Cellular Immune Modulation after a Single High Dose of Levamisole in Patients with Carcinoma

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The effect of a single high dose of Levamisole (200 mg/M^2) on delayed-type hypersensitivity (DTH) *in vivo* and on lymphocyte blastogenesis to mitogens and antigens *in vitro* was studied in 26 patients with carcinoma. Similar studies were conducted in 24 control patients. Levamisole had a moderate but significant enhancing effect on DTH to Dermatophytin detectable no earlier than eight hours and still present at 48 hours after the drug administration. A moderate but significant enhancing effect on lymphocyte blastogenesis to mitogens and antigens was also demonstrated during the same time sequence. Further clinical trials with Levamisole should be conducted with more attention paid to schedule of drug administration.

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CELLULAR IMMUNE DEFICIENCY has been documented in patients with various malignant diseases including most, if not all, histologic categories.[2–4,6,11,18–21] Clinical correlations frequently indicate that good general immunocompetence is a highly favorable prognostic factor in these neoplastic diseases.[2,6,11,20] A growing body of evidence now suggests that correction or reversal of some of the immunologic deficiencies associated with cancer can be achieved by the administration of immunotherapy, along with significant prolongation of chemotherapy- and surgery-induced remissions and overall survival in selected groups of patients.[11,14,15,20,33] The most widely used immunopotentiating agent in these clinical trials is BCG, although other biological (bacterial and nonbacterial) products are also being evaluated at present.[14,15,28,32,33,41,43]

The use of synthetic chemicals as immune modulators in the treatment of cancer is in its infancy. One such group of recently discovered substances is the phenyl-imidothiazole compounds. In particular, Levamisole, the levoisomer of tetramisole (2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b) thiazole hydrochloride) has received a great deal of attention in recent years, mainly because of its unique immunobiologic characteristics. Levamisole enhances antibody production in mice[38] and man[26] and it may protect against infection in rats[12] and man.[10] There are conflicting reports on the antineoplastic effect of Levamisole when given as a single agent.[35,39,42] However, it can prolong chemotherapy-induced remission and overall survival in animal tumor models.[8,46]

The effect of Levamisole on human cancer is currently being studied. Poor or absent delayed-type hypersensitivity (DTH) skin reactions to Dinitrochlorobenzene (DNCB) and PPD can be promptly restored in 25–45% of cancer patients and elderly people by a short administration of Levamisole[51,52,54] Even more striking is a recent demonstration that Levamisole, used as an adjuvant to potentially curative surgery in lung cancer patients, can effectively prolong the disease-free interval.[1] A study of optimal dose and schedule was urgently needed to achieve maximal antitumor effect. One of the major questions relevant to *in vivo* use of Levamisole as an immunomodulatory agent is the duration and intensity of its effect on the various components of the immune system. Therefore, we undertook a sequential study of *in vivo* and *in vitro* cellular immune parameters among patients with carcinoma who received a single maximum tolerated dose of Levamisole.

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Materials and Methods

Fifty patients with histologically proven carcinoma of the large bowel (31), breast (8), lung (6), stomach (2), small bowel, pancreas, and head and neck were studied during their evaluation and work-up in the clinic. All the patients had advanced cancer, with the exception of one patient with Dukes' "B" carcinoma of the colon. They were stratified according to previous treatment status (chemotherapy and radiotherapy vs. no treatment). Patients who previously received immunotherapy were excluded. No patient received chemo- or radiotherapy three weeks prior to or during the study. After an informed consent was obtained, 26 patients were randomized to receive Levamisole and the remaining 24 served as controls. The randomization plan was prepared by the statistician of the Ortho Research Foundation in the form of consecutively numbered and sealed envelopes containing cards indicating Levamisole or control. The envelopes were opened only after an informed consent was obtained. In analyzing the study we found that the distribution of sex and age (mean and range) and primary site of tumors was similar between treatment and control groups.

Levamisole (R 12564, Janssen R&D Inc., New Brunswick, NJ) in 25- and 150-mg tablets was given in a single dose of 200 mg/M^2. This dose was selected after a higher single dose of 265 mg/M^2 (based on animal toxicity data) was found to be intolerable for two patients. Sequential immunologic studies *in vitro* and *in vivo* were performed at these time points: Patients randomized to receive Levamisole were studied twice before Levamisole administration (−24 hours, 0 hours) and also at 4, 8, 24, and 48 hours after drug administration. Control patients were studied according to the same time schedule but without receiving Levamisole. In general, each study point consisted of a skin test application *in vivo* and lymphocyte blastogenesis *in vitro*.

