IMMUNOTHERAPY WITH LEVAMISOLE
Early Decrease of cAMP Levels in Lymphocytes from Treated Cancer Patients

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Lymphocyte cyclic nucleotide content was studied before and after Levamisole administration to cancer patients. Twelve patients with disseminated cancer received 100 mg/m^2 on two consecutive days; nine comparable patients with disseminated cancer served as controls. Endogenous cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were measured in lymphocytes before, 24, and 48 hours after ingestion of the first dose of Levamisole. A statistically significant decline in lymphocyte cAMP level was observed after drug administration and no significant changes were noted in cGMP levels. Further studies will be necessary to correlate this biochemical change in cyclic nucleotides with modulation of the functional level of cellular immune mechanisms.

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CHANGES IN LYMPHOCYTE CYCLIC NUCLEOTIDES have been demonstrated during the immune response.[2,6,20–22] Thus, a growing body of evidence now indicates that stimulation of lymphocytes *in vitro* by phytohemagglutinin (PHA) or concanavalin-A (Con-A) results in an early rise and subsequent decline of cyclic adenosine monophosphate (cAMP).[13] This has led to the theory that cAMP may be associated with early activation events.[17] Changes in cyclic guanosine monophosphate (cGMP) are evidently controversial. Watson demonstrated that during B-lymphocyte activation, the intracellular level of cGMP rises while that of cAMP tends to decline.[20] Hadden *et al.*[7,8] showed a significant rise in cGMP level after PHA stimulation which was associated with cellular changes in ionized calcium.[8] In contrast, studies by Parker *et al.* failed to show consistent changes in cGMP following PHA stimulation.[13]

The differences between changes in cAMP and cGMP during mitogenic stimulation have led to the dualism hypothesis[5] or the ratio hypothesis.[20] These hypotheses suggest that during the immune response the changes in the level of these nucleotides occur in opposing directions, *i.e.*, when one rises the other declines and vice versa.

Studies in mice have shown an increase in adenylate cyclase in lymphoid tissues during graft vs. host reactions.[16] Preliminary studies of mixed leukocyte reactions in man have shown a substantial and consistent elevation in cAMP concomitant with active blastogenesis and DNA synthesis,[10] strongly suggesting that cellular immune mechanisms in the human may also be associated with cyclic nucleotide metabolism.

Levamisole, a known modulator of cellular immune mechanisms[11,15,25] prolongs survival of patients with lung and breast cancer.[1,14] Its precise mechanism of action is still unknown, but upon incubation with mouse lymphocytes *in vitro*, a rise in cGMP and a decline in cAMP could be demonstrated.[6] We therefore undertook to study the effect of Levamisole immunotherapy on the endogenous cyclic nucleotide level in lymphocytes from treated cancer patients and found a significant decrease in cAMP after drug administration.

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Materials and Methods

Twenty-one patients with disseminated cancer were studied. Twelve patients (11 breast cancer, one rectal cancer) received Levamisole. Nine patients (eight breast cancer, one melanoma) served as controls. No patient received chemotherapy or irradiation during the study. Occasional patients continued regularly to take medications which were prescribed prior to the study. Those medications included digoxin and diuretics in one patient in each group, aspirin and Percodan in two patients in each group and Darvon (Propoxyphene hydrochloride) in one patient in the Levamisole group. The Levamisole and control groups were comparable in terms of age, sex, and extent of disease. Levamisole (100 mg/m^2/day, supplied as 25-mg tablets) was administered in three divided doses on two consecutive days.

Peripheral blood lymphocytes were sampled one day before drug administration as well as 24 and 48 hours after the first tablet was ingested. Control patients were studied according to the same time schedule but without the drug. For lymphocyte cAMP and cGMP determination, 35 ml of peripheral venous blood was defibrinated by swirling with glass beads. Lymphocytes (98–99% pure) were separated from phagocytic monocytes and granulocytes by incubation with carbonyl iron followed by centrifugation on ficoll-hypaque gradient as previously described.[3] Lymphocytes were washed twice in RPMI 1640 (Grand Island Biological Company, Grand Island, N. Y.) and incubated for 10 minutes in tris ammonium chloride buffer to remove remaining red blood cells. Cyclic nucleotides were extracted from the lymphocyte suspension by adding cold perchloric acid in a final concentration of 0.4M. The cells were immediately sonicated with a Biosonic IV sonicator (Bronwil Company, Rochester, N. Y.) for 2½ minutes at a fixed frequency of 20 ± 0.04 kHz and 75 watts. The supernatant was gradually neutralized with KOH and kept frozen until assayed. cAMP content in all the samples from each patient was measured on the same day in duplicates by a radioimmunoassay utilizing the method of Steiner[19] as modified by Williams.[24] cGMP was measured in triplicate by the radioimmunoassay as modified by Frandsen and Krishna.[4] The results were expressed in picomoles cAMP or cGMP/1 × 10^7 lymphocytes and analyzed statistically by the Wilcoxon-Mann-Whitney test for paired and unpaired measurements.[18]

