Effect of Short-Term Levamisole Therapy on Delayed Hypersensitivity

YASHAR HIRSHAUT, MD, CARL M. PINSKY, MD, IRENA FRYDECKA, MD, HAROLD J. WANEBO, MD, SHARON PASSE, MS, VALERIE MIKÉ, PHD, AND HERBERT F. OETTGEN, MD

A randomized trial of short-term Levamisole treatment was undertaken in a cancer population unresponsive to dinitrochlorobenzene (DNCB) to determine whether this agent increased delayed hypersensitivity. Of 100 patients entered, 50 received Levamisole (150 mg daily × 3) during DNCB challenge. The other 50 patients were challenged but not given the drug. The conversion rate to DNCB+ was 20% (10/50) for those treated and 12% (6/50) for controls. The difference is not significant. When all 100 patients were considered there was a statistically significant inverse relationship between extent of disease and the incidence of conversion to a DNCB reactive state. Levamisole as given does not appear to have a major influence on delayed cutaneous hypersensitivity.

Cancer **45:362–366, 1980.**

THE ONLY IMMUNE DEFICIT that has been established to be of prognostic significance in patients with malignancies is a failure by many of them to develop contact sensitivity to dinitrochlorobenzene (DNCB) after primary sensitization.[3,11] Studies by Eilber and Morton[3] and by Pinsky *et al.*[11] indicate that the prevalence of this deficit is related to tumor burden. Patients with widespread tumor are more likely to display an impaired response to DNCB than are those with local or regional disease. However, even when the cancer is sufficiently localized and completely resected, depressed immune reactivity may be evident in some patients. Failure to respond to DNCB sensitization is associated with a poor prognosis, both for those whose tumor has been surgically removed and those with residual disease.[4,11,12]

Levamisole, tetrahydro-6-phenylimidazothiazole, is an antihelminthic agent widely used outside the United States. In 1971, Renoux and associates[14] discovered its ability to stimulate a response in mice to Brucella vaccine. Since then this agent has been reported to exert a broad immunopotentiating effect on immunity in both animals and man.[2,8,9,13,17–19] Studies by Renoux *et al.*[16] and subsequently by Tripodi and co-workers[20] in the United States have suggested that Levamisole may be able to restore delayed cutaneous hypersensitivity in aged individuals and otherwise unresponsive cancer patients. Because cutaneous anergy implies early recurrence and short survival, its reversal by Levamisole may be of considerable importance to affected patients.

The current study was undertaken to investigate the influence of short-term Levamisole therapy on delayed cutaneous hypersensitivity in tumor-bearing individuals.

Materials and Methods

Skin Tests

DNCB (Lot 81800) was purchased from K and L Laboratories Inc., Plainview, NY. DNCB tests were carried out according to methods previously described.[11] Briefly, to determine a patient's ability to develop a *de novo* response to DNCB, a sensitizing dose of 2000 μg DNCB dissolved in 0.1 ml of acetone was applied to the inner aspect of the upper arm, inside a plastic ring 2 cm in diameter, and allowed to evaporate. Test doses of 100, 50 and 25 μg were applied in the same way and at the same time to the ipsilateral forearm. All sites were covered with dressings for 48 hours and kept dry. They were then examined for erythema and induration at 48 hours, 2 weeks, and whenever possible during the intervening time. The greatest diameter of erythema and/or induration observed was measured and recorded. For patients who showed no delayed hypersensitivity reactions at the test sites at 48 hours, erythema and induration beginning 9 to 10

From the Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

Supported by National Cancer Institute Contracts N01CB53970 and CA05826 as well as Grant IM-26A from the American Cancer Society.

Address for reprints: Yashar Hirshaut, MD, 1275 York Avenue, New York, NY 10021.

Accepted for publication February 13, 1979.

0008–543X/80/0115/0362 $0.75

days after sensitization were considered evidence of *de novo* acquisition of sensitivity to DNCB. Patients who showed no reaction after 14 to 16 days were tested again with 200, 100, 50, and 25 μg of DNCB. These tests were considered positive if there was definite induration of at least 10 mm in diameter at the site of DNCB application after 48 hours.