Skin Tests

The skin test antigens used and the technique of their application have been previously described.[20] All the skin test reactions were read 24 and 48 hours after application. Forty-four of 50 patients in the study were skin-tested initially with a battery of recall antigens including Dermatophytin as part of their clinical evaluation. However, Dermatophytin was the only antigen used for sequential immunologic evaluation throughout the study in all patients. This recall antigen was chosen because of its close correlation with reactivity to DNCB in solid tumor patients.* Positive delayed type hypersensitivity reactions were taken as ≥2 mm of induration and expressed by geometric mean of 2 right angle diameters.

Skin test data were analyzed in two ways: First, in terms of significant changes in mm of induration between study points. A positive change was defined as a conversion of skin tests from negative to positive or ≥100% increase in size of induration. A negative change was defined as conversion from positive to negative or ≥50% decrease in size of induration. No change was defined as <100% increase or <50% decrease in size of induration. The overall sequential changes within each group and the differences between Levamisole-treated and control patients at each study point were analyzed by the chi-square test.[44] Second, skin reactions expressed in mm of induration were compared between Levamisole-treated and control patients at each study point by the Wilcoxon-Mann-Whitney test for unpaired observations.[44]

Lymphocyte Blastogenesis

Concurrent with the application of the skin tests, venous blood was drawn for *in vitro* studies of lymphocyte blastogenesis. Blood was defibrinated and lymphocytes separated on ficoll-hypaque as previously described.[29] Lymphocytes were washed twice in Hank's balanced salt solution and resuspended in RPMI 1640 (Grand Island Biological Company, Grand Island, NY) supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2mM/ml of L-glutamine, and 10% pre-Levamisole autologous serum. The cell concentration was adjusted to 7.5 × 10^5/ml. Microcultures were set up in 6–12 replicates as described by Thurman *et al.*[49] with these modifications: 1.5 × 10^5 lymphocytes (0.3 ml) were distributed with a Hamilton repeating dispenser (Hamilton Company, Reno, Nevada) into each well of microculture plates (micro test II culture plates, Falcon Plastics, Oxnard, CA). In addition to unstimulated controls, lymphocyte cultures were stimulated with these mitogens and antigens: (0.02 ml) phytohemagglutinin (Difco, Detroit, MI) diluted 1:10 and 1:100, Concanavalin-A (Nutritional Pharmaceuticals Co., Cleveland, OH) diluted 1:10 and 1:100, pokeweed mitogen diluted 1:20 and 1:200 (Grand Island Biological Co., Grand Island, NY) Streptolysin-O (Difco) in full strength and 1:10 dilution. In addition, patients' lymphocytes were stimulated by viable cryopreserved normal donor lymphocytes in one-way mixed lymphocyte cultures (MLC).[13,27] The same stimulator lymphocytes were used for each patient throughout the study. For stimulator cells, donor lymphocytes were separated on ficoll-hypaque gradient and resuspended in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum, antibiotics, and L-glutamine. Aliquot doses of

* G. Mavligit, unpublished data.

4×10^6/ml viable lymphocytes were placed into glass ampules and frozen (Linde BF-4 biological freezing system) with 10% DMSO and stored in liquid nitrogen. For use in MLC, cells were rapidly thawed in 37 C and washed with RPMI 1640 supplemented with 10% heat inactivated fetal calf serum. After irradiation with 4000 rads, 1.5×10^5 stimulator lymphocytes were added to MLC cultures. MLC controls consisted of wells containing irradiated stimulator lymphocytes cultures with irradiated responder lymphocytes. After incubation for seven days in a humidified atmosphere of 5% CO_2 at 37 C, tritiated thymidine, 1 μCi (specific activity 1.9 Ci/mM, Schwartz-Mann, Orangeburg, NY) was added to all cultures for additional eight hours of incubation. Blastogenesis was terminated by cooling in a refrigerator, and the cells were harvested with the MASH device (Microbiological Associates, Bethesda, MD). The blastogenesis was estimated by thymidine uptake as measured by liquid scintillation counting and expressed as counts per minute per 1.5×10^5 lymphocytes (CPM). For the analysis of lymphocyte blastogenesis data, the mean CPM value of replicate experimental observations was used for each patient at each study point. The overall net blastogenic responses (experimental CPM–control CPM) were compared between the first and subsequent study points separately for Levamisole-treated and control patients by the Wilcoxon-Mann-Whitney test.[44] Data analysis were based on differences in CPM between two individual measurements. Differences in CPM of ≥25%, ≥50%, and ≥100% between two individual measurements were considered significant and defined as ≥25%, ≥50%, and ≥100% increase or ≥20%, ≥33%, and ≥50% decrease respectively.

