In view of the strong correlation between chemically induced cancer and mutagenicity by the corresponding compounds in the Salmonella test system, metabolites of *N*-hydroxy-2-AAF other than the sulfate ester may play a significant role in the carcinogenesis of *N*-hydroxy-2-AAF. At present, however, no conclusion about the relative carcinogenicity of these metabolites can be drawn.

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Effect of Levamisole on phytohemagglutinin-stimulated human lymphocytes*

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Levamisole† (1-tetramizole), a potent anthelminthic, has been shown to have immuno-stimulatory activity in animals and man [1]. It is currently being investigated clinically in the treatment of neoplastic disease [2], rheumatoid arthritis [3], systemic lupus [4], and other disorders presumed to be associated with impaired cellular immunity. Previous studies on the effect of Levamisole on lymphocyte transformation *in vitro* have produced conflicting results. Copeland *et al.* [5] found no stimulation of resting human lymphocytes *in vitro* by the drug, nor could they demonstrate either enhancement or inhibition of lymphocyte transformation by specific or non-specific mitogens; Pabst and Crawford [6] showed significantly greater responses in human lymphocytes to *Candida albicans*, measles virus and purified protein derivative of *Tubercle bacilli* in the presence of Levamisole. Wachi *et al.* [7] found suppression of RNA synthesis of human lymphocytes cultured with phytohemagglutinin (PHA) and Levamisole in 3-day cultures, but little or no effect on DNA synthesis. When these cultures were continued for 4 days, however, they reported a stimulatory effect on DNA synthesis. In order to further clarify the effects of Levamisole on human lymphocytes, studies were undertaken with both resting and phytohemagglutinin-stimulated normal and anergic lymphocytes, and a normal cell line which has been maintained in continuous culture.

Lymphocytes were obtained from normal healthy volunteers and a selected group of patients with diseases associated with anergy, after consent was obtained. One of these patients was a 53-year-old male with severe rheumatoid arthritis, who had not received cytotoxic drugs or corticosteroids, and from whom aspirin was withheld for 1 week prior to obtaining the cells [8]. There were two patients with Hodgkin's disease, one was a 42-year-old male with

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the lymphocyte depleted type, Stage II B, and the other was a 19-year-old female with the nodular sclerosis type, Stage III AS. Both patients were receiving radiotherapy at the time their lymphocytes were obtained. One patient, a 73-year-old male, had chronic lymphatic leukemia which had not required treatment.

Blood was collected in vacutainer tubes containing 357 USP units heparin/20 ml of blood. Lymphocytes were separated on ficoll-hypaque, washed twice with Hank's balanced salt solution and cultured at a density of 1.5×10^6 cells/ml in RPMI 1640 supplemented with glutamine (2 mM), penicillin (100 units) and streptomycin (100 μg/ml of culture) and 20% fetal calf serum. For studies of mitogen response, 2-ml cultures in triplicate were stimulated with 1–7 μg phytohemagglutinin/ml of culture and incubated at 37° for 72 hr. Purified phytohemagglutinin (Burroughs Wellcome, North Carolina) was dissolved in Hank's balanced salt solution and stored at −20°. Aliquots of the same lot were used for all the experiments. Levamisole was dissolved in Hank's balanced salt solution and was measured by incorporation of [^{3}H]thymidine (Amersham, 5 Ci/mmole), and 0.5 μCi/ml of culture was added 40 hr after phytohemagglutinin stimulation. Cultures were incubated for 20 hr at 37° and isotope incorporation was measured as previously described [9]. The synthesis of cellular protein and RNA was determined by the addition of 0.1 μCi [^{14}C]leucine (Amersham, 350 μCi/mmole) and 0.1 μCi [^{14}C]uridine (Amersham, 450 μCi/mmole), respectively, to 1.5×10^6 cells. The incorporated radioactivity was measured as above. A human lymphocyte cell line (Associated Biomedic Systems, 4098, spontaneous transformation) was grown in suspension culture in RPMI 1640 supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum. Cultures were washed with Hank's balanced salt solution and suspended at a density of 2.5×10^6 cells/ml in the same medium. DNA, RNA and protein synthesis were determined.