Table 1. cAMP Levels in Human Peripheral Blood Lymphocytes (picomole/l × 10^7 cells) before and after Levamisole Administration (100 mg/m^2) Compared by the Wilcoxon-Mann-Whitney Test*

	cAMP (picomoles/10^7 lymphocytes)		
		After Levamisole	
Patients	Before Levamisole (A)	24 hours (B)	48 hours (C)
Levamisole			
1	6.86	1.65	2.23
2	7.86	2.25	5.25
3	26.00	4.88	4.09
4	6.90	2.15	5.40
5	5.98	5.54	4.45
6	2.20	2.00	—
7	14.51	8.28	8.35
8	17.77	25.58	8.65
9	5.67	—	4.79
10	7.19	10.99	6.78
11	6.35	4.55	4.69
12	6.37	3.60	5.28
Median	7.05	4.55	5.25
	(D)	(E)	(F)
Controls			
1	8.72	9.01	11.97
2	10.05	5.21	11.43
3	11.85	11.30	8.72
4	20.32	40.36	21.10
5	6.98	11.32	6.66
6	4.54	4.23	12.18
7	56.41	18.74	—
8	9.77	2.88	9.14
9	4.26	5.91	5.00
Median	9.77	9.01	10.28

* The difference between		P-value:
A vs. B	—	0.12;
A vs. C	—	0.004;
A vs. D	—	0.3;
B vs. E	—	0.06;
C vs. F	—	0.004;
D vs. E	—	0.6;
D vs. F	—	0.6.

Results

cAMP levels in peripheral blood lymphocytes (picomoles/10^7 lymphocytes) are shown in Table 1. Prior to Levamisole administration, the median cAMP level was 7.05 picomoles/10^7 cells for the experimental group (Column A, range 2.2–26.0) and 9.77 picomoles/10^7 cells for the controls (Column D, range 4.54–56.41), P = 0.3. Twenty-four hours after Levamisole administration, a median cAMP level declined to 4.55 picomoles/10^7 cells in patients receiving the drug (Column B) and remained signifi-

cantly low (median 5.25 picomoles/10^7 cells) at 48 hours (Column C) as compared to the pre-Levamisole level (Column A) with P = 0.12, P = 0.004 respectively.

This sustained decline in lymphocyte cAMP among patients receiving Levamisole (Columns B, C) was also significant when compared to cAMP levels measured in the controls on parallel times (Columns E, F) with P = 0.06, P = 0.004 respectively. No significant changes were noted in the sequential studies among the controls (Column D, E, F).

No significant changes in cGMP were noted among patients receiving Levamisole. The median cGMP prior to Levamisole administration was 0.101 picomoles/10^7 lymphocytes and 0.090 both at 24 and 48 hours thereafter.

The recovery of radio-labeled cAMP and cGMP ranged between 68–94% and 75–92% respectively.

Discussion

This study clearly indicates that an early significant decline in the endogenous lymphocyte cAMP is a result of Levamisole immunotherapy in cancer patients. No significant changes occurred in regard to cGMP.

The relationship between changes in cyclic nucleotide metabolism, and those concerning cAMP in particular, and the immune response, is currently receiving a great deal of attention. Since Levamisole is apparently a potent immune modulator,[11,15,25] currently used for immunological restoration in patients with malignant disease following potentially curative procedures,[1,14] a connection between its immunologic stimulation and the changes in cyclic nucleotides can be postulated.

In vitro studies using mouse macrophages have shown similar results, *i.e.*, a decline in macrophage cAMP content following incubation with Levamisole.[12] Furthermore, Hadden *et al.*, using mouse lymphocytes have also demonstrated a decline in cAMP along with a rise in cGMP following *in vitro* incubation with Levamisole.[6]

In a more recent study, Wilkins and Olkowski have noted a rise in lymphocyte cAMP content among patients with malignant melanoma and squamous cell carcinoma of the head and neck who were studied 6–12 weeks after initiation of Levamisole immunotherapy.[23] This rise in cAMP may be a delayed phenomenon, whereas the initial decline noted in our study could represent an early activation event.

The direct effect of Levamisole on adenylate cyclase and phosphodiesterase, (both involved in the metabolism of cyclic nucleotides) is currently being investigated in our laboratory. Levamisole is evidently a potent inhibitor of alkaline phosphatase and 5′-nucleotidase.[9] It may also have an initial inhibitory effect on adenylate cyclase with a delayed inhibitory effect on phosphodiesterase resulting in an early decline and subsequent rise in cAMP. It is also possible that Levamisole may have an early activating effect on phosphodiesterase with delayed activating effect on adenylate cyclase which could explain the observed changes in cAMP. Time-sequenced combinations of inhibition and activation of these enzymes can also serve as a possible explanation. Further studies will be necessary to correlate these changes in cAMP induced by Levamisole with its restorative effect on cellular immune mechanisms.

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