The Wilcoxon-Mann-Whitney test was therefore performed according to the magnitude of the change by definition. Thus, when ≥25% increase or ≥20% decrease were considered, all the values that fell below these levels were annulled yet were included in the analysis with a ranking value of 0. Similarly, when only differences of ≥50% increase or ≥33% decrease were considered, again all the values below these levels would be annulled and receive a ranking value of 0 for the analysis. Finally, when only differences of ≥100% increase or ≥50% decrease were considered, all the values below these levels were again annulled and received a ranking value of 0 before performing the Wilcoxon-Mann-Whitney test.

Results

Maximum Tolerated Single Dose of Levamisole

Two of the first three patients receiving Levamisole at a dose of 265 mg/M², experienced severe side effects lasting approximately eight hours. These effects consisted of abdominal cramps in one and recurrent vomiting combined with extreme weakness in the other. Thus, Levamisole dosage was subsequently reduced to 200 mg/M², which was well tolerated. Table 1 summarizes the side effects observed in all the patients. These

TABLE 1. Side Effects and Degree of Toxicity after a Single Dose of Levamisole in 26 Carcinoma Patients

Side effect	Degree of toxicity (No. of patients)			
	Mild	Moderate	Severe	Overall
Nausea	8	2	3	13
Fatigue	1	3	2	6
Tremor	3	2		5
Vomiting	1	2		3
Dizziness	2	1		3
Abdominal pain	1	1	1	3
Mental depression	1	1		2
Hyperhydrosis	2			2
Nervousness	1			1
Polyuria	1			1
Hyperventilation	1			1
Facial flush	1			1

TABLE 2. Individual Data of Sequential Dermatophytin Skin Test Reactions (mm) in Association with Levamisole Administration to Cancer Patients

Skin test #	1*		2†		3‡		4§		5‖
Reading hours	24	48	24	48	24	48	24	48	24
Patient no.									
1	9	21	16	22	17	27	16	23	15
2	0	0	10	8	8	7	7	6	10
3	8	10	9	8	6	6	4	3	10
4	0	0	0	0	12	0	7	0	0
5	7	14	11	12	11	15	10	16	12
6	0	0	0	0	0	0	0	0	0
7	0	0	5	3	4	3	4	3	5
8	0	10	5	7	4	5	7	9	4
9	0	0	0	0	0	0	0	0	0
10	0	5	5	7	3	6	0	9	13
11	0	0	0	0	0	0	0	0	0
12	9	14	6	5	6	5	6	5	5
13	0	0	0	—	—	—	—	—	—
14	2	2	2	2	2	2	2	2	2
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	3	3	3	4	3	4	3
19	0	0	0	0	0	0	6	0	0
20	0	3	0	0	0	0	0	0	0
21	0	0	0	0	0	0	2	2	2
22	10	11	45	36	36	24	32	16	30
23	10	11	9	0	0	0	0	0	0
24	3	3	0	0	0	0	4	0	3
25	21	25	23	22	19	30	17	25	30
26	6	5	0	0	8	0	3	0	8

* 24 hours before Levamisole.
† At time 0.
‡ 4 hours after Levamisole.
§ 8 hours after Levamisole.
‖ 24 hours after Levamisole.

TABLE 3. Individual Data of Sequential Dermatophytin Skin Test Reactions (mm) among Control Cancer Patients

Skin test #*	1		2		3		4		5
Reading hours	24	48	24	48	24	48	24	48	24
Patient no.									
1	0	0	0	0	0	0	0	0	0
2	0	5	6	10	4	4	3	4	4
3	0	0	0	0	0	0	0	0	0
4	8	7	4	5	5	9	8	10	6
5	5	7	0	0	0	0	0	0	0
6	0	0	5	0	4	0	4	0	0
7	0	—	—	—	—	—	—	—	—
8	0	0	6	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	2	0	2	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	7	16	6	0	5	0	0	0	0
16	9	0	0	0	0	0	0	0	0
17	14	16	7	13	8	15	8	12	4
18	0	0	0	0	0	0	4	0	0
19	4	6	0	0	5	7	5	7	6
20	0	0	0	0	0	0	0	0	0
21	6	5	3	0	3	0	3	0	0
22	6	0	5	7	4	5	0	3	0
23	0	0	0	0	0	0	0	8	0
24	19	28	16	29	16	22	18	30	28

* See footnote to Table 2.

side effects usually appeared 60–90 minutes after ingestion of the drug and usually disappeared within two hours thereafter. One or more side effects, usually mild to moderate in severity, were observed in 15 of 26 patients. Nausea was the most common side effect observed in 13 out of 26 patients. In 10 of those, it was mild to moderate. Severe nausea was encountered in three patients. In two of those it was associated with impaired liver function. Other common side effects were fatigue and a fine tremor, both suggesting a direct effect of Levamisole on the central nervous system.

