

6-1982

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Recommended Citation

Page, Robert H.; Valdivia, Duilio A.; Talley, Robert W.; and Huseby, Robert A. (1982) "Effect of Levamisole on the Incidence of Spontaneous Mammary Tumors in C3H Mice," *Henry Ford Hospital Medical Journal* : Vol. 30 : No. 2 , 65-69.

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Effect of Levamisole on the Incidence of Spontaneous Mammary Tumors in C3H Mice†

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Spontaneous mammary carcinoma in C3H mice is preceded by premalignant hyperplastic alveolar nodules. When a primary tumor is evident, examination of the other mammae will reveal a number of microscopic precancerous lesions in various stages of transformation to overt malignant tumors. In an effort to inhibit the development of additional malignant tumors after surgical removal of the first apparent tumor, we treated female C3H/HeJ mice with subcutaneous injections of levamisole (0.6 mg per kg of body weight) on a twice weekly regimen. All further tumors were surgically excised when they became palpable. The surgical excision of bulk tumor burden, immunorestitution by levamisole, and the presence in the tumor cells of

immunogenic surface antigens related to the virus all might be expected to enhance immunodulation of tumor cell growth or immunorejection of tumor cells. Immune activity measured by assaying macrophage migration inhibition factor, extractable from splenocytes, was significantly greater in the levamisole-treated mice than in the controls throughout the experiment. Although fewer tumors developed in the treated animals during the first eight weeks, this apparent effect of immunoperturbation was only transitory. By the end of 14 weeks, there was no longer a statistically significant difference in cumulative tumor index between the two groups.

Evidence has accumulated that cancer patients have diminished immune defenses when compared to normal individuals (1). It has also been shown that a relationship exists between the general immune status of the host and the clinical course of cancer (2). Concomitant with immunodepression in the cancer patient, a possible tumor cell immunorejection mechanism might also be impaired (1), so that normalization of the immune function could become an important adjunct in cancer treatment.

Levamisole, an antihelminthic drug, has been shown to potentiate immune responses in animals and humans (3-5). Indeed, Tripodi, et al (5) reported immunorestitution in anergic cancer patients after levamisole was administered.

The development of spontaneous mammary tumors in mice is influenced by several factors, including the murine mammary tumor virus (MuMTV), specific genetic constitution, and defined hormonal stimulus (6). Mammary tumors and hyperplastic alveolar nodules (HANs),

which are the morphological precursor of most mammary tumors, develop with high frequency in C3H mice with vertically transmitted MuMTV (7). Although virgin and multiparous mice will have essentially the same final tumor incidence, in parous females malignancy generally appears earlier.

The development of humoral and cellular immunity directed against MuMTV in high mammary tumor strains of mice is well known. Recently, Tagliabue, et al (8) demonstrated that cellular immune activity against MuMTV was not detectable by an indirect macrophage migration inhibition factor (MIF) procedure in C3H/HeN mice until they were approximately 14 weeks of age,

Submitted for publication: August 7, 1981

Accepted for publication: October 6, 1981

†This work was supported in part by the Matilda R. Wilson Fund.

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after which it became detectable before visible primary tumors developed. In this test system, immune activity tended to disappear by 36 weeks of age, when these mice were presumed to have subclinical tumors. A possible explanation for this immunodepression was provided by Creemers and Bentvelzen (9), who demonstrated that cells that suppress lymphocyte proliferation appear during mammary tumor growth and reach peak activity when tumors attain a weight of 0.5 to 1.5 gm.

To evaluate the efficacy of levamisole-induced immunorestitution in tumor cell growth inhibition and/or tumor cell rejection, we felt that the C3H/HeJ mouse would be a good model. Once the primary tumor becomes evident, the mouse should not be in an immunoinactive state as it relates to the MuMTV cell surface antigens. By radically excising the macrotumor but leaving relatively small numbers of active tumor cells in other primary microtumors, it should be possible to create a favorable situation in which the residual tumor cells would be susceptible to heightened immune activity. Therefore, if levamisole were effective in restoring immune cognizance of the tumor cell surface antigens, this renewed activity would be sufficient to suppress or reject the minimal tumor cell burden or inhibit the HANs from being transformed into overtly malignant tumors.