All patients were also tested for pre-existing delayed hypersensitivity to common antigens, including dermatophytin "O" (1:100, a Candida antigen supplied by Hollister-Stier Laboratories, Yeadon, PA), mumps skin-test antigen (Eli Lilly & Co., Indianapolis, IN), tuberculin (intermediate strength, supplied by Parke-Davis Co., Detroit, MI), and streptokinase/streptodornase (SK = 4 units; SD = 1 unit; supplied by Lederle Laboratories, Pearl River, NY). These antigens were injected intradermally in volumes of 0.1 ml. The tests were read at 24 or 48 hours, and considered positive if the diameter of induration observed was more than 5 mm. Persons not reacting to tuberculin and SK/SD were rechallenged with tuberculin, second strength, and a ten-fold less dilute SK/SD preparation (SK = 40 units; SD = 10 units).

Drug

Levamisole was suppled by the Ortho Pharmaceutical Corporation (Raritan, NJ) in tablet form, 150 mg Levamisole per tablet.

Study Design

The plan for the study is shown in Figure 1. Patients attending regular sessions at the outpatient clinics of Memorial Hospital for Cancer and Allied Diseases, New York, NY, were tested for DNCB reactivity. All those not reacting to DNCB were asked to participate in a randomized study intended to determine whether Levamisole could induce responsiveness to this substance. Once consent was given, individuals were randomly selected to enter either the treatment or control group.

At a minimum interval of one week following the previous DNCB challenge, all subjects were rechallenged with DNCB using doses of 200, 100, 50, or 25 μg. Patients to be treated received Levamisole 150 mg for the first time 6 hours later. Additional doses were administered at 24 and 48 hours following rechallenge. Those in the control group received nothing. At 54 hours, 6 hours after the third Levamisole dose, the DNCB test result was read and recorded. Whenever DNCB was applied, the patient's responses to intradermal antigens were also tested.

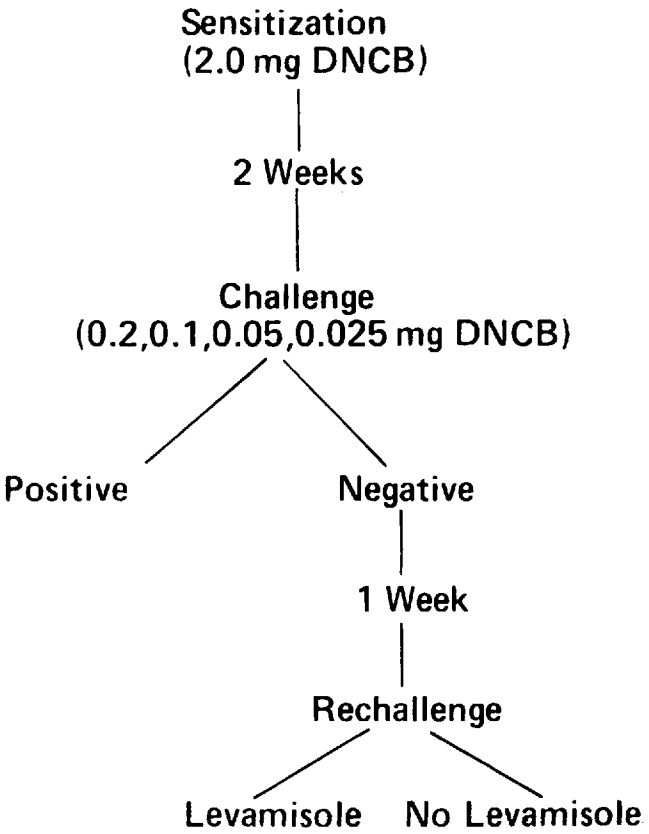

FIG. 1. Study design for evaluating the effect of Levamisole on the response of cancer patients to DNCB.

Statistical Methods

Contingency tables were analyzed using the chi-square statistic. The test for trend in proportions[1] was applied to ordered contingency tables. When comparing two proportions obtained from paired samples, McNemar's test[1] was employed. All reported P-values refer to two-sided tests.