TABLE 4. Dermatophytin Skin Test Reactions (24-Hour Reading) in Levamisole-Treated and Control Patients with Carcinoma

Skin test evaluation	Comparison between skin tests applied at study point							
	2* vs 1		3 vs 1		4 vs 1		5 vs 1	
	Lev	Con	Lev	Con	Lev	Con	Lev	Con
Patients studied	25	23	25	23	25	23	24	22
Positive change†	7	2	8	3	8	5	8	3
Negative change†	3	6	2	5	3	6	3	6
No change†	15	15	15	15	14	12	13	13

* Lev.—Levamisole-treated.
Con.—Controls.
† See definition in "Methods."

Delayed-Type Hypersensitivity Reaction

Delayed hypersensitivity to Dermatophytin was initially detected in 10 of 26 patients randomized to subsequently receive Levamisole and in nine of 24 control patients. Five of 16 initially unresponsive Levamisole-treated patients and eight of 15 controls remained unresponsive throughout the study. The results of sequential skin tests with Dermatophytin are shown in Tables 2 and 3 (individual data) and in Tables 4 and 5 (summary form). Positive changes (compared to the initial skin tests) in delayed hypersensitivity were more frequently observed among Levamisole-treated than among controls at all study points when results were read at 24 hours (Table 4). In contrast, negative changes occurred more frequently among the controls. These differences were not statistically significant, perhaps because more than half the patients in each group did not show any change in skin test reaction. No significant differences in skin test reactions were observed among the various study points after the administration of Levamisole in either group (*i.e.*, 3 vs. 2; 4 vs. 3, etc.). Also, similar analysis based on 48-hour reading of skin test reactions showed no significant differences between study points and groups of patients.

The time relationship between Levamisole administration and its enhancing effect on DTH is shown in Table 5. Levamisole has a statistically significant enhancing effect on DTH only when the skin test is applied no earlier than eight hours after the ingestion of the drug and read at 48 hours ($P = 0.03$). Significant enhancing effect was also noted if read at 24 hours when skin test was applied as late as 24 hours after Levamisole ($P = 0.012$).

Lymphocyte Blastogenesis

The possible stimulatory effect of Levamisole given *in vivo* on spontaneous lymphocyte DNA synthesis *in vitro* in the absence of mitogens, is shown in Table 6. Although the mean and median CPM of lymphocytes from Levamisole-treated patients show a tendency to rise following Levamisole administration, the values among the controls tend to decline. However, these differences within each group and between the groups at various study points are not statistically significant.

The effect of Levamisole on lymphocyte blastogenesis induced by PHA is shown in Tables 7 and 8 (individual data) and Table 9 (summary form). The individual differences in lymphocyte blastogenesis for the first three patients (Table 7) between the first and fourth study points (−24 hours vs. 8 hours) are −19%; +52% and −29% respectively. The difference of −19% is considered insignificant (see last paragraph in Methods)

TABLE 5. Dermatophytin Skin Test Reactions in Levamisole-Treated and Control Patients with Carcinoma

Skin test applied		Skin test in mm of induration (mean values) read at					
		24 hours			48 hours		
Study point	Time relationship to Levamisole administration (hrs)	Levamisole	Control	*P* value*	Levamisole	Control	*P* value
1	−24	3.1 N = 26	3.1 N = 24	0.1	5.0 N = 25	3.5 N = 23	0.4
2	0	5.7 N = 25	2.2 N = 23	0.2	5.4 N = 24	2.8 N = 22	0.16
3	4	5.6 N = 25	2.4 N = 23	0.09	5.3 N = 24	2.7 N = 22	0.2
4	8	4.9 N = 25	2.6 N = 23	0.36	4.8 N = 24	2.9 N = 22	0.03
5	24	6.1 N = 24	2.1 N = 22	0.012			

* Wilcoxon-Mann-Whitney test.

and therefore was ranked as 0, whereas the other two values are ranked according to their magnitude. Thus, in the Wilcoxon-Mann-Whitney test the ranking order will be 0; 29; 52;, etc. In other words, all the insignificant differences were annulled but included in the statistical evaluation with a ranking value of 0. If we considered only differences of ≥50% increase or ≥33% decrease as significant, the value of −29% would also be annulled and the ranking order would be 0; 0; 52.