Materials and Methods

Animals

We used female C3H/HeJ mice bred and raised in our own colony. All mice were mated, and after the first litter was weaned, they were placed in stock pens until a palpable mammary tumor developed. When primary tumors became grossly evident, the mice were randomized into one of four groups and treated as follows:

- 1) 1° tumor: Mice were killed by cervical dislocation and the spleens removed. The splenocytes were processed for the MIF assay to establish a baseline of MIF activity in the mice which developed tumors.
- 2) Postsurgery: The tumors were surgically excised, the animals kept for one week, and then the spleens were removed for MIF assay to determine the level of activity in the absence of the major tumor burden.
- 3) Surgical control: The primary, and all subsequent tumors arising in 90 days, were excised. The few mice that died during surgery had their spleens removed for MIF. The rest of the animals, 90 days after initial surgery, were killed by cervical dislocation and the spleens removed for MIF.

4) Levamisole-treated: Primary and subsequent tumors were resected as for the previous group. In addition, these mice were given twice weekly subcutaneous injections of levamisole that began two days after initial surgery and continued for 90 days. As before, at the time of accidental operative death or after 90 days, spleens were removed and used for MIF assay.

5) Pretumor: An additional 22 mice, with no gross evidence of tumor, were used at five months of age to assay for MIF in pretumorous animals.

Levamisole

Levamisole phosphate (Ripercoll L, made by American Cyanamid Co), in an 18.2% injectable solution (equivalent to 136.5 mg of levamisole hydrochloride per ml) was diluted to 0.06 mg per ml in sterile 0.9% sodium chloride solution. All mice in the treatment group (group 4) received subcutaneous injections twice weekly (0.6 mg per kg of body weight) that started two days after the first tumor was removed and continued throughout the study.

The pharmacology of levamisole has been studied extensively and the importance of the dose administered has been stressed (10,11). In preliminary studies, with normal BALB/c mice, levamisole at a dosage of 0.6 mg/kg stimulated *in vivo* production of MIF, and weekly assays showed that MIF at high levels was maintained for four weeks. However, at higher dosages of the drug (1.1, 2.2, and 3.3 mg/kg), *in vivo* production of MIF was stimulated early but was no longer apparent after two weeks.

MIF assay

Based on the demonstrations that the lymphokine MIF is produced *in vivo* during periods of immunologic response (12-14) and that it can be extracted from lymphoid cells (including splenocytes) during such periods (12,15-17), we carried out the following procedure to evaluate immune activity in the experimental animals. Peritoneal exudate cells (PEC) were induced in BALB/c mice by intraperitoneal injection of 3 ml of sterile mineral oil at three-four days before harvesting. Extracts of splenocytes were prepared as previously described (16), and PEC migration in Sykes-Moore chambers was assayed according to the method of Bloom, et al (18).

In brief, capillary tubes containing a 10% (V:V) suspension of PEC were centrifuged at 250 X g for 5 min. The capillary tubes were cut at the interface of packed cells and medium, and two packed tubes were placed in a Sykes-Moore chamber. One ml of medium plus 0.2 ml of splenocyte extract was added to each chamber, except

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for those for control migration, which contained medium only. After 20-22 hr of incubation at 37°C in a humidified 5% CO₂ incubator, migration areas were traced with a microscope viewing screen and the surface areas were determined. An index of PEC migration (MI) was calculated by the following formula:

$$\text{MI} = \frac{\text{Average migration of four replicates with splenocyte extract}}{\text{Average migration of four replicates with medium only}}$$

Values of ≤ 0.80 were generally considered positive for MIF.

Results

Splenocytes for MIF determination were obtained from at least 20 individual mice in each group. Migration indices are shown in Fig. 1.