Survival curves were obtained by the Kaplan-Meier method[6] with survival time measured in months from both time of diagnosis and time of entry into the study. The logrank procedure[10] provided the significance test for detecting differences in survival times of two or more groups.

TABLE 1. DNCB Reactivity in Population Screened

Diagnosis	Number of patients	% DNCB +
Carcinoma, head & neck	47	72
Melanoma	30	73
Carcinoma, testicle	13	62
Sarcoma	23	52
Carcinoma, breast	17	47
Non-hodgkin's lymphoma	12	42
Hodgkin's disease	38	42
Other	89	47
TOTAL	269	55

TABLE 2. Response to Intradermal Antigens in Screened Population

Antigen	Number of patients	% Reactive
Mumps	275*	59
Dermatophyton "O"	275	47
PPD, standard	276	41
SK/SD	276	35

* Some patients did not return for DNCB challenge.

TABLE 3. Patient Entry

Total DNCB negative	127
Entered in Levamisole study	100
Not entered	27
Reasons for exclusion from study:	
Travel difficult	6
Too ill to consent	6
Refused	7
Expired	4
Entered nursing home	1
Failed to return	3
	27

Results

The DNCB reactivity of 269 patients screened for this study is shown in Table 1. A total of 55% displayed a delayed hypersensitivity response to one of the test doses. The highest prevalence of responsiveness was among those with melanoma and carcinoma of the head and neck. Persons with lymphoma and Hodgkin's disease were the least reactive. For the same population the results of tests with intradermal antigens are shown in Table 2. The highest prevalence of response was to mumps antigen, to which 59% of 275 patients responded. Only 35% of these patients reacted to SK/SD.

To enter 100 patients in the protocol investigation, 127 DNCB-negative individuals were identified. The reasons for exclusion from study are indicated in Table 3.

Of 50 persons receiving Levamisole during DNCB rechallenge, 10 (20%) converted from negative to positive (Table 4). Among the 50 patients in the control group, six (12%) converted their skin test reactivity in a similar fashion. The difference in the conversion rates between the two groups is not statistically significant. (Given a sample size of 50 in each group and true conversion rates of 5% and 25%, say, a statistically significant difference at $P = .05$ would be observed with probability .75. For conversion rates of 5% vs. 15%, the same probability is only .25.)

TABLE 4. Levamisole as Related to DNCB Reactivity

Status	Number of patients	Number reactive on rechallenge	% Conversion
Treated	50	10	20
Control	50	6	12

Changes in reactivity to intradermal antigens are shown in Table 5. Levamisole administration did not significantly affect responsiveness to any of these substances. For each skin test, McNemar's test for paired data indicated no significant differences between the proportions of positives before and after treatment for either the treated or the control group. (Because of missing values, totals for each group do not sum to 50.) There was also no correlation between acquisition of reactivity to DNCB and alteration in response to intradermal skin tests.

Conversion of DNCB reactivity was not associated with age, sex, or extent of disease for either the treated or the control group. However, when combining the two groups, a trend of decreasing conversion rate with increasing disease involvement was noted which was statistically significant, with $P < .05$ (Table 6). The relation of DNCB test conversion to surgery, radiation, or chemotherapy 2 weeks or more prior to entry into the study was also analyzed for each group. No evidence of a dependence between test conversion and prior treatment was noted.

At the time of final evaluation, there were 24/49 treated patients and 28/50 control patients still alive. Survival analysis showed no indication of a better prognosis for patients receiving short-term Levamisole whether time of diagnosis or first therapy (Fig. 2) was used as the starting point. The treated and control groups were further broken down by age (≤40 years vs. >40 years), sex, extent of disease, and absence of treatment or treatment by any modality prior to drug administration. No statistically significant differences in the survival patterns of the respective subgroups were revealed. Survival as related to conversion of DNCB activity is shown in Figure 3. Persons acquiring DNCB responsiveness following Levamisole treatment did not demonstrate an improved prognosis. The six patients spontaneously converting from DNCB-negative to DNCB-positive appeared to have a slightly better prognosis early in the trial but the sample size is too small for definite conclusions.

