

## Effect of Levamisole on the *In Vitro* Immune Response of Spleen Lymphocytes (38686)

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Stimulation of the immune system by chemical agents such as poly I:poly C (1-3), polyanions (4), tilorone (5), pyran copolymer (6), and levamisole (7-9) have been demonstrated by *in vivo* methods. The chemicals poly I:poly C (10) and levamisole (11) are active *in vitro* stimulators of spleen cell cultures, and levamisole has been shown to be a stimulator of peripheral blood lymphocytes (12). We have used the *in vitro* mixed lymphocyte reaction to demonstrate a direct stimulatory action of levamisole on cells involved in allogeneic recognition reactions and report here that low concentrations of levamisole induce blastogenic stimulation of spleen cell cultures as well as enhance allogeneic stimulation in mixed lymphocyte cultures.

**Methods and Materials.** Inbred mouse strains. C57B1/6 and DBA/2 male mice, 6-8 wk of age, were used throughout the study.

**Levamisole.** L-2, 3, 5, 6-tetrahydro-6-phenylimidazole [2, 1-b] thiazole hydrochloride (LMS, obtained from Janssen Pharmaceuticals, Beerse, Belgium) was dissolved in distilled water at a concentration of 1 mg/ml. Appropriate dilutions were made in RPMI-1640 medium containing 20% fetal bovine serum, 100 units/ml penicillin and 1  $\mu$ g/ml streptomycin (RPMI-FBS). The drug was added to cultures in 100  $\mu$ l volume at the initiation of the cultures.

**Mixed lymphocyte culture.** The one way mixed lymphocyte reaction (MLR) was employed, using mitomycin C (75  $\mu$ g/ml final concentration) as target cell blocking agent (13). Spleens were removed and minced finely in RPMI-1640; single cell suspensions were prepared by forcing the pieces through a 16-gauge needle and passing the suspension through gauze pads. The

cells were washed twice in 50 ml RPMI-1640 with centrifugation at 980 g for 5 min in a PR2 refrigerated centrifuge. Target cells were inactivated by treating an aliquot of the cell suspension with mitomycin C (Cal. Biochem., San Diego, CA) for 30 min at 37° followed by two 50 ml washes of RPMI-1640. Effector cells were diluted in RPMI-FBS to a density of  $1 \times 10^6$  cells/ml; target cell suspensions were diluted in RPMI-FBS to contain  $2 \times 10^6$  cells/ml. One ml of both target and effector cells were mixed in plastic 12  $\times$  75 mm tubes containing 100  $\mu$ l of the appropriate LMS concentration, and the cultures were placed in a humidified 5% CO<sub>2</sub>-in-air atmosphere at 37° for 1, 2, or 3 days. Four hours before termination, 2  $\mu$ Ci <sup>3</sup>H-methyl thymidine (<sup>3</sup>H-TdR) (New England Nuclear, sp. ac. 2 Ci/mole) was added to each culture in 50  $\mu$ l vol. The cells were collected on glass-fiber pads, washed with phosphate buffered saline, pH 7.2, and the DNA precipitated on the pad with 10% cold TCA. The pads were dried and placed in 10 ml Aquasol (New England Nuclear, Inc., Boston, MA) in scintillation vials. <sup>3</sup>H-TdR incorporation was determined by liquid scintillation spectrometry in a Packard TriCarb Liquid Scintillation counter, using the channels ratio method for determining <sup>3</sup>H counting efficiency. Stimulation indices are reported as the ratio of disintegration/min (d/m) in cultures with allogeneic target cells  $\div$  d/m in cultures with isogeneic target cells.