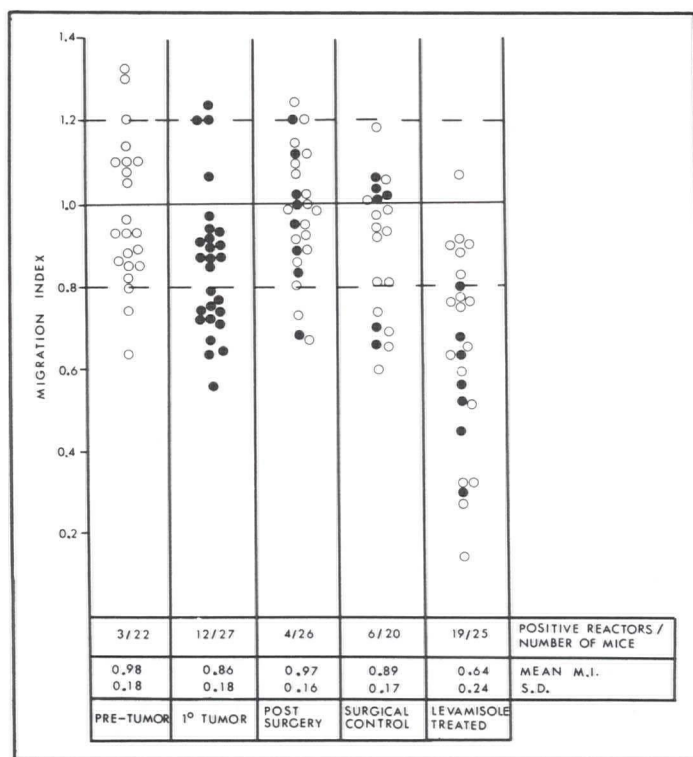


Fig. 1

Comparison of migration indices for the five groups of C3H/HeJ mice studied. Open circles indicate mice with no gross evidence of tumor; closed circles denote mice which had a palpable tumor at the time spleen cells were harvested for MIF assay.

Minor differences between the pretumor group, the one-week postsurgical, and control groups were not statistically significant, by the two-sample t test. The difference between the primary tumor group and both the

pretumor and one-week postsurgery groups was of borderline significance ($p < 0.02$), while the differences between the levamisole-treated animals and all other groups was highly significant ($p < 0.001$).

TABLE I

Comparison of Average Values for Age of Mice at First Primary Tumor, Numbers of Second Primaries, and Immuno-activity Index

	Control Group	Treated Group	p* Value
Age (in weeks) at first tumor	30.94 (± 5.74)	31.70 (± 5.65)	NS**
Number of weeks evaluated	10.10 (± 3.15)	10.61 (± 4.45)	NS
Number of additional tumors per mouse	4.23 (± 3.37)	3.60 (± 2.60)	NS
Number of additional tumors per mouse per week	0.46 (± 0.40)	0.37 (± 0.28)	NS
Macrophage migration inhibition index	0.89 (± 0.17)	0.64 (± 0.24)	0.0001

* Two = sample t test

** NS = not significant

Tumor incidence

The numerical values shown in Table I indicate that mice in control and treated groups were well matched in all respects, except for MIF values.

Because some mice died during surgery and some were arbitrarily sacrificed at different times in order to monitor their activity, cumulative tumor incidence (CTI: number of tumors/mouse weeks survived) was used to correct for the variance of numbers in the two groups. At weeks 7 and 8 (Fig. 2), the levamisole-treated animals had a significantly lower tumor incidence than did controls ($p < 0.001$). By week 9, treated animals were developing more additional tumors than were controls. They continued to do so until the study ended at 14 weeks; at this point, the difference in CTI was no longer statistically significant at the 0.05 level.

Autopsy findings were essentially similar in levamisole-treated and surgical control mice: two instances of lung metastases were noted, one in each group.

Discussion

After the first appearance of a spontaneous mammary tumor, treatment of C3H/HeJ mice by surgical tumor

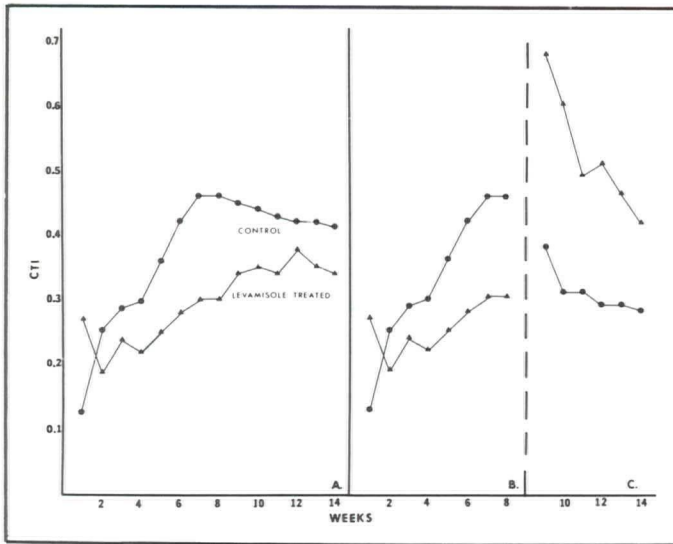


Fig. 2

Cumulative Tumor Index

$$CTI = \frac{\text{accumulated number of tumors}}{\text{accumulated number of mouse weeks at risk}}$$