Discussion

The current study demonstrates that repeat DNCB challenge in persons initially not reacting to this contact allergen is associated with a modest conversion rate from DNCB-negative to DNCB-positive. When Levamisole is administered during a DNCB challenge period, a somewhat higher but not significantly different conversion rate is noted. Levamisole, therefore,

TABLE 5. Effect of Levamisole on Reactivity to Intradermal Antigens

Antigen	Treated				Control			
	+/+*	−/−	+/−	−/+	+/+	−/−	+/−	−/+
Derm. "O"	5	35	5	3	4	34	2	4
Mumps	3	33	7	6	6	26	6	6
PPD	6	38	3	2	7	32	3	5
SK/SD	7	38	1	3	7	33	2	3

* Pre/post last challenge with intradermal antigens. No significant alterations in responsiveness noted.

does not appear to exert any major influence on patient responsiveness to DNCB.

Overall, the conversion rate observed in the entire study population was inversely related to extent of disease and was higher for those with limited or not evident disease. Interpretation of present results must, therefore, be tempered by the consideration that Levamisole might be more effective if given early during the course of disease to those whose immune status has not already been seriously compromised by malignancy.

Using an identical treatment protocol, Tripodi and associates[20] reported a 45% conversion rate after Levamisole administration among 20 patients who were initially DNCB-negative. There were no conversions observed in a smaller control group. Their overall study design differed from ours, however, in several important respects. First, Tripodi and associates' study did not have randomized controls. Patients were challenged twice instead of just once to establish their DNCB-negative condition. The second challenge included a 200-μg dose of DNCB, the first did not. Finally, Tripodi and co-workers chose to disregard the results of the 200 μg challenge in their final calculations.

Their control and experimental groups indicated some potentially important differences. The median age of the treated patients was 53.4 years. That of the controls whose ages were known was 64.6 years. The ages of three of the 14 control subjects are not given, suggesting that they were not evaluated as carefully as the experimental patients. Those destined to receive Levamisole but not as yet treated showed a 45% spontaneous conversion rate after the second DNCB challenge, using a dose of 200 μg. In contrast, the control patients had a spontaneous conversion rate of only 23%. Furthermore, ⅓ of those who spontaneously converted in the experimental group developed sensitivity to the 50-μg dose of DNCB, while none of the control patients developed a reaction to a challenge dose below 200 μg. Reactions to PPD before Levamisole were also greater in the experimental than in the control population. Differences between the experimental and control groups

TABLE 6. Relationship between Extent of Disease and DNCB Conversion Rate

Extent	No change	Conversion	Total	% Conversion
Free of disease	6	4	10	40
Local	5	2	7	29
Regional	13	3	16	19
Distant	60	7	67	10
TOTAL	84	16	100	16

noted by Tripodi *et al.* may, therefore, have resulted from factors introduced by failure to randomize patient entry rather than the use of Levamisole. For example, younger, more immunologically intact patients are more likely to be affected by repeated DNCB sensitization. The additional challenge received by the patients of Tripodi *et al.* may also explain their higher conversion rate.

Perhaps most important in distinguishing between

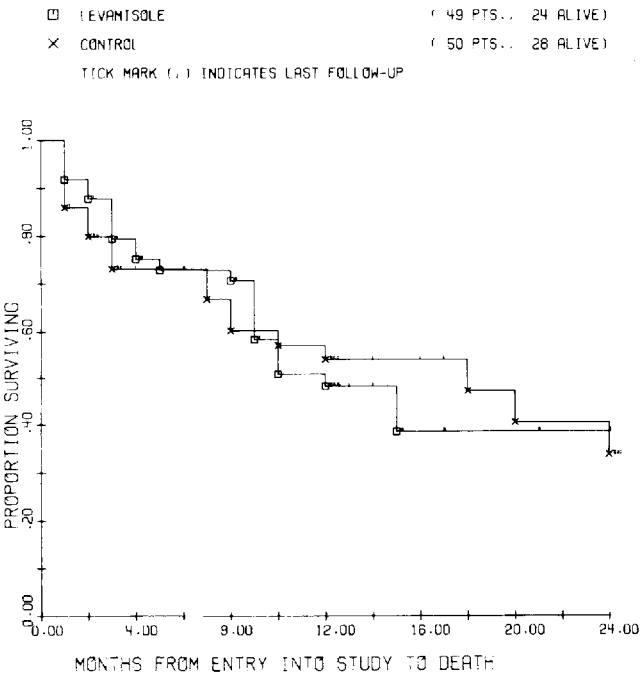

FIG. 2. Survival curves for the Levamisole and control groups.

