal emanating from an event subsequent to the binding of the lectins. It is not known whether this occurs intracellularly or by configurational alterations of the cell surface. In this connection it may be pertinent to mention a preliminary observation suggesting that Ete treated cells are capable of interfering with the mitogenic response of untreated cells. In that experiment Ete treated cells were washed and mixed with fresh lymphocytes exposed 2 hr previously to PHA. The latter cells showed no increased uptake of thymidine.

It was previously demonstrated that Ete can abrogate *in vivo* cell mediated immunity as measured by GVH reactions and allograft rejections. Together with the present findings of the inhibition of responses to PHA and Con A the results argue in favor of Ete being a T cell suppressor. Suppression of T cells can also be invoked as an explanation of the inhibition of the antibody production to a T cell dependent antigen, sheep red blood cells (1). However, the temporal conditions underlying maximal interference with humoral and cellular immunity suggest that the effect may have been also directed against B cells. Evidence for direct activity against B cells has been obtained in preliminary experiments. Work is currently in progress on the isolation of the active principle responsible for the activities of Ete.

Summary. Aqueous ethanol extract of a tunicate which was previously found to exert antitumor and immunosuppressive activities *in vivo* was tested for its effect on normal human lymphocytes *in vitro*. The extract suppressed the uptake of tritiated thymidine by lymphocytes stimulated with mitogen. This suppressive effect did not require continuous presence of the extract. Treatment of lymphocytes prior to mitogenic stimulation resulted in suppressive effect. The fact that suppression by the extract could also be achieved 24 hr after exposure to mitogen, an interval which was found to suffice for the attainment of maximal commitment for blastogenic transformation indicates that Ete can act at a stage subsequent to the binding of the lectin and elicitation of a mitogenic signal(s).

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