Accordingly (Table 9) at a dilution of 1:10, stimulation by PHA was significantly enhanced ($P < 0.01$) only in the Levamisole group and only at the fourth study point (*i.e.*, eight hours following drug administration) as compared with the first (pre-Levamisole) study point. This significant enhancement of the response to PHA was only observed when a divergence of 25% or more between two individual measurements (CPM) was considered significant for statistical analysis (see foot note to Table 9).

In contrast, at a dilution of 1:100, Table 9) stimulation by PHA was significantly enhanced at three study points: the third, fourth, and sixth of the Levamisole group. The most vigorous reaction was observed at the fourth point when statistical significance ($P = 0.04$) was preserved despite the upgrading of the analysis by utilizing a divergence of 100% or more between two individual measurements. It is noteworthy that at the third study point there is also a significant increase in response to PHA in the control group ($P = 0.01$). However, this statistical significance no longer exists ($P = 0.2$) upon upgrading the analysis using a divergence of 100% or more between two individual measurements. This is in contrast to a ($P = 0.08$) in a parallel analysis for the Levamisole-treated group.

Essentially similar results of lymphocyte blastogenesis were observed with Concanavalin-A (Table 10). At a dilution of 1:10, stimulation by Con-A was significantly enhanced at two study points (fourth and sixth) in the Levamisole group. In both study points, statistical significance was still preserved ($P = 0.01$) upon upgrading the analysis using a divergence of 50% or more between two individual CPM. This is in contrast to the finding of significant enhancement of Con-A-induced blastogenesis ($P = 0.04$) at the fourth study point in the control group and only when analysis was based on a divergence of 25% or more between two individual CPM.

In the analysis of Con-A-induced blastogenesis (dilution 1:100), a spurious discrepancy exists between the expression of results in terms of mean and median CPM and the *P* values calculated by the Wilcoxon-Mann-Whitney test. This is to say, that while mean and median CPM values at the first study point are sometimes higher than at subsequent study points, the Wilcoxon-Mann-Whitney test may indicate a statistically

TABLE 6. Effects of Levamisole on Spontaneous DNA Synthesis by Lymphocytes in the Absence of Mitogens (H^3—Uptake, CPM)

Treatment group	Time relationship between Levamisole administration (↓), study points ( ) and CPM					
	hours					
	−24 (1)	0 ↓ (2)	4 (3)	8 (4)	24 (5)	48 (6)
Levamisole						
N	24	26	24	22	23	22
Mean	738	760	881	732	794	857
Median	557	612	645	627	621	585
Control						
N	24	24	22	19	21	21
Mean	890	745	691	809	826	527
Median	727	491	488	520	437	535

TABLE 7. Individual Data of Sequential Lymphocyte Blastogenesis (CPM) Induced by Phytohemagglutinin (1:10 Dilution) in Patients Receiving Levamisole

	Hours					
Study point	−24	0	4	8	24	48
Patient no.						
1	8332	9223	10080	6732	11228	9286
2	21437	32913	22012	33617	×	43100
3	27802	17317	23524	19503	17142	—
4	60672	78657	90286	—	44810	32244
5	28311	31420	38964	55399	—	33940
6	26595	32811	48390	48339	49477	65047
7	17729	34419	33627	26875	14374	18868
8	17574	20006	19753	44315	19679	—
9	17232	62858	52096	47600	54200	40462
10	39258	41871	—	—	—	—
11	×	47160	71023	36433	37600	21192
12	×	37691	40557	—	25746	18385
13	23258	28485	23398	20280	33651	15542
14	21621	38963	×	18618	14208	14250
15	11918	11733	9924	8560	9961	4603
16	45694	28524	51618	35769	11265	23194
17	11169	29380	14453	14279	18983	12073
18	12907	9972	12493	6608	5098	7941
19	19431	29184	22205	18492	21529	20464
20	12664	10037	29147	18795	7599	16764
21	11683	14020	19321	10549	8718	11518
22	8533	20277	15391	20580	30367	27530
23	50387	23033	19039	74193	22180	4118
24	60454	28548	39420	44185	—	—
25	7547	18687	14156	11523	17108	10249
26	42040	41426	26212	×	34395	31603

— No culture performed.
× Culture contaminated and discarded.

significant enhancement of lymphocyte blastogenesis compared with the first study point. This discrepancy can be accounted for by the following analytical considerations: although means and medians are linearly derived from individual CPM data, greater importance is given in the Wilcoxon-Mann-Whitney test to significant rather than to insignificant divergences between two individual CPM; the latter are automatically annulled.

