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The Effect of Levamisole on Cell-mediated Immunity and Suppressor Cell Function[1]

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SUMMARY

The immunopotentiating drug, levamisole, was found to augment human lymphocyte responses to allogeneic cells and plant mitogens *in vitro*. The effect was critically dose dependent and, at high doses, suppression rather than augmentation of the immune response was observed.

Our hypothesis that augmentation of immune responses by the drug is due to the selective impairment of immunoregulatory suppressor activity was tested in a model using human splenic and thymic suppressor cells. Contrary to expectation, the drug was found to be capable of augmenting suppressor activity rather than abolishing it.

It is concluded that levamisole is a nonspecific stimulator of lymphocyte function, irrespective of the role played by these cells in the human response.

INTRODUCTION

Levamisole, *l*-2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)-thiazol monohydrochloride, is an anthelmintic drug which has been shown to potentiate both *in vitro* (8–10) and *in vivo* (8, 13, 18) immune responses in animals and man. As a result, there has been considerable interest in the drug as a possible therapeutic agent that might be of value in the treatment of cancer by the augmentation of immune responses to tumors. Encouraging results have been observed in laboratory animals in the remission of both solid tumors (12) and leukemia (2). In addition, the drug has been shown to increase impaired cellular immunity in cancer patients (18).

However, there are some conflicting reports that show that the drug has no effect on *in vitro* responses (5) and no effect on animal tumor models (11).

The purpose of this study was to define more accurately dose-response effects of levamisole on cell-mediated immunity using an *in vitro* model and, further, to test our hypothesis that the drug augments immune responses by selectively impairing suppressor cell function. The *in vitro* models used were the MLC[3] and the response of lymphocytes to stimulation by plant mitogens.

For the suppressor cell studies, a previously described model involving the use of stimulated splenic or thymic cells was used (15, 16). It was found in this model that splenic cells stimulated with PHA were capable of suppressing MLC, and rodent studies have suggested that a subpopulation of such cells plays an important role in the regulation of immune responses (7, 17). The effect of levamisole on the ability of these cells to suppress the MLC was tested.

MATERIALS AND METHODS

MLC's were set up between random normal volunteers. Heparinized venous blood was drawn and lymphocyte suspensions were prepared by density gradient separation using Ficoll-sodium metrizoate, (specific gravity, 1.08) (Pharmacia Fine Chemicals, Inc., Piscataway, N. J., and Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N. Y.). Cell counts were adjusted to 1×10^6/ml in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated human AB serum, 100 units penicillin per ml, 100 μg streptomycin per ml, 1% L-glutamine (200 mM; Microbiological Associates, Bethesda, Md., and 2.5% *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (1 M; Grand Island Biological Co. Cells used as stimulators were treated with Mitomycin C (Laboratory & Educational Supplies, Inc., Los Angeles, Calif.), 25 μg/ml for 30 min at 37°. Aliquots of 1×10^5 responding and stimulating cells were placed in the wells of Linbro microtiter plates (Linbro Chemical Co., New Haven, Conn.) and incubated for 6 days at 37° in an atmosphere containing 5% CO_2.

A stock solution of levamisole was made up (levamisole hydrochloride, R 12514, Batch SM 18276; Janssen Pharmaceutica, New Brunswick, N. J.), and graded doses of drug were added to wells at the time of setting up the MLC to provide final concentrations of between 0.2 and 3.0 μg/ml.

At Day 6, 2 μCi [^{3}H]thymidine (New England Nuclear, Boston, Mass.) were added to each well, and after a further incubation of 6 hr the cells were harvested with the multiple automated sample harvester (Mash II Model, Microbiological Associates) onto Whatman glass fiber discs (H. Reeve Angel & Co., Clifton, N. J.). These were then dried and immersed in scintillation fluid and counted on a Nuclear-Chicago counter. All cultures were run in triplicate. The effect of the levamisole on the MLC was expressed as a simple ratio

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[3] The abbreviations used are: MLC, mixed lymphocyte culture; PHA, phytohemagglutinin-P.

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$$\text{Augmentation} = \frac{\text{cpm for MLC + levamisole}}{\text{cpm for MLC alone}}$$

If the resulting number was greater than 1, then augmentation of the MLC had occurred; while if it were below 1, then the levamisole was suppressing the MLC.