A: CTI calculated weekly over the 14-week observation period; B: CTI calculated weekly through week 8; C: CTI beginning at end of week 8 and continuing through week 14.

excision and twice weekly subcutaneous injections of levamisole had a marked effect upon immune activity, as measured by the *in vivo* MIF throughout the 90-day period of observation. This treatment did not significantly diminish the final incidence of further tumor development as compared to mice treated by sequential surgical excision of tumors only. However, in the levamisole-treated mice, there was an apparent inhibitory effect on tumor incidence for the first eight-week period after immunotherapy was started. By the ninth week, this group of mice exhibited a sudden increase in the number of additional tumors, while the tumor incidence in the surgical control group began to decline. By the end of 14 weeks, CTI was essentially similar in the two groups.

Fidler and Spitler (19), using two different transplantable mouse tumors, have reported that levamisole injection before tumor transplantation enhanced the early appearance of neoplasms but had no effect on the overall incidence or course of tumor growth when compared to controls. In a study of immune response effect of levamisole on young adult and aged mice, Bruley-Rosset, et al (20) found that this compound was immunorestorative in aged mice, prolonged their survival, and seemed to reduce the incidence of spontaneous tumors by the time they were 24 months old. Padarathsingh, et al (21) also demonstrated that a single injection of levamisole (0.5 mg/kg of body weight) was sufficient to restore immunocompetence in BALB/c mice after plasmocytoma and drug-induced immunodepression. Sampson and Lui (22)

found that levamisole was a nonspecific stimulator of human lymphocyte function; later, however, Sampson, et al (10,11) demonstrated that high-dose levamisole was immunosuppressive in rats, while low dose levamisole was immunopotentiating. They also showed that inhibition of growth of 7, 12-dimethylbenz (a) anthracene-induced breast tumors in Sprague-Dawley rats was dose related; a high dose of levamisole (8 mg/kg) did not inhibit tumor growth, while a low dose (2 and 4 mg/kg) inhibited good tumor growth.

In this study, while tumor growth inhibition did not continue beyond the eighth week of treatment, MIF values obtained at weekly intervals were consistently positive in the levamisole-treated mice and negative in the surgical control group throughout the study.

Other studies that yielded positive, negative, and/or contradictory results have been carried out in animal models by the administration of levamisole before challenge with a transplanted tumor burden, although Fidler and Spitler (19) point out that a better model might be an animal with a spontaneous tumor which then receives the drug therapeutically. The C3H/HeJ mouse, with a high incidence of spontaneous mammary tumors in breeding females, is such a model. This selection might be criticized because the C3H/HeJ mouse is known to have a macrophage defect that results in hyposensitivity to almost every known biologic effect of bacterial endotoxin (23). In addition, these macrophages demonstrate poor tumoricidal activity *in vitro* (24,25) and respond poorly to the lymphokine, migration inhibition factor (26). However, it has been shown in this strain that *in vivo* infection with *Mycobacterium bovis* BCG activates the macrophages (24); Vogel, et al (27) showed that BCG stimulation restores practically all biologic effects of endotoxin except for the production of antibody to bacterial lipopolysaccharide.

Our results indicate that levamisole did, indeed, have a general stimulatory effect on immune activity of the C3H/HeJ mouse. Transient tumor growth inhibition was observed. It has been recognized for some time that administration of immunostimulatory agents to experimental animals, or to human patients with malignant disease, produces many immunological changes in the host. Certainly, the effect of these agents on tumor growth inhibition appears to depend on a balance of immune reactions that are as yet unknown.

This study indicates a real need for further investigations to elucidate more specific factors involved in immune system perturbation before such agents can be expected to contribute significantly to a more effective therapy of human cancer patients.

Acknowledgment

The authors wish to thank Ms. D'Anna Moltmaker for excellent technical assistance.

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