Accordingly, at a dilution of 1:100, stimulation of lymphocyte blastogenesis by Con-A appeared to be significantly enhanced by Levamisole only at the fifth study point ($P = 0.05$). At the third study point, Con-A-induced lymphocyte blastogenesis was equally enhanced in both Levamisole-treated and control groups. In addition, a statistically significant increase in Con-A-induced blastogenesis was observed in the Levamisole group at the second study point. This Levamisole-unrelated finding may be associated with the effect of skin testing (using recall antigens) on lymphocyte blastogenesis in general,[48] and was reported elsewhere.[22]

Sequential lymphocyte blastogenic responses to PWM are shown in Table 11. At a dilution of 1:20, the blastogenic response to PWM in the control group is characterized by periodic dips from a baseline reaction. These dips were noted at the fourth and—with statistical significance ($P = 0.01$)—at the sixth study point. This phenomenon may represent a normal occurrence of periodic transient changes in the sensitivity of specific subpopulations of lymphocytes reacting with PWM. It could also be related to the suppressive effect of skin testing with recall antigens.[48] These periodic dips in blastogenic responses to PWM have been minimized or completely aborted by the administration of Levamisole, suggesting again an immunostimulatory effect of the drug. Levamisole had no effect on blastogenesis induced by PWM in a dilution of 1:200.

Studies of lymphocyte blastogenesis induced by the antigen streptolysin-O (SLO) (not shown in a table) have again shown a significant enhancing effect by Levamisole at the fifth study point ($P < 0.01$) but no such enhancement was noted in the control group. Sequential studies of mixed lymphocyte cultures (MLC) have not been modulated by Levamisole administration.

In addition to net CPM, the stimulation index (SI) was also calculated for the entire study as the ratio

TABLE 8. Individual Data of Sequential Lymphocyte Blastogenesis (CPM) Induced by Phytohemagglutinin (1:10 Dilution) among Control Patients

	Hours					
Study point	−24	0	4	8	24	48
Patient no.						
1	27399	28104	30664	31328	54979	26604
2	50994	54605	68797	51588	50661	32462
3	21412	25836	29221	35271	24342	18878
4	67171	107347	84834	68365	80922	60950
5	49416	39114	41496	55427	48615	—
6	26896	32607	23884	17821	—	26617
7	17237	4960	10531	5360	24165	26700
8	12951	12094	13740	7119	—	9513
9	13473	43158	—	—	—	—
10	42033	30630	35998	28772	25379	9561
11	14577	14191	39586	35766	33118	40768
12	9799	10061	8402	11952	12310	×
13	16421	46024	38415	53041	10789	21945
14	18277	17511	19543	14441	10433	11806
15	35926	27565	19738	29101	20432	18400
16	27091	19182	×	—	17610	16187
17	18830	17606	—	—	21194	23654
18	40947	53240	64307	80121	39120	76971
19	36716	45717	—	—	31938	54693
20	18194	14399	15953	—	18424	11123
21	34159	22508	28200	9016	17586	31051
22	36611	13617	25918	41441	15538	8521
23	12719	16283	15687	15133	11525	13980
24	5507	10426	6973	17068	12720	9812

— No culture performed.
× Culture contaminated and discarded.

between net CPM and CPM of unstimulated lymphocyte cultures. However, because Levamisole apparently has a direct stimulatory effect on the spontaneous lymphocyte DNA synthesis in addition to its effect on mitogen-induced blastogenesis, the expression of data in terms of SI becomes less meaningful than the expression of data in terms of net CPM. Therefore, SI results were omitted from this report.

Discussion

A single dose of Levamisole administered to cancer patients was shown to enhance: (1) delayed-type hypersensitivity reactions (DTH) to Dermatophytin *in vivo*; (2) lymphocyte blastogenesis to mitogens and antigens *in vitro*. Both of these parameters represent cellular immune responses, which have been shown to correlate well with the stage of disease and prognosis in cancer patients.[2,6,11,20]

In addition to its enhancing effect on DTH to Dermatophytin, Levamisole was shown to enhance DTH to PPD and to DNCB.[51,52,54] The enhancing effect of Levamisole on DTH to Dermatophytin in the present study was evident only when subsequent skin tests were compared to the first baseline (−24 hours) skin reaction, but not when compared with the second baseline (0 hours). Furthermore, once the first significant increase in DTH was noted, there was no additional augmentation upon subsequent skin testing. Studies on the effect of repeated skin testing on the intensity of DTH indicate that individuals who were initially reactive usually became less reactive upon subsequent testing.[22,36,47] This suggests an immunosuppressive effect induced through repeated testing by antigen excess, which could explain the lack in our study of subsequent increments in DTH, once enhancement was achieved. Furthermore, our observation of significant enhancement in DTH usually when test sites were read at 24 hours, but not at 48 hours, is in agreement with studies showing accelerated reactions and early declines in DTH following repeated testing.[36,45]