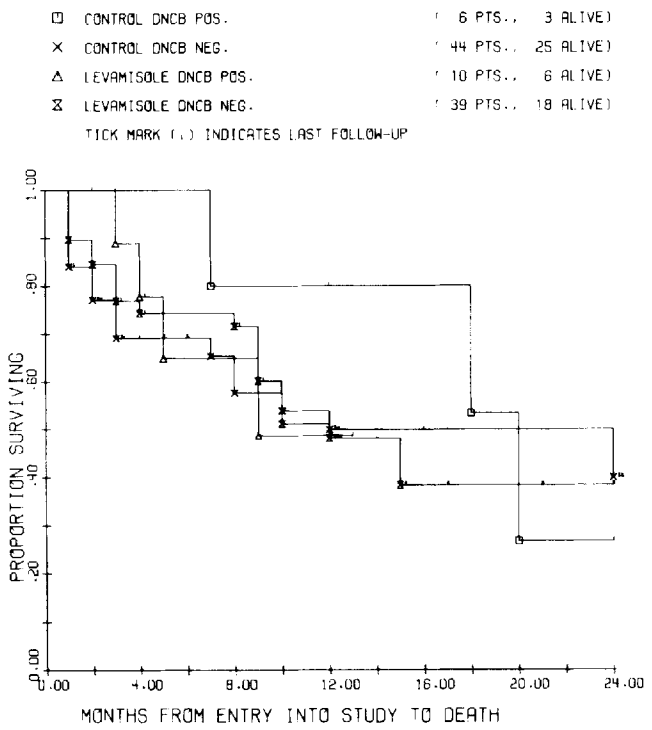

FIG. 3. Survival curves by treatment and DNCB conversion status.

this study and that of Tripodi *et al.* is the recognition that for the current study all persons reactive to 200 μg of DNCB were considered DNCB-positive and therefore excluded from entry. The trial of Tripodi and coworkers included persons spontaneously responding to a 200-μg challenge dose. This means that some of the patients reported as having become responsive to DNCB in the other study were already reactive, although to a higher dose. When first tested with 200-μg DNCB, nine of 20 patients in the treatment group were DNCB reactive but were permitted to remain in this group and were treated later with Levamisole.

The absence of a readily detected effect of Levamisole on DNCB reactivity is consistent with findings previously reported by Hirshaut *et al.*[5] These indicate that using a fairly broad spectrum of tests both for humoral and cell-dependent immunity, it is difficult to show that Levamisole has any effect on function of the immune system when administered to patients with advanced cancer. Similar observations have been made by Lichtenfeld[7] in a group of patients with carcinoma of the lung. Some encouragement regarding Levamisole's efficacy has come from reports that this agent may be capable of affecting some *in vitro* immune parameters.[15,18] These findings remain to be confirmed. In the long run, a more complete understanding of the value of immunopotentiators such as Levamisole will have to await better definition of the selective immunosuppressive effects that occur in those with malignancy.

REFERENCES

1. Armitage, P.: Statistical Methods in Medical Research. New York, John Wiley and Sons, 1971.

2. Brugmans, J., Schuermans, V., De Dock, W., Thienpont, D., Janssen, P., Verhaegen, H., Vannimmen, L., Louwagie, A. C., and Stevens, E.: Restoration of host defense mechanisms by levamisole. *Life Sci* 13:1499–1504, 1973.

3. Eilber, F. R., and Morton, D. L.: Impaired immunologic reactivity and recurrence following cancer. *Cancer* 25:362–367, 1970.