**Results and Discussion.**  $1 \times 10^6$  spleen cells in 2 ml RPMI-FBS were incubated with increasing concentrations of LMS in order to confirm the effect of LMS on spleen cell blastogenesis seen previously (11). The enhancing effect of LMS on DNA synthesis of the spleen cell cultures is seen in Table I. Maximum stimulation was con-

sistently observed at 10–100 ng/ml and occurred on the first day in culture in two of three experiments; the stimulation of effector cells alone always preceded any effect on allogeneic stimulation and occurred at lower concentrations of LMS. DNA synthetic response of allogeneic-stimulated C57B1/6 effector cells was elevated slightly on days 2 and 3 in the presence of 1  $\mu$ g/ml LMS. Lower doses of LMS were not consistently active. The response of DBA/2 effector cells to allogeneic target cells was not affected by LMS (Table II).

The time course of allogeneic lymphocyte stimulation was not altered by the presence of LMS; the time of maximal stimulation was the third day after culture initiation, indicating that recognition of, and blastogenic reaction to, histocompatibility antigen was not initiated faster in the presence of LMS.

The blastogenic reaction of spleen cells (Table I) in the presence of LMS suggests that stimulation of the immune response could be primarily due to initial nonspecific proliferation of lymphoid cells, leading to

an increased number of cells capable of reacting specifically to the antigen presented. Such a mechanism would be useful in overcoming depleted immune cell compartments in immunosuppressed animals, allowing restoration of resistance to infection or return of immune surveillance in depression due to malignancy. Evidence for this activity has been obtained by several investigators. Perk *et al.* (personal communication) have found that germinal centers and lymphoid follicles of spleens from immunosuppressed mice returned to normal more rapidly in animals treated with LMS. In addition, Tripodi *et al.* (14), have demonstrated that LMS stimulates immune responses in anergic tumor patients, and Lichtenfeld *et al.* (12) have reported enhanced immunologic stimulation of peripheral lymphocytes from tumor patients in the presence of LMS. We are presently investigating the mechanism of action of LMS on the various compartments of the reticuloendothelial system and have obtained evidence that while lymphoid cells are susceptible to the blastogenic activity

TABLE I. EFFECT OF LEVAMISOLE ON DNA SYNTHESIS OF MURINE SPLEEN CELL CULTURES. SPLEEN CELL SUSPENSIONS WERE PREPARED IN RPMI-FBS AND DISPENSED IN 2 ml VOLUMES AT A DENSITY OF  $5 \times 10^5$ /ml. LMS WAS ADDED IN 100  $\mu$ l VOLUME AT INITIATION OF CULTURES. 2  $\mu$ Ci  $^3$ HTdR WAS ADDED 4 HOURS BEFORE TERMINATION AFTER 1, 2, OR 3 DAYS *in vitro*. LABELED CELLS WERE COLLECTED ON GLASS-FIBER FILTERS AND THE DNA PRECIPITATED WITH COLD 10% TCA.

LMS conc $\mu$ g/ml	Mouse strain	Day 1		Day 2		Day 3	
		Ave $d/m^a \pm$ SE	% Control	Ave $d/m \pm$ SE	% Control	Ave $d/m \pm$ SE	Control
0	C57B1	4055 $\pm$ 156	100	5651 $\pm$ 2051	100	6132 $\pm$ 1314	100
0.001		4908 $\pm$ 764	121	6741 $\pm$ 474	119	6513 $\pm$ 862	106
0.01		8955 $\pm$ 486 <sup>b</sup>	220	7221 $\pm$ 233	127	10040 $\pm$ 985 <sup>d</sup>	163
0.1		4209 $\pm$ 356	103	5735 $\pm$ 275	101	8799 $\pm$ 794	143
1.0	DBA	5069 $\pm$ 1747	125	5343 $\pm$ 67	94	5403 $\pm$ 1069	88
0		6745 $\pm$ 371	100	11099 $\pm$ 953	100	31356 $\pm$ 5313	100
0.001		7319 $\pm$ 832	108	13168 $\pm$ 578	117	27860 $\pm$ 2634	88
0.01		9859 $\pm$ 506 <sup>c</sup>	145	18036 $\pm$ 1032 <sup>c</sup>	162	30762 $\pm$ 2453	98
0.1		8451 $\pm$ 514 <sup>d</sup>	125	17793 $\pm$ 2254	160	34001 $\pm$ 2582	108
1.0		9017 $\pm$ 335 <sup>c</sup>	133	17477 $\pm$ 1799 <sup>d</sup>	157	38625 $\pm$ 239	123

<sup>a</sup> Average disintegrations/min. of three replicate cultures  $\pm$  standard error of the mean.