For the mitogen studies peripheral blood lymphocyte suspensions were prepared as for the MLC but 0.2×10^6 cells were added to each well. Separate studies were carried out using PHA, 6.0 μg/ml (Difco Laboratories, Detroit, Mich.), concanavalin A (Difco), 50 μg/ml, and pokeweed mitogen (Grand Island Biological Co.), 30 μg/ml. Cultures were incubated for 3 days prior to counting. The effect of levamisole was determined as for the MLC by the addition of graded doses of the drug at the beginning of the culture and expressed as a ratio

$$\text{Augmentation} = \frac{\text{cpm for culture + levamisole}}{\text{cpm for culture alone}}$$

For the suppressor cell studies, suspensions of human spleen or thymus cells were obtained from material discarded at operation. The thymus specimens were obtained from patients undergoing cardiac surgery for ischemic heart disease, and the specimens used were those removed to gain access to the coronary vessels. Splenic tissue was obtained from patients undergoing laparotomy for splenic rupture due to trauma, to gastric surgery in which the spleen was incidentally removed, or from patients undergoing splenectomy prior to renal transplantation. In a previous report (16) it was shown that the suppressor activity of splenic cells obtained from uremic patients did not differ from those obtained in otherwise normal patients undergoing splenectomy for trauma. The system has been described in detail elsewhere (15, 16); briefly, the cell suspensions were prepared by homogenizing the tissue concerned and making up a cell suspension in prepared Roswell Park Memorial Institute medium similar to that used for the MLC. The cells were then cultured for 3 days with PHA, and following repeated washing, the cells were added to the MLC; the MLC was terminated after 6 days. The degree of suppression provided by the cells was calculated from the formula

$$\%\ \text{suppression} = 100 - \frac{\text{cpm for MLC + stimulated suppressors}}{\text{cpm for MLC + unstimulated suppressors}} \times 100$$

The suppressors were divided into 2 groups. One group was prepared as described above, and the 2nd group was incubated with levamisole, 1 μg/ml, during the 3-day incubation period with PHA. The effect of the 2 groups of cells was compared in paired experiments, and the differences in subsequent suppression of the MLC was evaluated using the paired *t* test.

As a control for all of the above experiments, cell suspensions were incubated with levamisole alone and counted after 6 days.

RESULTS

Levamisole alone was without effect on lymphocyte suspensions kept in culture for 6 days. This is illustrated in Table 1. It demonstrates that, in the absence of stimulation, the cultures took up little [^{3}H]thymidine, and graded doses of levamisole did not significantly alter the measured activity.

Table 1
Effect of levamisole on unstimulated cultured lymphocytes

Experiment	cpm/culture after following dose of levamisole					
	0.0 μg/ml	0.2 μg/ml	0.6 μg/ml	0.8 μg/ml	1.2 μg/ml	1.6 μg/ml
1	172[a]	302	427	582	79	163
2	200	191	236	359	154	176
3	138	167	197	222	123	160
4	355	302	258	283	393	260
5	1141	1203	473	2315	860	572

[a] Values are the mean of triplicate observations.

The effect of the drug on the MLC is shown in Table 2. The derived ratio of counts of the MLC with the drug to the counts of the MLC alone is plotted against the dose of drug in the culture medium in Chart 1. The values given are the means for between 3 and 18 experiments. An augmentation of the MLC was observed in the dose range of approximately 0.4 to 1.2 μg/ml. At higher doses suppression of the MLC was noted and at 3.0 μg/ml intense suppression of the MLC was seen.

Table 3 demonstrates the effect of levamisole on mitogen-induced responses; Chart 2 illustrates the derived ratios of levamisole-treated to untreated cultures. The pattern of augmentation of the responses was similar to that obtained with the MLC studies. All points represent the mean of 6 experiments. Again, at higher doses, the drug appears to depress mitogen responses.

The effect of levamisole on the suppressor activity of splenic or thymic cells is documented in Table 4, and the calculated percentage suppression for treated and untreated suppressor cell is shown in Chart 3. In these paired experiments, cells incubated with levamisole consistently showed greater suppressive ability than cells not exposed to the drug, the mean values for suppression of the 2 groups being 74 and 60%, respectively ($p < 0.05$, using paired *t* test).

In all the experiments described, levamisole did not have any effect on cell viability, as judged by the eosin Y dye exclusion test.

DISCUSSION

The dose levels of levamisole used in the study were chosen to reflect recommended therapeutic levels. The MLC was certainly augmented at a level of between 0.4 and 1.2 μg/ml. This agrees very well with figures given in an earlier study (9), and further, we found the 3- to 4-fold increase in activity of the MLC to be similar to that reported in the earlier study.