4. Eilber, F. R., Nizze, J. A., and Morton, D. L.: Sequential evaluation of general immune competence in cancer patients. Correlations with clinical course. *Cancer* 35:660–665, 1975.

5. Hirshaut, Y.: Phase I evaluation of immune effects of levamisole. *In* Progress in Cancer Research and Therapy, vol. II. M. S. Chirigos, Ed. New York, Raven Press, 1977; pp. 147–158.

6. Kaplan, E. L., and Meier, P.: Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* 53:457–481, 1958.

7. Lichtenfeld, J. L., Wiernik, P. H., and Shortridge, D. G.: Phase I trial of levamisole in the treatment of nonresectable bronchogenic carcinoma. *In* Progress in Cancer Research and Therapy, vol. II, M. A. Chirigos, Ed. New York, Raven Press, 1977; pp. 135–145.

8. Lods, J. C., Dujardin, P., and Halpern, G.: Action of levamisole on antibody protection after vaccination with anti-typhoid and paratyphoid A and B. *Ann Allergy* 34:210–212, 1975.

9. Pabst, H. F., and Crawford, J.: L-tetramisole: Enhancement of human lymphocytes to response to antigen. *Clin. Exp. Immunol.* 21:468–473, 1975.

10. Peto, R., and Pike, M. C.: Conservatism of the approximation $\Sigma (O - E)^2/E$ in the logrank test for survival data or tumor incidence data. *Biometrics* 29:579–584, 1973.

11. Pinsky, C. M., El Domeici, A., Caron, A. S., Knapper, W. H., and Oettgen, H. F.: Delayed hypersensitivity reactions in patients with cancer. *In* Recent Results in Cancer Research, vol. 47, O. Mathe and R. Weiner, Eds. Berlin, Springer-Verlag, 1974; pp. 37–41.

12. Pinsky, C. M., Oettgen, H. F., El Domeici, A., Old, L. J., Beattie, E. J., and Burchenal, J. H.: Delayed hypersensitivity reactions in patients with cancer. *Proc. Am. Assoc. Cancer Res.* 12:100, 1971.

13. Renoux, G., and Renoux, M.: Action stimulante de derives du phenylimidothiazole sur les cellules splenique formatrices d'anticorps. *CR Acad. Sci.* 274:756–757, 1972.

14. Renoux, G., and Renoux, M.: Effet immunostimulant d'un imidothiazole dans l'immunisation des sauns contre l'infection par Brucella abortus. *CR Acad. Sci.* 272:349–350, 1971.

15. Renoux, G., and Renoux, H.: Thymus-like activities of sulfur derivatives on T-cell differentiation. *J. Exp. Med.* 145:466–471, 1977.

16. Renoux, G., Renoux, M., Morand, P., Dartigues, P.: Action immunostimulante du levamisole sur les personnes agres. *Rev. Med. Tours.* 7:797–801, 1973.

17. Spreafico, F., Vecchi, A., Mantovani, A., Poggi, A., Franchi, G., Anaclerio, A., and Garattini, S.: Characterization of the immunostimulants levamisole and tetramisole. *Eur. J. Cancer* 11: 555–563, 1975.

18. Sunshine, G., Lopez-Corrales, E., Hadden, E. M., Coffey, R. G., Wanebo, H., Hadden, J. W., and Rojas, A.: Levamisole and imidazole: In vitro effects in mouse and man and their possible mediation by cyclic nucleotides. *In* Modulation of Host Immune Resistance in the Prevention or Treatment of Induced Neoplasias, M. A. Chirogos, Ed. Washington, D. C., DHEW NIH 77-893, 1977; pp. 31–37.

19. Symoens, J., and Brugmans, J.: The effects of levamisole on host defenses. A review. *In* First International Conference on Modulations of Host Immune Resistance in the Prevention or Treatment of Induced Neoplasias, M. A. Chirigos, Ed. Washington, D. C., Fogarty International Center Proceedings, U. S. Government Printing Office; in press.

20. Tripodi, D., Parks, L. C., and Brugmans, J.: Drug-induced restoration of cutaneous delayed hypersensitivity in anergic patients with cancer. *N. Engl. J. Med.* 289:354–357, 1973.