<sup>b</sup> Significant at the  $P < 0.01$  level by Student's  $t$  test, as compared to value obtained in untreated cultures.

<sup>c</sup> Significant at the  $P < 0.025$  level by Student's  $t$  test, as compared to value obtained in untreated cultures.

<sup>d</sup> Significant at the  $P < 0.05$  level by Student's  $t$  test, as compared to value obtained in untreated cultures.

TABLE II. EFFECT OF LEVAMISOLE ON ALLOGENEIC STIMULATION OF DNA SYNTHESIS IN MLR.  $10^6$  EFFECTOR CELLS AND  $2 \times 10^6$  MITOMYCIN TREATED CELLS WERE MIXED IN 2 ml RPMI-FBS. LMS WAS ADDED IN 100  $\mu$ l VOLUME AT CULTURE INITIATION.  $^3$ HTdR LABELING AND DNA PRECIPITATION AS IN TABLE I.

LMS conc.	Target cell	Effector cell	Day 1		Day 2		Day 3	
			Ave. $d/m^a$	Stim. <sup>b</sup> index	Ave. $d/m \pm$ SE	Stim. Index	Ave. $d/m \pm$ SE	Stim. index
0	DBA	C57Bl	4478 $\pm$ 396	0.89	10862 $\pm$ 236	4.93	34568 $\pm$ 1382	7.90
0.001			3929 $\pm$ 405	1.10	6382 $\pm$ 1052 <sup>d</sup>	2.03	29148 $\pm$ 5736	8.11
0.01			5324 $\pm$ 403	1.26	10095 $\pm$ 581	3.80	39786 $\pm$ 168 <sup>d</sup>	11.07
0.1			5340 $\pm$ 521	1.04	11087 $\pm$ 1567	4.17	34785 $\pm$ 3604	9.87
1.0			5414 $\pm$ 378	0.83	12521 $\pm$ 87 <sup>c</sup>	3.49	42404 $\pm$ 2488 <sup>d</sup>	14.24
0	C57Bl	DBA	12090 $\pm$ 897	1.51	20278 $\pm$ 666	2.18	51362 $\pm$ 3888	2.81
0.001			13086 $\pm$ 617	1.69	20928 $\pm$ 2385	2.33	46119 $\pm$ 1508	4.43
0.01			13911 $\pm$ 949	1.62	21936 $\pm$ 907	2.46	50715 $\pm$ 4956	3.36
0.1			13539 $\pm$ 1719	1.69	19592 $\pm$ 790	2.11	52386 $\pm$ 2578	3.24
1.0			14520 $\pm$ 675	1.58	19329 $\pm$ 1726	2.03	42565 $\pm$ 3320	1.66

<sup>a</sup> Average disintegrations/min of three replicate cultures  $\pm$  standard error of the mean.

<sup>b</sup> Stimulation index = Ave  $d/m$  with allogeneic target cells/Ave  $d/m$  with isogenic target cells.

<sup>c</sup> Significant at the  $P < 0.025$  level by Student's  $t$  test, as compared to value obtained in untreated cultures.

<sup>d</sup> Significant at the  $P < 0.05$  level by Student's  $t$  test, as compared to value obtained in untreated cultures.

of LMS, macrophages are not stimulated to mitosis by the drug.

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Received September 6, 1974. P.S.E.M.B. 1975, Vol. 148.