TABLE 9. Effect of Levamisole on Lymphocyte Blastogenesis (CPM) Induced by Phytohemagglutinin

Treatment group	Time Relationship between Levamisole Administration (↓), Study Points and CPM Hours: −24 (1)	0 ↓ (2)	4 (3)	8 (4)	24 (5)	48 (6)
	PHA 1:10 dilution					
Levamisole						
Mean	25176	29946	31132	28238	22131	21926
Median	20434	28866	23461	20430	18983	17574
P						
Δ CPM ≥ 25%*		0.2	0.2	<0.01	0.2	0.2
Δ CPM ≥ 50%				0.07		
Control						
Mean	27282	29448	31058	32006	27704	26199
Median	24154	24172	27059	29101	21194	21945
P						
Δ CPM ≥ 25%		0.2	0.2	0.2	0.2	0.2
	PHA 1:100 dilution					
Levamisole						
Mean	4922	10059	9979	7920	5632	5252
Median	2709	5548	4259	4638	2482	2398
P						
Δ CPM ≥ 25%		0.01	0.02	<0.01	0.2	0.04
Δ CPM ≥ 50%		0.2	0.04	0.01		0.2
Δ CPM ≥ 100%			0.08	0.04		
Control						
Mean	10046	10013	11101	8480	6725	10577
Median	6154	6164	7082	4785	4320	8291
P						
Δ CPM ≥ 25%		0.2	0.01	0.1	0.1	0.12
Δ CPM ≥ 50%			0.03			
Δ CPM ≥ 100%			0.2			

* By Wilcoxon-Mann-Whitney test. A difference (ΔCPM) of ≥25% in CPM between two individual measurements is defined as ≥25% increase or ≥20% decrease. A difference of ≥50% is defined as ≥50% increase or ≥33% decrease. A difference of ≥100% is defined as ≥100% increase or ≥50% decrease in CPM.

TABLE 10. Effect of Levamisole on Lymphocyte Blastogenesis (CPM) Induced by Concanavalin-A

Treatment group	Time relationship between Levamisole administration (↓), study points ( ) and CPM Hours: −24 (1)	0 ↓ (2)	4 (3)	8 (4)	24 (5)	48 (6)
	CON-A 1:10 dilution					
Levamisole						
Mean	8227	9698	9338	9046	7936	8921
Median	6121	6969	6349	7834	8150	8361
P						
ΔCPM ≥ 25%*		>0.2	>0.2	<0.01	0.2	0.03
ΔCPM ≥ 50%				0.01		0.01
ΔCPM ≥ 100%				0.04		>0.2
Control						
Mean	7700	9238	9036	9699	8196	10487
Median	6031	8166	6317	8556	5783	10752
P						
ΔCPM ≥ 25%		>0.2	>0.2	0.04	0.2	>0.2
ΔCPM ≥ 50%				0.2		
	CON-A 1:100 dilution					
Levamisole						
Mean	5549	5325	6477	6928	4037	4450
Median	4574	3021	3280	4290	3185	3741
P						
ΔCPM ≥ 25%		<0.01	0.05	>0.2	0.05	0.1
ΔCPM ≥ 50%		<0.01	0.12		0.05	
ΔCPM ≥ 100%		<0.01			>0.2	
Control						
Mean	11204	5143	5923	6806	6475	7046
Median	6448	3672	3348	3355	1910	2917
P						
ΔCPM ≥ 25%		>0.2	0.05	0.18	>0.2	>0.2
ΔCPM ≥ 50%			0.15			

* See footnote to Table 9.

TABLE 11. Effect of Levamisole on Lymphocyte Blastogenesis (CPM) Induced by Pokeweed Mitogen