To study the effect of Levamisole on lymphocytes obtained from anergic patients, lymphocytes isolated from both normal and anergic patients were suspended in complete medium. Levamisole at a concentration of 50 μg/ml of culture was added to one half of each culture and the other half was left untreated. All cultures were incubated at 37° for 45 min. At the termination of incubation, untreated and Levamisole-treated cultures were again divided in half. One half was centrifuged, washed with Hank's balanced salt solution and resuspended in the same medium, and the other half was not washed. All cultures were then stimulated with PHA and treated as above.

Incubation with Levamisole for 72 hr at concentrations of 10, 25, 50, 100, 150 and 300 μg/ml of resting lymphocyte cultures had no effect on DNA synthesis. Similar concentrations of Levamisole added to lymphocyte cultures simultaneously with PHA also were without effect on DNA synthesis (Table 1). Levamisole incubated with resting cells at the above concentrations for shorter periods, 24 and 48 hr, did not alter DNA synthesis, and negative results were also obtained when the drug was added 24 and 48 hr after phytohemagglutinin stimulation. RNA and protein synthesis in both resting and phytohemagglutinin-stimulated lymphocytes were unchanged by Levamisole at the above concentrations. When it was added at concentrations of 500 μg/ml of cell culture, there was suppression of synthesis of these macromolecules, and in concentrations above 1 mg/ml of culture, the cells were no longer viable. The addition of increasing amounts of phytohemagglutinin (1, 3, 5 and 7 μg/ml) to cell cultures in the presence of 50 μg Levamisole/ml of culture neither enhanced nor inhibited DNA synthesis (Table 2).

Table 1. Effect of increasing concentrations of Levamisole on DNA synthesis in resting and PHA-stimulated lymphocytes

Levamisole (μg/ml of culture)	[^{3}H]thymidine incorporation* (cpm/1 × 10^6 lymphocytes) Resting	PHA-stimulated
0	1100	11,000
10	1000	9800
25	1200	10,000
50	1150	11,000
100	950	12,000
150	1000	10,500
300	900	9000

* Each figure represents the mean of triplicate determinations.

Table 2. Effect of Levamisole on DNA synthesis with increasing concentrations of PHA

PHA (μg/ml of culture)	[^{3}H]thymidine incorporation* (cpm/1 × 10^6 cells) Control	Levamisole-treated
1	5700	5400
3	11,000	10,500
5	11,500	11,000
7	10,000	9500

* Results represent the mean of duplicate determinations.

Incubation of Levamisole and phytohemagglutinin for 72 hr with lymphocytes from the patients resulted in no change in DNA synthesis. When Levamisole-treated, and untreated, anergic lymphocytes were incubated for 45 min in complete medium, washed, and then stimulated with phytohemagglutinin, there was marked enhancement of DNA synthesis. This was not found in cells obtained from the normal subjects and the patient with chronic lymphatic leukemia (Table 3).

Incubation with Levamisole for 3 hr and preincubation for 2, 4 and 24 hr, in concentrations of 10, 25, 50, 100, 250 and 500 μg/ml in a normal lymphocyte line (maintained in continuous culture) resulted in no change in DNA, RNA or protein synthesis. Removal of the compound by centrifugation and washing before the addition of the isotopes did not alter the result. Levamisole at concentrations above 1 mg/ml was cytotoxic.

The results of this study show that Levamisole did not enhance DNA, RNA or protein synthesis in resting or phytohemagglutinin-stimulated normal human lymphocytes even when sub-optimal concentrations of phytohemagglutinin were employed. Synthesis of these macromolecules was inhibited only at high concentrations of the drug which approached cytotoxic levels. Similar results were also obtained with a spontaneously transformed human lymphocyte cell line.