Table 2
Effect of levamisole on MLC

Experiment	cpm/culture after following dose of levamisole								
	0 μg/ml	0.2 μg/ml	0.4 μg/ml	0.6 μg/ml	0.8 μg/ml	1.0 μg/ml	1.2 μg/ml	1.4 μg/ml	3.0 μg/ml
1	2,286[a]	2,567	20,223	51,824	35,334	19,218	18,681	1,824	
2	7,963	2,862	15,677	30,322	30,202	17,397	22,543	3,719	
3	3,563	2,891	23,397	36,432	37,820	35,166	21,996	3,189	
4	28,540	28,952	20,830	27,543	36,589	36,991	1,041		
5	37,289	38,347	32,701	35,745	34,733	37,375	1,898		
6	31,264	30,963	24,813	37,765	46,754	39,750	931		
7	47,846	39,869	36,578	44,052	48,316	45,400	36,095	34,108	
8	61,944	54,400	44,059	55,094	63,378	50,106	42,746	46,288	
9	38,127	40,687	49,729	42,376	44,271	53,637	44,506	30,974	
10	30,451	28,814	32,006	26,681	19,979	20,703	30,585		
11	9,640	30,697	22,224	14,395	11,312	20,726	27,495		
12	38,552	54,826	35,681	42,662	29,499	32,427	30,122		
13	9,036	5,597	2,486	3,285	3,417	5,478	5,936	12,873	
14	5,719	8,882	6,220	6,782	6,608	7,416	9,150	13,204	
15	10,960				8,663				539
16	7,253				7,513				1,130
17	7,120				10,933				1,233

[a] Values are the mean of triplicate observations.

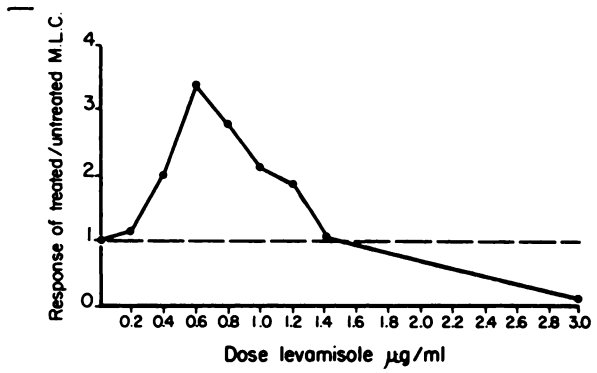

Chart. 1. The effect of levamisole on the MLC. A sharp augmentation of the mixed lymphocyte reaction occurred at a levamisole dose level of 0.6 μg/ml, while doses in excess of 1.4 μg/ml suppressed the MLC. *Points*, the mean of between 3 and 18 experiments.

However, the effective dose range is a narrow one, and at 3.0 μg/ml we observed suppression of the MLC. Others have reported suppression at high doses (5, 8). This clearly has important clinical implications, since it has been suggested that the drug merits clinical trials in the treatment of patients with cancer (3, 4), and it is conceivable that a series of conflicting reports on the efficacy of the drug may arise simply due to small variations in dose protocols. In addition, the possibility arises that cancer patients may indeed be made worse if dose levels are such that immunosuppression rather than immunopotentiation was likely to occur. Consequently, it would seem prudent to monitor clinical trials of the drug by *in vitro* assay of cell-mediated responses of the patient to be sure that the desirable dosage was, in fact, being achieved.