Treatment group	Time relationship between Levamisole administration (↓), study points ( ) and CPM Hours: −24 (1)	0 ↓ (2)	4 (3)	8 (4)	24 (5)	48 (6)
	PWM 1:20 dilution					
Control						
Mean	21276	21686	19129	17805	20478	15242
Median	22069	19581	16469	15608	17112	13873
P						
ΔCPM ≥ 25%*		0.08	0.2	0.1†	0.2	<0.01†
ΔCPM ≥ 50%		0.16		0.1		0.01
ΔCPM ≥ 100%				0.15		0.01
Levamisole						
Mean	20896	27172	23962	23852	20462	19241
Median	19065	22038	21465	20848	18373	16410
P						
ΔCPM ≥ 25%		>0.01	>0.2	>0.2	0.12	>0.2
ΔCPM ≥ 50%		0.15				
	PWM 1:200 dilution					
Control						
Mean	14851	14095	10902	10375	9767	10218
Median	8076	9517	5424	9230	7504	8561
P						
ΔCPM ≥ 25%		0.15	>0.2	>0.2	>0.2	0.01
ΔCPM ≥ 50%						0.03
ΔCPM ≥ 100%						0.1
Levamisole						
Mean	7259	8673	8106	9016	6583	6458
Median	4652	4799	3837	6111	3817	5117
P						
ΔCPM ≥ 25%		>0.2	>0.15	>0.2	0.09	>0.2
ΔCPM ≥ 50%					0.14	
ΔCPM ≥ 100%					0.2	

* See footnote to Table 9. † (1) > (4); (1) > (6).

The enhancing effect of Levamisole on DTH appears to begin as early as eight hours after drug administration. This prompt effect of Levamisole on DTH was previously reported by Tripodi *et al.*,[50] who demonstrated conversion of DTH to DNCB from negative to positive within 24 hours after administration of the drug. Since in our study the enhancing effect of Levamisole on DTH was still present 48 hours after drug administration, we can conclude that the duration of this effect is for no less than 48 hours.

The enhancing effect of Levamisole given *in vivo* on lymphocyte blastogenesis to mitogens and antigens *in vitro* lends further support to the potentiation of cellular immune mechanisms by this drug. Similar to its effect on DTH, Levamisole begins to manifest its enhancing effect on blastogenesis as early as eight hours after drug administration. This effect was still present 48 hours later. This is in total agreement with studies reported by other investigators using Levamisole *in vivo*.[50,52]

The enhancing effect of Levamisole on blastogenesis was noted both for T-lymphocyte and B-lymphocyte mitogens. This is also in agreement with studies using Levamisole *in vitro*, which showed its enhancing effect on lymphocyte blastogenesis to mitogens and antigens capable of stimulating both T and B lymphocytes.[5,24,30,34] However, all studies of lymphocyte blastogenesis, using Levamisole either *in vivo* or *in vitro*, suggest that different concentrations of each mitogen or antigen and different times of incubation are required in order to demonstrate the enhancing effect of this drug. An earlier study which did not consider these variables failed to show this enhancing effect.[9]

We could not demonstrate a significant enhancing effect of Levamisole on the spontaneous DNA synthesis in cancer patients' lymphocytes. This is at variance with results obtained by others.[31,55] In those studies, however, Levamisole was not given *in vivo* as in our study but rather added to the cultures *in vitro*.

The mechanism of action of Levamisole is unclear. A growing body of evidence derived from studies using Levamisole *in vitro* now suggests that Levamisole primarily modulates thymus-dependent lymphocyte function.[34,37,40,53,56] Using Levamisole *in vivo*, we confirmed the stimulatory effect of this drug on thymic-related lymphocyte function. Our studies also suggest that this drug may enhance B-cell function as manifested by the blastogenic response to PWM and SLO. However, controversy still exists as to the specificity of PWM as well as that of PHA as B-cell or T-cell mitogens, respectively.[7,30]

Recent evidence emphasizes the possible association, at the molecular level, between cyclic nucleotides and the stimulatory effect of Levamisole on lymphocytes and macrophages. Thus, Levamisole induces a rise in the intracellular level of 3′,5′-cyclic guanosine-monophosphate (cGMP)[16] and a decline in 3′,5′-cyclic adenosine monophosphate (cAMP) of lymphocytes[16,23] and macrophages.[25] These activated macrophages also became more phagocytic. Because similar changes in cyclic nucleotides occur during stimulation of lymphocyte blastogenesis by mitogens *in vitro*,[17] it appears that the immunostimulatory effect of Levamisole *in vivo* is mediated through changes in cyclic nucleotides in the various cellular components of the immune system, and forms the basis for its clinical use.

The efficacy of Levamisole immunotherapy in the treatment of cancer remains to be proven. Clinical trials are now under way, and preliminary results appear to be rather promising.[1] The results of the present study may contribute information related to the toxicity, the maximum tolerated single dose, and the duration of Levamisole-induced immune stimulation.

The latter, being no less than 48 hours, tend to suggest that Levamisole should be given no more often than twice a week for optimal results. Moreover, daily compared to discontinuous administration of Levamisole resulted in lower level of DTH to DNCB.[54]

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