These findings are in agreement with the report of Copeland *et al.* [5], but do not support the findings obtained by Pabst and Crawford [6] and Wachi *et al.* [7]. It seems unlikely that these differences could be due to variations in experimental design, since essentially the same methodology was used in all of the studies. The employment of different mitogens might account for some of the observed discrepancies. Wachi *et al.* [7] used phytohemagglutinin and pokeweed. Pabst and Crawford [6] used *C. albicans*, measles virus, and purified protein derivative of *T. bacilli*, and both of these groups reported an enhancement of DNA synthesis by Levamisole. Copeland *et al.* [5] used pokeweed, phytohemagglutinin, streptolysin-*O*, streptokinase-streptodornase and mixed lymphocyte reactions, and reported no enhancing effect on DNA synthesis in the presence of Levamisole with any of the above mitogens.

Table 3. Effect of Levamisole and phytohemagglutinin on DNA synthesis

Lymphocytes	[^{3}H]thymidine incorporation* (cpm/1 × 10^6 cells) Incubated without Levamisole + PHA	Incubated with Levamisole + PHA	Incubated with Levamisole + PHA	Incubated without Levamisole + PHA
Hodgkin's (nodular sclerosis)	2600	2800	5200	5500
Hodgkin's (lymphocyte depleted)	1700	1640	3200	3400
Chronic lymphatic leukemia	3500	3200	3000	3300
Severe rheumatoid arthritis	3200	3000	8000	8200
Normal	11,000	10,850	10,700	11,000

* Results represent the mean of triplicate determinations.

Since we employed phytohemagglutinin as the sole mitogen, our results are directly comparable only to the data obtained with phytohemagglutinin in the previously published studies. We have no explanation for the disparity between the positive results obtained with phytohemagglutinin and Levamisole by Wachi *et al.* [7] and the negative findings of Copeland *et al.* [5] and ourselves.

We studied the effect of phytohemagglutinin stimulation on anergic lymphocytes from two patients with Hodgkin's disease, one with severe rheumatoid arthritis, and one with chronic lymphatic leukemia, and compared the response to that from lymphocytes obtained from normal subjects in the presence and absence of Levamisole. In none of these cultures were we able to demonstrate any enhanced response to phytohemagglutinin stimulation after incubation with the drug for 72 hr, or when it was present for 45 min prior to the addition of phytohemagglutinin. When incubation was followed by removal of the Levamisole by washing prior to phytohemagglutinin stimulation, there was enhancement of DNA synthesis in the lymphocytes obtained from the two patients with Hodgkin's disease and the patient with severe rheumatoid arthritis. To control the above experiments, lymphocytes from the same donors were incubated in medium without Levamisole, washed, and stimulated with phytohemagglutinin. This also resulted in an enhancement of the phytohemagglutinin response comparable in magnitude to that observed when Levamisole was present (Table 3).

We conclude that with the exception of the leukemic cells, incubation of the anergic lymphocytes in culture medium in the presence or absence of Levamisole, when followed by washing, enhances DNA synthesis in response to phytohemagglutinin stimulation. This suggests that, under these experimental conditions, new receptor sites amitogen may be exposed by the process of incubation in medium and washing, and that this does not require the presence of the chemical agent. Further studies will be undertaken to elucidate the mechanism by which incubation and washing of anergic lymphocytes *in vitro* results in enhancement of DNA synthesis after stimulation with phytohemagglutinin. The failure to observe enhancement in DNA synthesis in the lymphocytes from the patient with chronic lymphatic leukemia treated in this manner is consistent with the previously reported observation that the anergy of these cells is not due to the inability of the cells to bind phytohemagglutinin, but to the failure of intra-cellular processes involved in the maturation of ribosomal RNA[10].

Stimulation of lymphocytes *in vitro* with phytohemagglutinin tests only the functional integrity of the isolated lymphocyte to a non-specific mitogen. It lacks the interaction of various humoral and cellular components involved in the antibody response *in vivo* to specific antigens [11]. Thus, the lack of an enhancing effect of Levamisole *in vitro* on mitogen-stimulated lymphocytes is not incompatible with the reports of stimulation of skin test responses in patients given the drug [12].

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