The effect of the drug on mitogen-induced responses was

Table 3
Effect of levamisole on lymphocyte response to plant mitogens

Experiment	Mitogen	cpm/culture after following dose of levamisole					
		0 μg/ml	0.2 μg/ml	0.6 μg/ml	0.8 μg/ml	1.2 μg/ml	1.6 μg/ml
1	Pokeweed mitogen	7,730[a]	52,786	61,442	63,499	3,658	6,002
2	Pokeweed mitogen	18,649	17,207	14,205	16,088	14,273	16,189
3	Pokeweed mitogen	1,060	1,405	1,324	869	975	887
4	Pokeweed mitogen	7,393	6,272	6,761	6,192	5,860	6,545
5	Pokeweed mitogen	51,077	69,158	74,550	82,199	73,751	61,833
6	Pokeweed mitogen	11,959	9,601	12,630	11,920	12,068	10,649
7	Concanavalin A	74,055	71,364	79,440	98,799	70,680	79,059
8	Concanavalin A	5,321	18,311	17,908	11,340	1,456	2,416
9	Concanavalin A	1,143	2,087	1,639	904	907	796
10	Concanavalin A	43,097	35,629	33,748	42,227	34,458	43,093
11	Concanavalin A	51,778	36,290	45,972	42,610	45,586	38,975
12	Concanavalin A	11,817	8,650	11,414	13,635	11,301	10,572
13	PHA	177,097	164,279	165,197	166,568	174,043	142,041
14	PHA	9,803	25,910	16,112	18,420	7,516	13,715
15	PHA	9,882	12,792	12,309	8,355	6,684	7,264
16	PHA	88,730	82,407	68,816	82,181	80,468	88,750
17	PHA	78,843	58,344	69,107	67,113	76,052	57,884
18	PHA	5,792	4,512	7,086	3,771	5,786	4,977

[a] Values are the mean of triplicate observations.

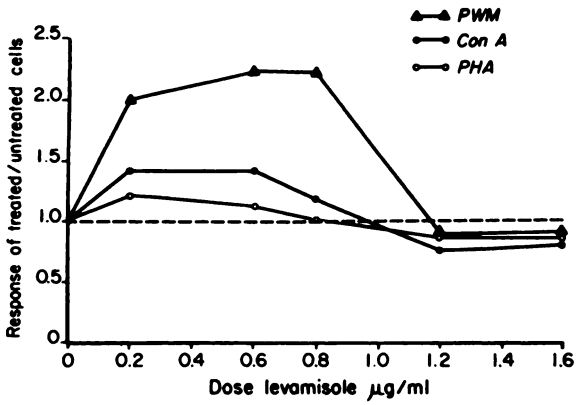

Chart 2. The effect of levamisole on mitogen responses. Levamisole potentiated responses to mitogens in a similar way to that seen in the MLC. Each *point* is the mean of 6 experiments. *PWM*, pokeweed mitogen; Con A, concanavalin A.

Table 4

Effect of levamisole on suppressor cell function

Experiment	cpm/culture		
	Control MLC	Control MLC + suppressors	Control MLC + levamisole-treated suppressors
1	1,559	1,012	188
2	1,790	1,421	1,154
3	3,269	1,906	1,377
4	26,107	13,575	10,342
5	25,422	3,891	2,408
6	15,610	5,210	3,714
7	34,691	1,572	1,211
8	15,560	4,818	3,522
9	22,501	3,889	2,401

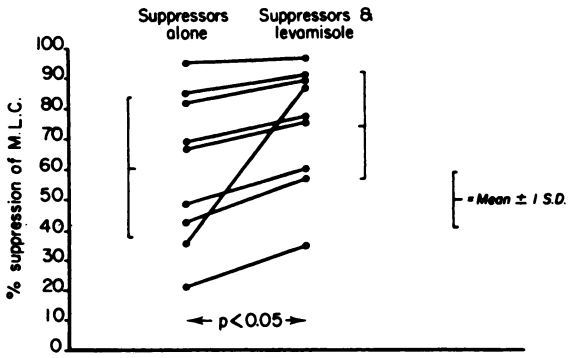

Chart 3. The effect of levamisole on suppressor cell function. In paired experiments, levamisole consistently potentiated suppressor cell activity ($p < 0.05$, derived from paired t test).

qualitatively similar to that observed in the MLC but was not as marked. However, the therapeutic dose range seems to be much the same, but again, suppression of responses was seen at higher doses. Of the 3 mitogens tested, it appeared that the response to pokeweed mitogen was augmented to the greatest degree, and this would suggest that the drug acts more selectively on the B-cell population than on the T-cell population (1). However, such distinctions may not be justified (6).

It has been suggested that some immune-deficient states and some naturally occurring cancers are associated with excessive suppressor cell activity (14, 19), and it seemed an attractive hypothesis that levamisole might act by specifically impairing suppressor cells, thus accounting for its known immune-potentiating effects, and some of the tumor regression seen experimentally. However, our observations do not support this hypothesis and, in fact, potentiation of suppressor activity was consistently observed in the paired experiments. Extrapolating the *in vitro* observations to *in vivo* situations, it would seem that on balance, although the drug is capable of increasing the activity of immunoregulating suppressor cells, the drug clearly potentiates helper activity to a greater degree, and the net result is immunopotentiation.

It is concluded that the drug is able to augment several types of lymphocyte function, irrespective of the part played by the cell in the immune response.

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